VIBRIO TOXINS: PERTURBATIONS OF MEMBRANE FUNCTION

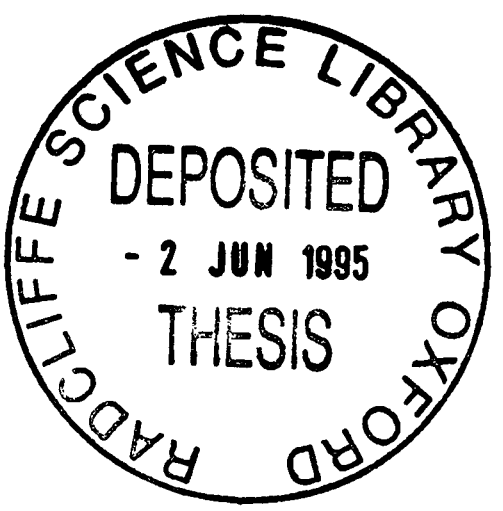
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ABSTRACT

Vibrio toxins: perturbations of membrane function

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Many bacterial toxins are important virulence factors, capable of instigating marked changes in the physiology of susceptible cells and tissues. Mechanisms of membrane attack by *Vibrio* toxins were examined on target cells, using cell physiological techniques, in particular, assays of haemolysis and radioisotope movement.

Kanagawa haemolysin (KH; commercially available preparation of the thermostable direct haemolysin (TDH)) of *V. parahaemolyticus* caused lysis of human (but not horse or hagfish) erythrocytes that occurred (a) after colloid osmosis due to raised cation permeability, (b) independently of the KH:red cell ratio, and (c) with a monovalent cation selectivity series (reversed Eisenman VIII with a small K^+ anomaly). The binding phase of KH was longer than the 1 - 2 minutes suggested by other workers.

The KH-induced cation leak was (a) rapid in onset, (b) of a magnitude higher in the first ten minutes of treatment than subsequently, (c) of a multi-hit nature, (d) unaffected by a variety of membrane-active agents, and (e) inhibited by Zn^{2+}, Cd^{2+} or mixing of toxin with dibutyl phthalate. Neuraminidase treatment of HRBC enhanced KH-induced cation influx and haemolysis, suggesting that additional receptors for TDH were unmasked by this treatment. In the presence of subhaemolytic KH, physiological levels of extracellular Ca^{2+} increased K^+ influx by the Gardos channel, and Mg^{2+} (1.5 mM) decreased flux by the $Na^+/K^+/2Cl^-$ cotransporter. There were no significant changes to sodium pump activity.

TDH and El Tor haemolysin (ETH) were purified from culture supernatants of *V. parahaemolyticus* and non-O1 *V. cholerae*, respectively. Highly purified TDH was derived from KH and used to confirm that the identified features of KH action were attributable to TDH. Although ETH also caused haemolysis by colloid osmosis secondary to increased cation permeability, it differed from TDH in its lability to air/vibration, relative magnitude of induced influx with respect to time, and selectivity series of induced lesion. Concentrated supernatants, from *V. cholerae* strains deleted of known virulence factors, caused morphological changes of Chinese hamster ovary cells, suggesting the presence of unidentified factors capable of perturbing cell physiology.

Confirming its potential as an enterotoxin, KH (albeit at a high dose) increased the efflux rate constant for $^{86}Rb^+$ from rabbit jejunocytes. These findings are discussed in the context of a possible pathogenic role for TDH in the gastrointestinal tract.

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"Un poème n'est jamais achevé - c'est toujours un accident qui le termine, c'est-à-dire qui le donne au public." Paul Valéry (1871-1945), in Littérature (1930).

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ABBREVIATIONS

aa	amino acid
ATZ	anilinothiozolinone
A_x	absorbance at *x* nanometres
BAPTA	1,2*bis*(2-aminophenoxy)ethane N, N, N', N'-tetracetic acid acetomethoxyester
BCA	bicinchoninic acid
BSA	bovine serum albumin
CA	carbonic anhydrase
CHO	Chinese hamster ovary
CP	centrifugation protocol
CT	cholera toxin
DBP	di-n-butyl phthalate
DIDS	4,4'-diisothiocyano-2,2' stilbene disulphonic acid
DMSO	dimethylsulphoxide
DNP	dinonyl phthalate
DTL	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(β-aminoethylether)N,N,N'-tetraacetic acid
EMEM	Eagle minimum essential salts medium
ETH	El Tor haemolysin
FCS	foetal calf serum
FPLC	fast performance liquid chromatography
GCS	gut cell solution
G_t	tissue conductance
H^{50}	time taken (mins) for 50% haemolysis to occur
Hct	haematocrit
HEPES	N-2-hydoxyethylpiperazine-N'-2-ethane sulphonic acid
HPLC	high performance liquid chromatography
HRBC	human red blood cells
HU	haemolytic unit
I_{SC}	short circuit current
IU	international unit
K_{av}	fraction of stationary gel volume available for diffusion of solute
kd	kilodalton
K_d	distribution coefficient
KH	Kanagawa haemolysin
LYZ	lysozyme
MBS	MOPS buffered saline
MOPS	3-[N-Morpholino]propane sulphonic acid
MSA	methyl sulphate
MWCO	molecular weight cut-off
OVB	ovalbumin
PAGE	polyacrylamide gel electrophoresis
P*b*	phosphorylase *b*
PCV	packed cell volume

PITC	phenylisothiocyanate
PMSF	phenylmethylsulphonyl fluoride
PTC	phenylthiocarbamyl
PTH	phenylthiohydrazine
R_f	relative mobility
r.p.m.	revolutions per minute
SDS	sodium dodecyl sulphate
STI	soybean trypsin inhibitor
TCA	trichloracetic acid
TDH	thermostable direct haemolysin
TPB	tryptose phosphate broth
Triton X-100	octylphenoxypolyethoxy ethanol
UV	ultraviolet
v/v	volume per volume
V_e	elution volume
V_o	void volume
V_s	stationary volume
V_t	total volume of packed gel fitration bed
w/v	weight per volume
YE	yeast extract

subscripts: [distinguish $_o$, $_s$ and $_t$ from above]

b	blank
i	intracellular
o	extracellular
s	serosal
sa	sample
st	standard
t	transepithelial

CHAPTER 1

Introduction

'To have begun is half the job...'
Horace, *in* Epistles (transl.), I.ii.40

Diarrhoea caused by infectious agents is a major cause of mortality and morbidity, especially in the Third World. Children are severely affected, particularly when diarrhoeal illness is compounded with malnutrition. Estimation of the global problem of acute diarrhoeal disease has been undertaken by analysis of morbidity and mortality data from epidemiological investigations in Africa, Asia (excluding China) and Latin America (Snyder & Merson, 1982). In these countries there are thought to be between 5 and 10 million deaths from diarrhoea per year (Walsh & Warren, 1979). Children under the age of five years suffer between 2.2 and 3 diarrhoeal illnesses per year with a total mortality of 7 %. In industrialised countries, although life-threatening diarrhoeal infections still occur in the young and in institutions, the majority of such illness is mild and causes only minor inconvenience (Evans, 1986). Nevertheless, diarrhoeal disease remains an important cause of lost production and morbidity.

Bacterial pathogens which cause diarrhoea are usually ingested in food or water, or as a result of faeco-oral transmission. Many such bacteria elaborate exotoxins which perturb the physiological function of the gut (Evans, 1986; Nair & Pal, 1989). Members of the *Vibrio* genus are important contributors to this spectrum of diarrhoeal disease; *V. cholerae*, in particular, has caused devastating outbreaks, especially manifest as cholera pandemics (Barua & Burrows, 1974), and the extended outbreak (1992 onwards) of Bengal cholera (Ramamurthy *et al.*, 1993; Albert *et al.*, 1993; Nair *et al.*, 1993), recently designated the eighth cholera pandemic since 1817. The unprecedented emergence of a novel toxigenic strain of *V. cholerae* (*V. cholerae* O139) (Nair *et al.*, 1993) emphasises the continued importance of research into pathogenic mechanisms. Other *Vibrio* species associated with human gastrointestinal disease (Janda *et al.*, 1988) include *V. parahaemolyticus* (Blake *et al.*, 1980; Joseph *et al.*, 1982) and various others (Janda *et al.*, 1988) listed in Table 1.1. At least one major exotoxin is associated with each pathogenic species of the *Vibrio* genus. Of the non-cholera *Vibrio*s, the most important is the predominantly food-borne pathogen *V. parahaemolyticus*. This is especially true in Japan and Taiwan (Sakazaki & Balows,

bacterium	reference	? major exotoxin(s)	reference
V. cholerae O1	Pierce & Mondal, 1974	cholera toxin	De, 1959 Moss & Vaughan, 1986, 1988a,b, 1989 Rappuoli & Pizza, 1991
V. cholerae non-O1, ctxA$^-$	Morris & Black, 1985	*zonula occludens* toxin (*zot*)	Fasano *et al.*, 1991
		accessory cholera enterotoxin (ace)	Trucksis *et al.*, 1993
		El Tor haemolysin (ETH)	Hall & Drasar, 1990
		other factors	Michalski *et al.*, 1993
V. cholerae non-O1, (O139), ctxA$^+$	Ramamurthy *et al.*, 1993 Albert *et al.*, 1993 Nair *et al.*, 1993	cholera toxin	Hall *et al.*, 1993
V. parahaemolyticus	Fujino *et al.*, 1953 Joseph *et al.*, 1983	thermostable direct haemolysin (TDH)	Honda & Iida, 1993 Takeda, 1983
V. mimicus	Davis *et al.*, 1981 Shandera *et al.*, 1983	cholera toxin	Spira & Fedorka-Cray, 1983
		enteroxins similar to those from *V. cholerae* non O1	Nishibuchi & Seidler, 1983
V. hollisae	Hickman *et al.*, 1982 Morris *et al.*, 1982	cytolysin similar to TDH	Nishibuchi *et al.*, 1990
V. fluvialis	Lee *et al.*, 1981 Morris & Black, 1985	*V. fluvialis* enterotoxin	Sanyal *et al.*, 1980 Lockwood *et al.*, 1982
		V. fluvialis cytotoxin	Wall *et al.*, 1984
V. furnissii	MMWR 1969a,b Brenner *et al.*, 1983	enterotoxin similar to that of *V. fluvialis*	Nishibuchi & Seidler, 1983
V. metschnikovii	Jean-Jacques *et al.*, 1981	*V. metschnikovii* cytolysin	Miyake *et al.*, 1988, 1989
V. vulnificus	Blake *et al.*, 1979 Morris & Black, 1985 Klontz *et al.*, 1988	vulnificolysin	Kreger *et al.*, 1988
V. alginolyticus	Sakazaki, 1968 Joseph *et al.*, 1983	collagenase	Takeuchi *et al.*, 1992
V. damsela	Love *et al.*, 1981 Morris *et al.*, 1982	damselysin	Kothary & Kreger, 1985 Kreger *et al.*, 1988
V. cincinnatiensis	Bode *et al.*, 1986 Brayton *et al.*, 1986	? - but close ancestry with *V. mimicus*, *V. fluvialis* & *V. metschnikovii*	Brayton *et al.*, 1986

Table 1.1 Pathogenic species of the *Vibrio* genus and associated exotoxins with selected references. Species are grouped according to the site affected by infection; the top eight cause gastrointestinal disease, whereas the bottom four are associated with extraintestinal disease only. References given for exotoxins are different from those given for bacteria.

1981; Kudoh *et al.*, 1988; Spriggs & Sack, 1990), where it accounts for more than 50 % of hospital admissions due to food-poisoning (Kudoh *et al.*, 1988).

Physiological studies of intestinal function (Field, 1981; Chang & Rao, 1991; Schultz, 1991) have led to important therapeutic progress; most obviously the combination of glucose and salts in rehydration solutions has made possible the oral replacement of fluid lost in diarrhoea (Hirschorn & Greenough, 1991). This protocol is of particular importance in the Third World (Field, 1981; Hirschorn & Greenough, 1991).

In seeking to understand the pathogenic mechanisms instigated by diarrhoea-causing bacteria, it is important to characterise the action of their toxins, the eventual goal of such work being the pharmacological blockade of toxin-induced diarrhetic processes (Mynott *et al.*, 1991, 1993). The mode of action of cholera toxin has been exceptionally well defined (Moss & Vaughan, 1986, 1988a,b, 1989) and the molecular pathophysiology of *Vibrio cholerae* is well understood (Field & Semrad, 1993). Recently, attention has turned to the actions of other diarrhoeal toxins, in particular the *E. coli* heat-stable enterotoxins STa (Field *et al.*, 1978; Schultz, 1991; Field & Semrad, 1993) and STb (Kennedy *et al.*, 1984; Evans, 1986), shiga toxin (Cleary *et al.*, 1985; Evans, 1986), *C. difficile* toxins (Evans, 1986; Fiorentini & Thelestam, 1991; Fiorentini *et al.*, 1993; Shoshan *et al.*, 1993), and *Ciguatera* toxin (Fasano *et al.*, 1991). Moreover, as a result of knowledge so gleaned, our understanding of physiological alimentary function has been greatly advanced (Field, 1981; Evans, 1986; Field & Semrad, 1993). The use of well-characterised toxins, for example cholera toxin (Barnes & Conn, 1993; Ramamoorthy *et al.*, 1993), pertussis toxin (Sa & Fox, 1994; Shigematsu *et al.*, 1994), and *Clostridium perfringens* δ-toxin (Jolivet-Reynaud *et al.*, 1982, 1988, 1993; Cavaillon *et al.*, 1986) to probe cell physiology is an emergent facet of such research (Braun & Focareta, 1991; Bhakdi *et al.*, 1993; Read & Stein, 1993).

Toxins from the *Vibrio* genus have only recently attracted a similar level of interest (Nishibuchi *et al.*, 1992). The present study focusses on toxins derived from culture supernatants of the *Vibrio* genus, in particular the thermostable direct haemolysin (TDH) and the El Tor haemolysin (ETH), from *V. parahaemolyticus* and cholera toxin negative ($ctxA^-$) *V. cholerae*, respectively. Although these bacteria are major causes

of gastroenteritis (Blake *et al.*, 1980; Joseph *et al.*, 1982; Morris & Black, 1985; Janda *et al.*, 1988), the actions of their associated putative virulence factors (TDH and ETH respectively) are poorly understood. In their classification of cytolytic peptides, Bernheimer & Rudy (1986) ascribed these *Vibrio* cytolysins to an 'individualistic agents' category, in which no mechanism of action was defined. The importance of TDH is underscored by the recent discovery that this agent and closely related molecules (accorded to the TDH family of toxins (Honda *et al.*, 1992; Honda & Iida, 1993)) are not confined to *V. parahaemolyticus*, but are also produced by *V. cholerae* non-O1 (Honda *et al.*, 1986a; Yoh *et al.*, 1986a; Baba *et al.*, 1991; Terai *et al.*, 1991), *V. hollisae* (Yoh *et al.*, 1986b; Yoh *et al.*, 1989; Yamasaki *et al.*, 1991) and *V. mimicus* (Terai *et al.*, 1990; Yoshida *et al.*, 1991). In addition, the structural gene encoding ETH has been shown to possess considerable homology to that for vulnificolysin (Yamamoto *et al.*, 1990c), the potent cytolysin associated with *V. vulnificus*. Thus, an understanding of the mechanisms of toxicity for these two toxins may have even wider implications than originally envisaged.

As is demanded by the multifarious and sometimes (apparently) conflicting functions of the alimentary canal (eg a barrier function against parasites and toxins (Walker & Owen, 1990), yet an absorptive function for nutrients), the gut is a complex and highly organised structure (Ham & Cormack, 1987; Castro, 1989; Wood, 1991), in which neurobiological, endocrine and immunological systems are integrated. It is noteworthy that the number of immune cells in the gut is said to equal the total in the remainder of the body (Wood, 1991). There are many possible sites for toxin action within the alimentary canal. Toxin action may be mediated by routes other than a direct action on the intestinal epithelium, such as by means of inflammatory or neurological mediators. For example, the enteric nervous system is responsible *in vivo* for most of the change in fluid transport induced by cholera toxin (Cassuto *et al.*, 1982, 1983; Jodal *et al.*, 1993). Nevertheless, the cell membrane acts as the primary structure for cellular compartmentalisation from the external environment and is - with the exception of toxins which attack extracellular components (eg *zonula occludens* toxin (ZOT) that affects tight junctions (Fasano *et al.*, 1991)) - the barrier that a toxin must either attack or bypass to intracellular sites.

In seeking to make an initial assessment of effects on membrane function, the

responses of red cells (erythrocytes constitute a well characterised model membrane system) have often been studied to analyse mechanisms of membrane perturbation. Many different agents and imposed extracellular conditions can perturb erythrocyte membrane function, including bacterial toxins (Bernheimer & Rudy, 1986; Kreger, 1991), divalent cations (Ellory *et al.*, 1992; Yang *et al.*, 1993), low ionic strength (Bernhardt *et al.*, 1991), oxidative stress (Deuticke *et al.*, 1983, 1984, 1991), and cellular parasite infection (Dunn *et al.*, 1969; Bookchin *et al.*, 1980; Ginsburg *et al.*, 1988; Kirk *et al.*, 1991, 1994). In the case of bacterial toxins (Freer, 1986; Harris, 1986; Braun & Focareta, 1991; Kreger, 1991), such studies form the basis of further experimentation on more physiological preparations. This approach has been adopted in this study. The actions of *Vibrio*-derived toxins, in particular TDH (Takeda, 1983; Honda & Iida, 1993) and ETH (Honda & Finkelstein, 1979; Hall & Drasar, 1990; Yamamoto *et al.*, 1990b,c), have been comprehensively characterised on erythrocytes (Chapters 4, 5, 7 and 8), before attempting analysis of TDH on isolated enterocytes. Use of cell physiological techniques is intended to bridge the gap in our understanding between the molecular biology of toxins and the pathogenesis of *Vibrio parahaemolyticus*. By appreciating the role of such toxins as virulence factors, their action may be set in the context of overall pathogenic schema.

The specific aims of this investigation and the methods employed to achieve them are described in Chapter Three. Investigations performed to define the nature of membrane attack by Kanagawa haemolysin (KH) as evinced by aspects of the haemolytic reaction are detailed in Chapter Four. Experiments examining the nature and consequences of the KH-induced lesion, using isotopic flux methodology, are given in Chapter Five. Data derived from microbiological experiments are presented in Chapter Six, examining: (i) production and purification of the thermostable direct haemolysin (TDH) of *V. parahaemolyticus*, (ii) production and purification of the El Tor haemolysin (ETH) of *V. cholerae*, and (iii) the production of accessory factors by ctxA$^-$ *V. cholerae*. Erythrocyte membrane attack by a highly purifed TDH preparation is the subject of Chapter Seven; these experiments were performed to assess the contribution of this toxin to membrane attack by KH. The nature of erythrocyte membrane attack by ETH is examined in Chapter Eight. Analysis of toxin action on isolated intestinal cells is described in Chapter Nine; these data assess the plausibility

of pathophysiological roles for these toxins. Finally, the findings of Chapters Four to Nine are summarised and their implications discussed in the light of previous and current research (Chapter 10); an emphasis for continued research is suggested.

The following chapter is a synopsis of pertinent background theory. It outlines: (i) membrane structure and function, (ii) intestinal function in electrolyte absorption and secretion, (iii) bacterial toxinology, with particular emphasis on (a) the role of toxins in bacterial pathogenesis, and (b) mechanism of action, (iv) disease caused by *V. parahaemolyticus* and cholera toxin-negative *V. cholerae*, and (v) membrane physiology of target cells.

CHAPTER 2

Background theory: membranes, intestinal function and bacterial toxins

'Of course the first thing to do was to make a grand survey of
the country she was going to travel through'
Lewis Carroll *in* Through the Looking Glass

2.1 INTRODUCTION

As has already been made clear, the disruption of membrane function by toxins is a crucial element in many pathogenic processes (Chapter 1). This project examines the action of toxins from *Vibrio parahaemolyticus* and *Vibrio cholerae*, exploring both mechanistic details of membrane attack and sequelae to this action, and attempts to set these findings in the context of an overall pathogenic schema. To accomplish these aims, an extensive general background theory is required, involving several general physiological fields, which are reviewed in this chapter: (i) membrane structure, (ii) membrane function, (iii) intestinal function, and (iv) bacterial toxins.

This chapter then contains rather more specific details of pertinent history, clinical relevance and current knowledge of (a) *V. parahaemolyticus*, and the thermostable direct haemolysin (TDH) and related haemolysins, and (b) *V. cholerae* and toxins other than cholera toxin. Finally, the membrane physiology of cells used as target systems is discussed.

2.2 MEMBRANE STRUCTURE

Membrane structure has been the subject of many extensive reviews and excellent monographs (see Stein, 1986, 1990; de Robertis & de Robertis, 1987; Stryer, 1988; Lee, 1990; Singer, 1992; Yeagle, 1992) - only an overview is given here. The pivotal role of all membranes, whether cell (plasma) membranes, or of subcellular origin, is selective compartmentalisation. Upon this tenet are based all the specialised functions that are ascribed to particular membranes. Membranes are thin structures (6 - 10 nm across) comprised of three principal components: lipid, protein and carbohydrate. The carbohydrate is always bonded to either lipid (as glycolipid) or protein (as glycoprotein). The ratios of these components vary according to the membrane studied. The cardinal role of the lipid bilayer is as a barrier to diffusion of polar substances between

intracellular and extracellular environments (though some species of lipid have more subtle roles, for example the inositol lipids form part of a second messenger cascade). It is the protein components that are predominantly responsible for the specific functions of the membrane.

In all biological membranes these components are assembled in a similar way that conforms, in part, to the structure depicted by the fluid mosaic model (Singer & Nicolson, 1972; Figure 2.1), in which proteins are pictured as floating, and freely diffusing, in a two-dimensional bilayer sea of lipid molecules. The concept that membranes have a fluid structure has had considerable influence on ideas of organisation in the plane of the membrane; however, there have been important modifications to this model over the last two decades (Singer, 1992). In particular, the Singer-Nicolson model:

(i) underestimates the ratio of the masses of membrane protein:membrane lipid (Singer, 1992).

(ii) fails to appreciate that some proteins have limited mobility in the plane of the membrane. Proteins vary in this mobility, which may be measured using the technique of fluorescent recovery after photobleaching (Elson, 1986). Typically, it is found that some proteins are relatively unrestricted in their movement through the membrane phase. Others, however, are completely immobile (Axelrod, 1983). It is believed that this immobilisation results from interactions with other membrane proteins and proteins that belong to the cytoskeleton (Figure 2.2). The restriction of lateral diffusion of some proteins in the plane of the membrane is important for the formation of specialised regions in a cell membrane eg the tight junctions of epithelia which serve to create a distinctive protein composition for the apical and basolateral membrane domains (Contreras *et al.*, 1992).

(iii) does not appreciate the existence of lipid domains, detected using fluorescent phospholipids, even in the fluid membrane phase (Glaser, 1993; Rodgers & Glaser, 1993).

2.2.1 Membrane lipid structure

Most of the cell components are dissolved in water, the universal solvent. Lipids, however, are insoluble in water (but are highly soluble in organic solvents such as chloroform or methanol). Despite their chemical diversity, membrane lipids are all amphipathic, that is, they possess distinct hydrophobic and hydrophilic regions. This property enables lipids to provide the basic structural framework of the membrane. The

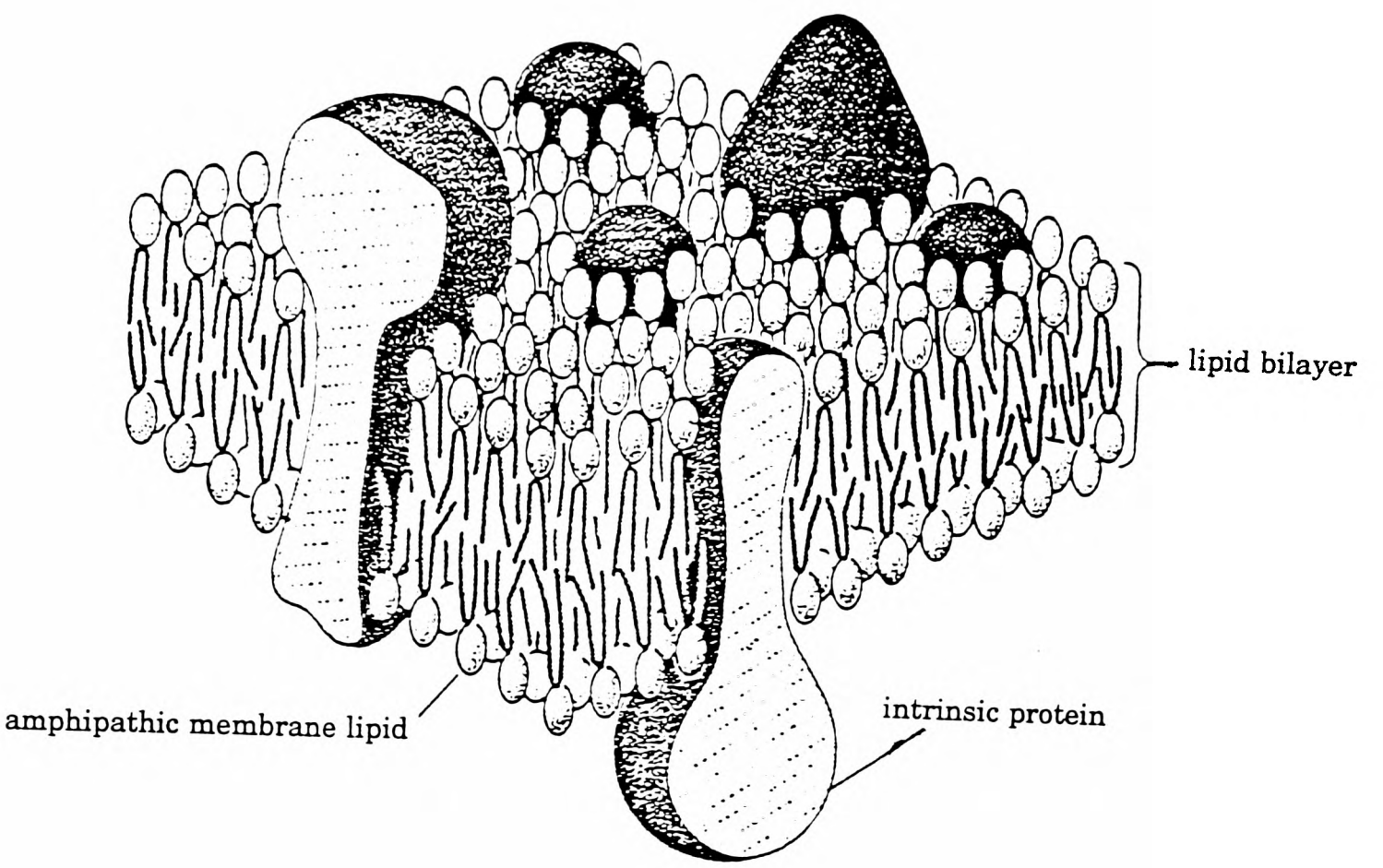


Figure 2.1 The fluid mosaic model of membrane structure. After Alberts *et al.* (1990).

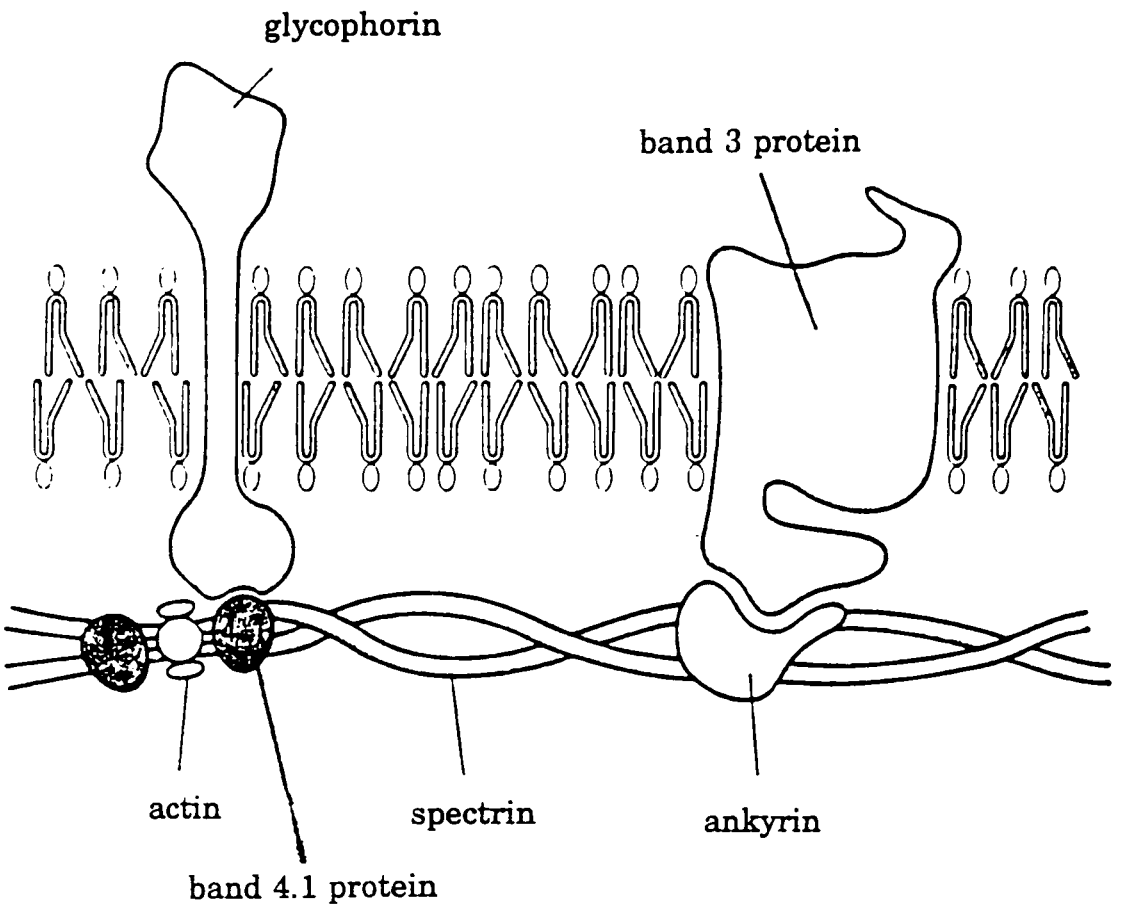


Figure 2.2 Interactions between membrane proteins and proteins of the cytoskeleton. Two intrinsic membrane proteins, glycophorin and Band 3, interact with cytoskeletal elements that lie beneath the cell membrane. Spectrin chains interact with ankyrin, Band 4.1, and actin to form a supercomplex. After Evans and Graham (1989).

lipids of importance for membrane structure are phospholipids, glycolipids and cholesterol.

Phospholipids are based on either glycerol (the glycerophospholipids) or on sphingosine (the sphingolipids). The former are the most common and consist of three parts: a glycerol backbone, a phosphorylated alcohol making the lipid headgroup, and two fatty acyl chains. The fatty acyl chains and phosphate ester form the hydrophobic and hydrophilic portions of the molecule respectively. Corresponding phospholipids with one fatty acyl chain (the lysophospholipids) are intermediates in cell metabolism and are not considered normal components of biological membranes. Both the fatty acid and alcohol moieties are variant entities, so there is a wide variety of chemical structures. The fatty acids of the membrane lipids of animal cells are generally unbranched, with an even number of carbon atoms, between 14 and 24 (Lee, 1990). The acyl chains may be either saturated or unsaturated; an unsaturated chain contains one or more C=C double bond in a *cis* configuration. The chemical structures of the most common fatty acids are given in Table 2.1. Esterification of two fatty acyl chains to a molecule of glycerophosphate gives phosphatidic acid which can link in turn to the bases choline, ethanolamine, serine or inositol to give, respectively, phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI) (Figure 2.3). The phosphate groups of all the phospholipids have a single negative charge at physiological pH; choline and ethanolamine contain amino groups which are positively charged (thus PC and PE are zwitterions with no net charge). Serine however has both one negative and one positive charge at pH 7, and hence PS bears a single net negative charge. The inositol group is uncharged, so PI carries a single net negative charge - the charge on the phosphoinositides will depend on the number of phosphate groups on the inositol ring. Cardiolipin (diphosphatidylglycerol (DPG)) has four fatty acyl chains and two negative charges, and in animals, is found only in the inner mitochondrial membrane. Plasmalogens are close structural relatives of phospholipids. They differ in that one of the fatty acyl chains is linked to the glycerol backbone via an ether (as opposed to an ester) linkage (Figure 2.4).

Glycolipids are comprised of lipid entities with an hydrophilic sugar headgroup. The major class of glycolipid is the glycosphingolipids; these are structurally similar to the phospholipids but consist of a carbohydrate chain attached at a right angle to the lipid ceramide. The carbohydrate chain therefore lies along the outside of the cell surface, roughly perpendicular to the hydrocarbon tails. It is thereby exposed to the extracellular

Structure	Notation	Name
$Me(CH_2)_{14}CO_2H$	16:0	palmitic acid
$Me(CH_2)_{16}CO_2H$	18:0	stearic acid
$Me(CH_2)_5CH{=}CH(CH_2)_7CO_2H$	16:1	palmitoleic acid
$Me(CH_2)_7CH{=}CH(CH_2)_7CO_2H$	18:1	oleic acid
$Me(CH_2)_4(CH{=}CHCH_2)_2(CH_2)_6CO_2H$	18:2	linoleic acid
$Me(CH_2)(CH{=}CHCH_2)_3(CH_2)_6CO_2H$	18:3	linolenic acid
$Me(CH_2)_4(CH{=}CHCH_2)_4(CH_2)_2CO_2H$	20:4	arachidonic acid

Table 2.1 Structures of fatty acids commonly found in phospholipids. The shorthand notation (Me = methyl- group) gives the number of carbon atoms in the chain followed by the number of double bonds.

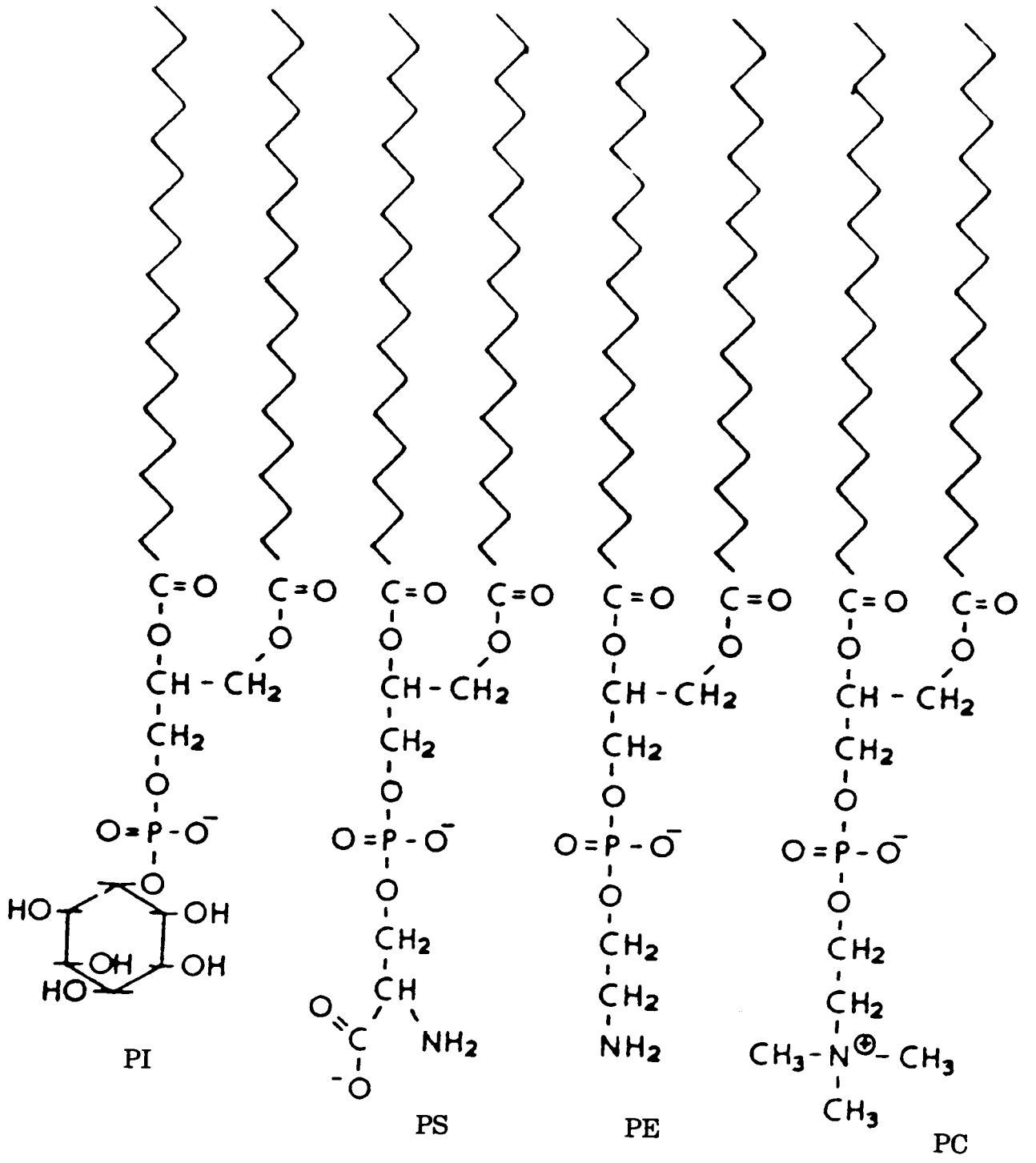
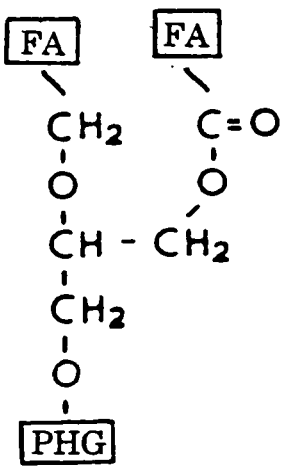


Figure 2.3 Structures of the most common membrane phospholipids. PC = phosphatidylcholine; PE = phosphatidylethanolamine; PS = phosphatidylserine; PI = phosphatidylinositol. After Finean and Michell (1981).



N.B. The ether (as opposed to ester) group may link the fatty acyl chain of either the C_1 or the C_2 (as given) of the glycerol molecule.

Figure 2.4 General structure of plasmalogens. FA = fatty acid group; PHG = polar headgroup.

environment unless it is partially obscured by proteins or other glycosphingolipids embedded in the membrane nearby. Most, but not all, glycosphingolipids conform to one of four basic core structures (Figure 2.5; Hakomori, 1986), which are defined according to the identity and bonding of the sugar residues closest to the ceramide. In each of the four core structures, ceramide is linked to glucose and the glucose is bonded to galactose. In the ganglio-series, the chain is continued with a sugar called N-acetylgalactosamine, to which another galactose is attached (Table 2.2). The internal galactose and the terminal galactose are each linked to an acidic sugar called sialic acid. In the globo-series, the initial glucose-galactose chain is continued with another galactose and N-acetylgalactosamine. In the lacto-series the initial chain continues with a sugar residue of N-acetylglucosamine and galactose, and depending on the position of the last bond, the lacto-series molecules are further classified as type 1 or type 2. There is considerable variation in both the ceramide portion and the sugar-based headgroup which can be elongated and branched - approximately 130 varieties of glycosphingolipid have been characterised. About 40 belong to the ganglio-series, 10 to the globo-series, and 60 are classified in the lacto-series (the remaining 20 are unclassified). The lacto-series include the clinically important blood-group antigens of the ABH system (Hakomori, 1986). Gangliosides, the general structure of which are shown in Figure 2.6, are defined by the presence of sialic acid in their carbohydrate chains (and are therefore negatively charged). The commonest sugar headgroups are shown in Table 2.2. Gangliosides appear to be important in cell function as receptors for hormones and toxins (see section 2.3.3; Hakomori, 1984, 1986).

The last important membrane lipid is cholesterol (Figure 2.7) which is the major sterol found in mammalian cells. Although its structure is totally different to those of the other membrane lipids, cholesterol is also amphipathic; the steroid ring is hydrophobic and the hydroxyl group hydrophilic.

2.2.2 Lipid composition of membranes

The relative proportions of the lipids described above vary considerably, both between different membranes of the same cell and analagous membranes of different cells. Relatively high levels of SM and cholesterol are found in the plasma membrane, whereas cardiolipin is found solely in mitochondria. There is also considerable interspecial variation in the composition of the same membrane, for example, although the sum of the

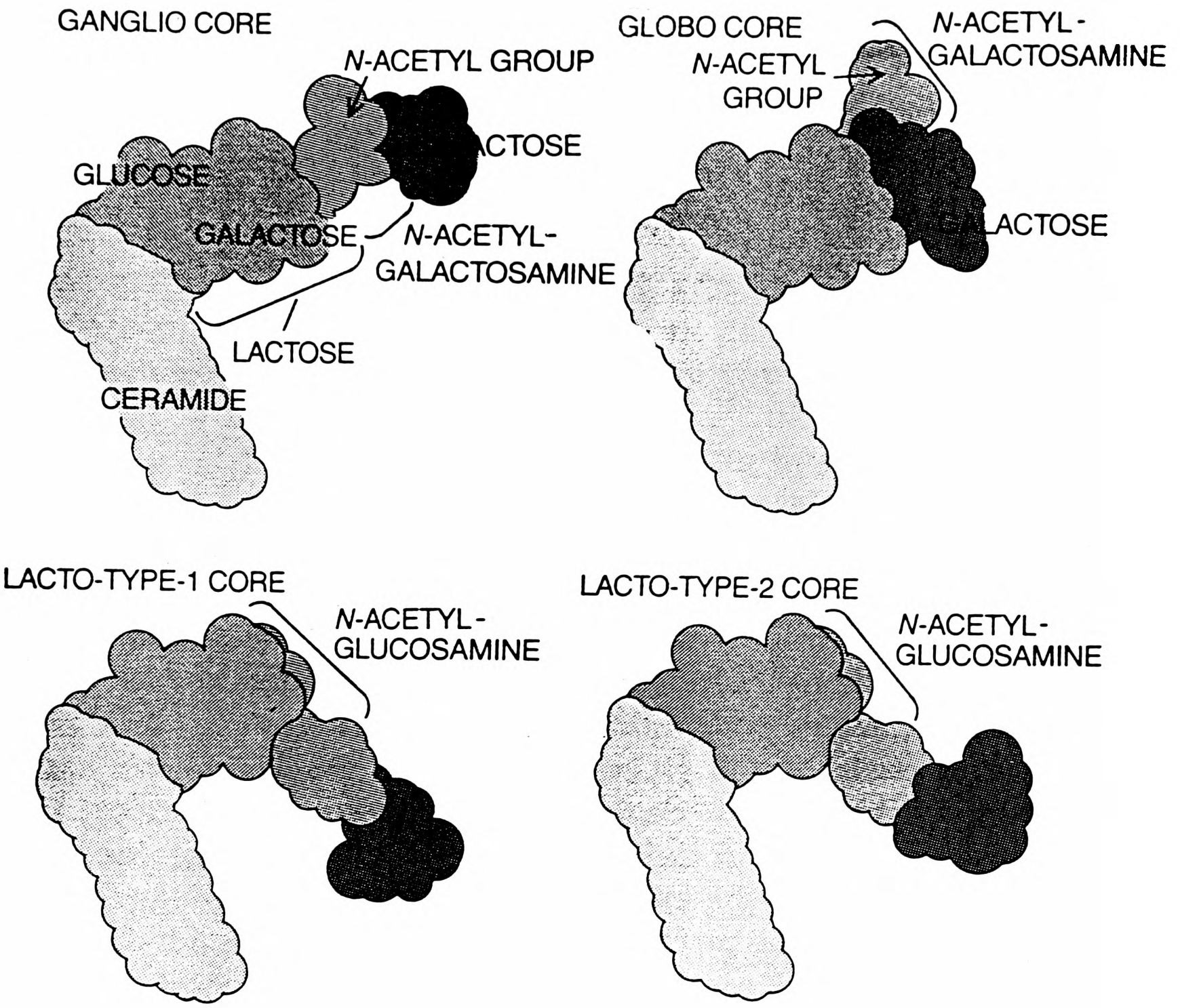


Figure 2.5 Core structures of the glycosphingolipids. After Hakomori (1986).

Sugar residue	Designation
-Glc-Gal(-NANA)	G_{M3}
-Glc-Gal(-NANA)-GlcNAc	G_{M2}
-Glc-Gal(-NANA)-GlcNAc-Gal	G_{M1}
-Glc-Gal(-NANA)-GlcNAc-Glc(-NANA)	G_{D1}
-Glc-Gal(-NANA-NANA)-GlcNAc-Gal(-NANA)	G_{T1}

Table 2.2 Structures of commonly occurring ganglioside headgroups. After Lee (1990). Abbreviations: Glc, glucose; Gal, galactose; GlcNAc, N-acetyl-D-glucosamine; NANA, sialic acid.

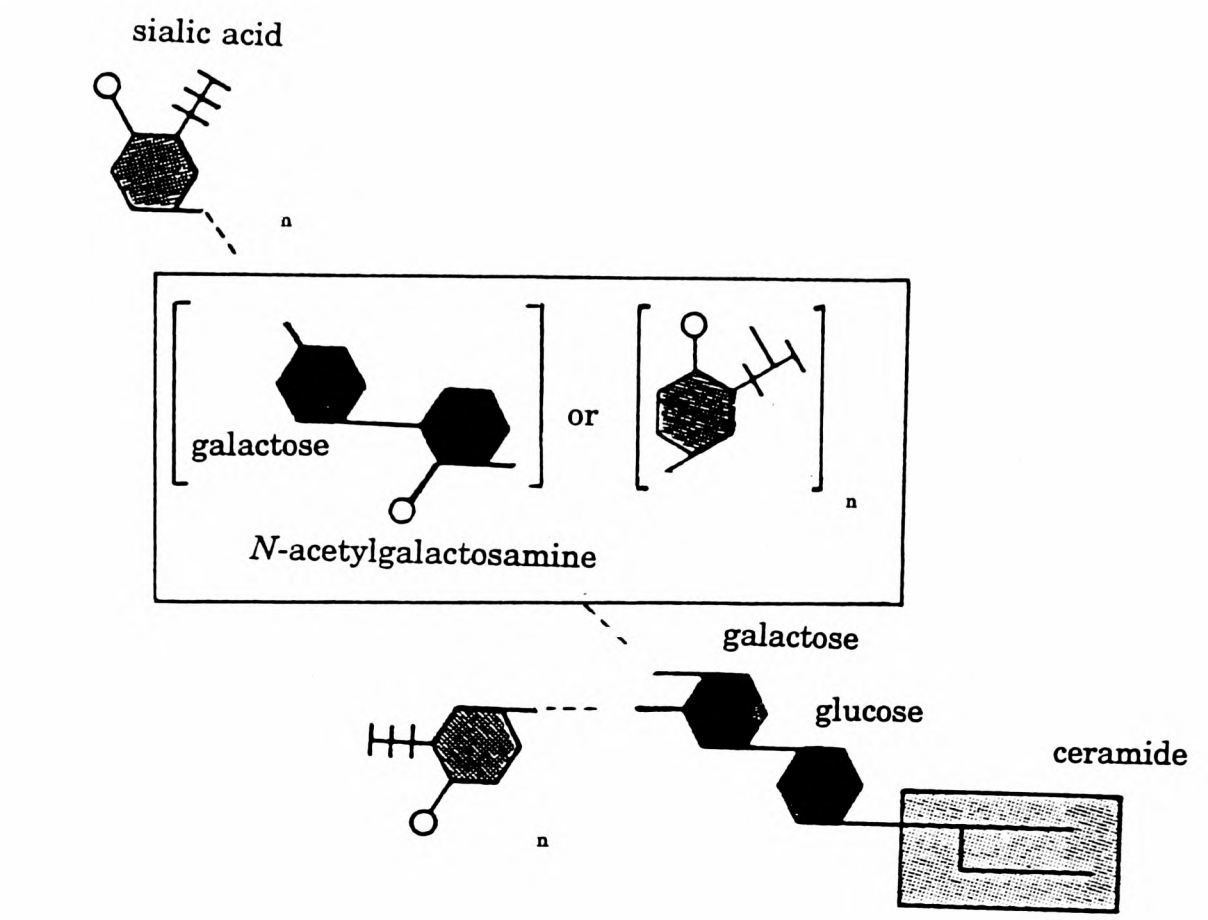


Figure 2.6 General ganglioside structure. See Table 2.2 for particular headgroups. After Hakomori (1986).

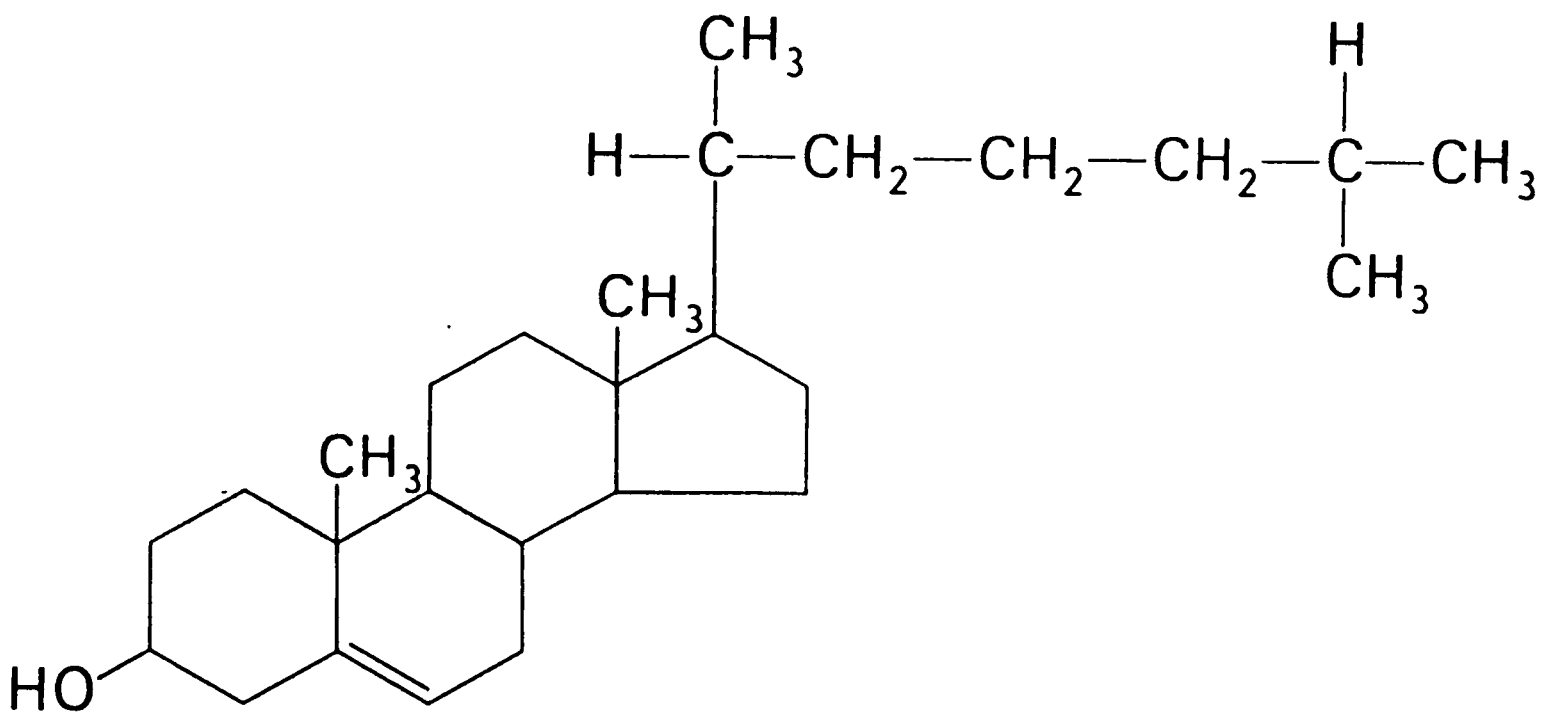


Figure 2.7 Cholesterol. This neutral lipid has a hydrophilic hydroxyl group at C-3. After Stryer (1988).

PC and SM contents of erythrocyte membranes of rat, pig, ox and sheep are all 40 - 45 %, the relative amounts of these two phospholipids differ considerably (Quinn, 1976; Figure 2.8).

Biological membranes exhibit a profound lipid asymmetry with respect to the axis running between the leaflets of the bilayer (Op den Kamp, 1979; Dressler *et al.*, 1983). Figure 2.9 shows the relative distributions of phospholipids from the human erythrocyte membrane (Rothman & Lenard, 1977; Op den Kamp, 1979; Dressler *et al.*, 1983). SM is located predominantly in the outer leaflet, whereas PE is concentrated in the cytoplasmic leaflet.

The distribution of the fatty acyl chain between membranes is even more variable (Hill *et al.*, 1965). Table 2.2 illustrates the different acyl chain compositions of the various classes of phospholipid found in the human erythrocyte membrane. It is, however, evident that the proportion of saturated to unsaturated fatty acids is close to one. Typically, in animal cell membranes, the saturated and unsaturated fatty acid chains are found at the 1 and 2 positions of phospholipids. There is, however, no evidence that a particular fatty acyl chain content is especially suited to a particular function. For instance, the fatty acyl chain compositions of sarcoplasmic reticulum from rabbit, rat, chicken, human and lobster are all very different (Marai & Kuksis, 1973; Lee *et al.*, 1986).

The phospholipid composition of animal cell membranes changes in response to maturation, disease, dieting and drug treatment. Changes in the phospholipid contents of animal cell membranes have been reported for a variety of disease states. These changes are most often small and of uncertain validity. Nonetheless, there are clear cases of defects in lipid metabolism having significant effects, for example defective sphingolipid metabolism in nervous tissue (Guroff, 1980) leads to an accumulation of a variety of lipids including SM (Niemann-Pick disease), gangliosides (Tay-Sachs disease) and cerebrosides (Gaucher's disease) (Lee, 1990). Modifications in membrane lipid content can also affect membrane transport function (Deuticke & Haest, 1987; Carruthers & Melchior, 1988), for example cholesterol depletion of the human erythrocyte membrane causes changes in sodium pump activity (Claret *et al.*, 1978; Lucio *et al.*, 1991).

2.2.3 The lipid bilayer

As described above, the common feature of all lipid molecules is their amphipathicity (Figures 2.3 - 2.7). This dichotomous structure is the basis of the role of lipids in

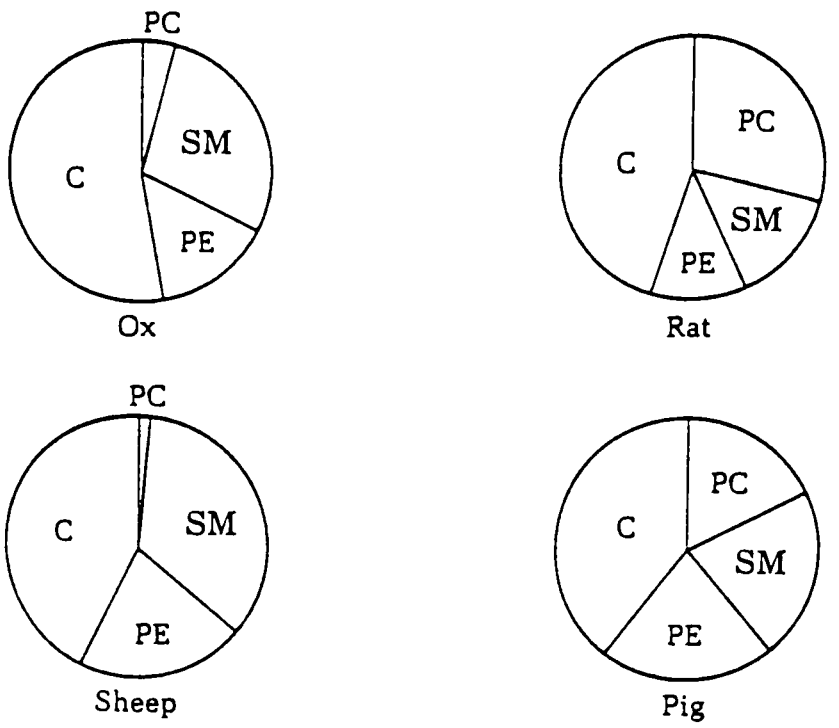


Figure 2.8 The relative contributions of individual phospholipids to the composition of erythrocyte membranes from rat, pig, ox and sheep. PC = phosphatidylcholine; PE = phosphatidylethanolamine; SM = sphingomyelin; C = cholesterol. After Quinn (1976).

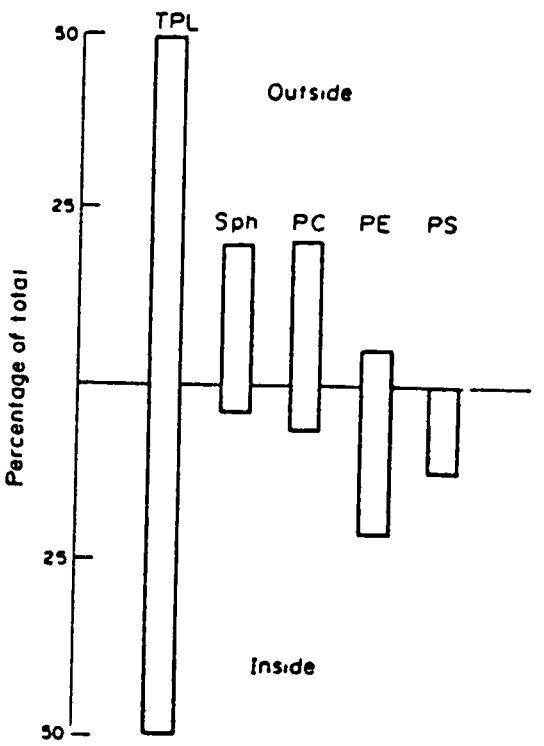


Figure 2.9 The distribution of phospholipids in the two leaflets of the human erythrocyte membrane. TPL = total phospholipid; Sph = sphingomyelin; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PS = phosphatidylserine. After Verkleij *et al.* (1973).

membrane formation; the polar end of the molecule will mix with water whilst the non-polar region will not. Thermodynamic considerations strongly favour lipids adopting a structure in which the polar headgroups interact with water on the outside of the bilayer, whilst the non-polar acyl chains are sequestered within the interior (see Figure 2.1). These bilayer sheets are the basic structural element of the membrane. Bilayer formation is a self-assembly process because no work is done to the system for the lipids to adopt this configuration; the driving-force for bilayer formation arises from the thermodynamic properties of the interaction between amphipathic lipids and water (Stryer, 1988).

Phospholipid bilayers can exist in one of two phases, depending on the temperature. At lower temperatures, a rigid gel phase may be adopted in which the acyl chains are packed into an extended, all-*trans* array, whereas at higher temperatures, the bilayer exists in a fluid, liquid phase (still two-dimensional) in which the fatty acyl chains are highly disordered with relatively free rotation about C-C bonds. If a bilayer contains only a single species of phospholipid, the fluid transition (Overath & Thilo, 1978) between the liquid and gel phases is a sharp, first-order transition, the transition temperature depending on the nature of both the headgroup and acyl chains (Lee, 1983). Introduction of one *cis* double bond into a saturated fatty acyl chain causes a marked decrease in the transition temperature; the introduction of a further double bond causes another (but smaller) decrease in the transition temperature. This effect can be understood in terms of the difficulty of packing unsaturated chains into a close-packed gel phase (see Figure 2.10). In biological systems at physiological temperatures, bilayers exist in the liquid phase. It appears that this is a requirement for cell survival: not only is the membrane 'brittle' in the gel phase, the interactions with membrane proteins are also considerably perturbed (Lee, 1990). An important property of the liquid phase is its 'fluidity'. There are several modes of mobility within the bilayer: rotational and lateral diffusions of the lipid entities and flexing of both the polar headgroup and the acyl chain. Flip-flop motion, in which a phospholipid moves from one side of the bilayer to the other, is rare because it involves the movement of the polar headgroup through the hydrophobic core (Houslay & Stanley, 1982). The infrequency of this motion facilitates the maintenance of the asymmetric phospholipid distribution. Because each bilayer phospholipid has acyl chains bonded to a relatively immobile headgroup, there is a gradient of motion within the membrane, in which both the rate and amplitude of movement increases along the fatty acyl chain from

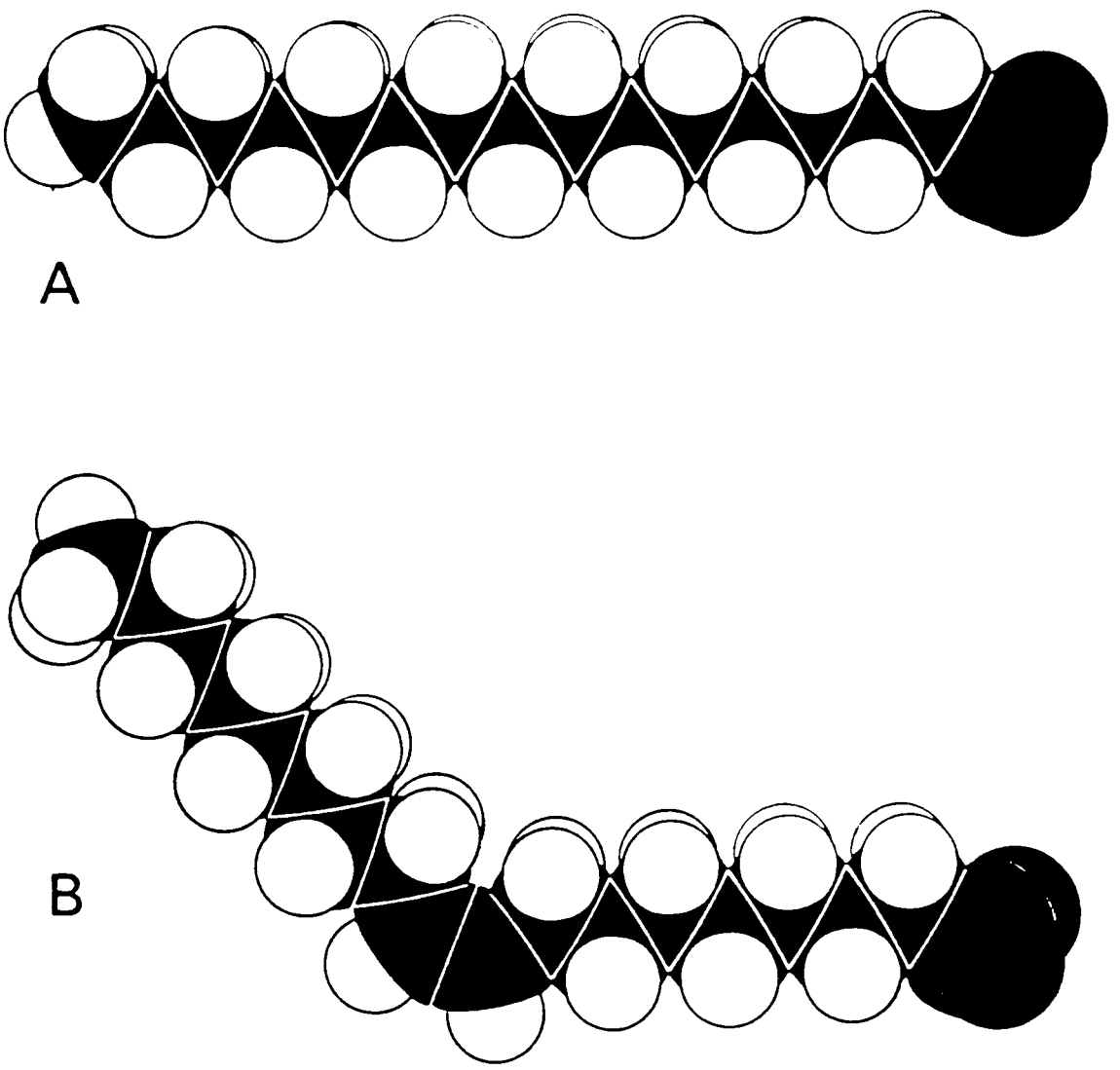


Figure 2.10 Space-filling models of (A) palmitate (C_{16}, saturated), and (B) oleate (C_{18}, unsaturated). The *cis* double bond in oleate produces a bend in the hydrocarbon chain. After Stryer (1988).

the carboxyl to the methyl end (Lee, 1983). Unsaturation affects the profile of motion within the bilayer. Because the C=C bond is relatively rigid, the area around the double bond will be more ordered than areas near the methyl end of the molecule (Seelig & Waespe-Sarcevic, 1978). With polyunsaturated chains, these effects are less marked. Addition of cholesterol to a lipid bilayer in the liquid phase decreases fluidity from its original value to one between those of the normal liquid crystalline and gel states. The rigid sterol ring system diminishes flexing of structures in the hydrophobic region of the membrane. Because cholesterol is found in the outer plasma membrane, it is commonly proposed that the biological role of cholesterol, in this context, is to reduce the membrane permeability to small solutes by increasing the viscosity of the membrane (Lee, 1990).

2.2.4 Types of membrane proteins

Whilst the lipid compartment of the membrane provides the primary permeability barrier between cytoplasmic and extracellular compartments, proteins confer distinctive functions. Membranes vary in their relative proportions of lipid and protein. A 'typical' plasma membrane has a molar ratio of about 100:1, about half the membrane surface being occupied by protein and about half by lipid (Lee, 1990). Membrane proteins are either extrinsic (peripheral) or intrinsic (integral) (see Figure 2.11). Extrinsic proteins appear to be attached to the membrane by charge-charge interactions with either other membrane proteins or charged phospholipids. They can be removed by washing with buffers of high ionic strength. Intrinsic proteins are more firmly embedded in the membrane and harsher procedures are required to remove them from the membrane, such as detergent treatment. Detergents are amphipathic but have more polar groups than the membrane lipids and are hence more water-soluble. The choice of the best detergent for a particular isolation remains very much a matter of trial and error (Racker, 1985). Intrinsic membrane proteins generally span the lipid bilayer, although it is possible that some only penetrate part of the way into the hydrophobic core (Figure 2.11). The region of the membrane protein in contact with the fatty acyl chains in the interior of the bilayer must be hydrophobic, in contrast with the regions of the protein exposed to water which are predominantly polar.

2.2.5 Membrane protein structure

Functional asymmetry of the membrane is a result of asymmetric distribution of membrane proteins. Intrinsic membrane proteins are arranged such that all copies face

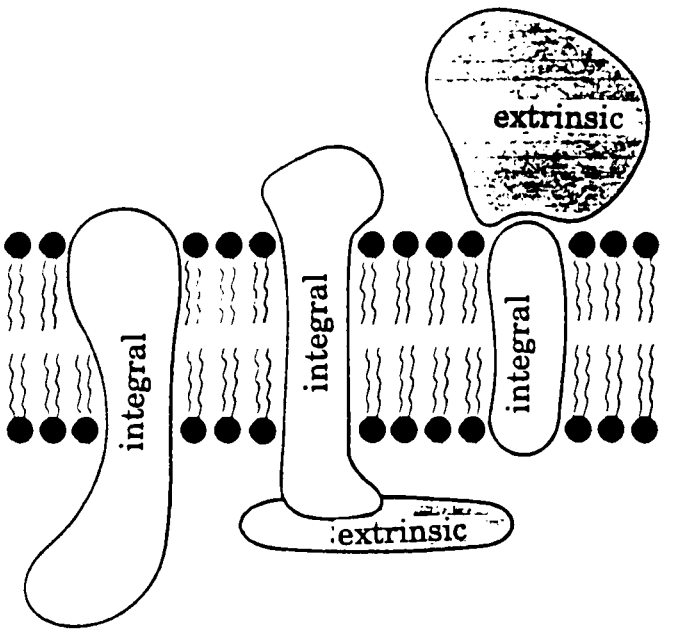


Figure 2.11 Classification of membrane proteins. Intrinsic (integral) membrane proteins interact extensively with the hydrophobic region of the bilayer. Nearly all integral proteins span the bilayer. Extrinsic (peripheral) membrane proteins bind to the surface of integral membrane proteins.

the same way and extrinsic proteins exist on only one side of the membrane. This asymmetry has been established for many membrane proteins using membrane-impermeant labelling reagents or proteolytic digestion techniques (Maddy, 1976).

Establishing three-dimensional protein structure is an arduous task, using techniques such as freeze-fracture electron microscopy and X-ray diffraction of protein crystals. However, consideration of the thermodynamic interactions that different portions of an integral membrane protein undergo with water, membrane lipid headgroups and the hydrophobic interior of the bilayer, serves as a predictive basis for protein structure. The membrane penetrant part of a integral membrane protein must expose only hydrophobic side-groups to the hydrophobic milieu of the bilayer. It appears that the membrane-spanning portion of many integral membrane proteins is an alpha-helix running perpendicular to the membrane surface (Lee, 1990). An alpha-helical structure is favoured within the hydrophobic core of the membrane because the polar regions of the peptide backbone are maximally and internally hydrogen-bonded. Transfer of a hydrogen-bonded C=O and N-H pair in a protein backbone from water to a non-polar environment gives a favourable free energy ΔDG_{trans} of -5.9 kJ(mol.)$^{-1}$ compared to the unfavoured change +17.1 kJ(mol.)$^{-1}$ for a non-hydrogen bonded pair (Martonosi, 1985). Because of the lack of directly-determined protein structures, much of the modelling of tertiary structure is based on predictions from amino acid sequences (Chou & Fasman, 1974; Kabsch & Sander, 1983). Classical methods of amino-acid sequencing are generally difficult for membrane proteins, particularly for the hydrophobic regions. However, methods involving the sequencing of cDNA cloned from the relevant messenger RNA are revealing. Derivation of a possible structural model for such a protein starts with a search for very apolar segments, long enough to span a lipid bilayer (typically c. 24 - 26 amino acids long). A quantitative measure of the relative hydrophobicities of amino acid side-chains can be made by consideration of the free energies of transfer of the side chain from water to a non-covalent solvent such as ethanol or dioxane (Reithmeier, 1985). These energies can be calculated in terms of the solvent-accessible surface area of the side chain and the polarity of the atoms comprising the side chain. The free energy of transfer of a particular residue from a random coil structure in water to an α-helical structure in a lipid bilayer can then be calculated in terms of the accessible surface area, the unsatisfied hydrogen bonds and the free energy required to neutralise charged groups (Guy, 1984; Reithmeier, 1985; Argos

& MohanaRao, 1986). Hydrophobic sequences thought to span the membrane are often flanked by charged amino acids. However, not all hydrophobic α-helical segments need span the membrane. Furthermore, the presence of charged amino acids in an otherwise hydrophobic sequence does not preclude this sequence being buried within the membrane since charged amino acids could interact with other suitably charged residues on adjacent alpha-helical segments, or line a water-filled pore. Furthermore, other motifs (eg β-sheets) can allow membrane penetration, with appropriate internal shielding of hydrophilic portions of the peptide chain (Lee, 1990). Structural predictions must be recognised for what they are (predictions) and treated with caution. For some membrane proteins, the membrane-penetrant sequences are likely simply to act as anchors, tagging the protein to the membrane, with little or no effect on the function of the protein.

2.2.6 Lipid-protein interactions

As is clear from the outline above, membrane composition is complex and variable. A typical membrane may contain several hundred chemically distinct species of lipid, and the description of membrane function at the molecular level would be extremely difficult. Equally arguable is that the diversity of lipids, within limits needed for a functional membrane, means that precise overall lipid compositon is unimportant and merely reflects the current availability of fatty acids, headgroups *etc.* in the cell. Certainly, many chemically distinct lipid species can be used to produce a functionally competent membrane. However, the functions of various membrane proteins do appear to be sensitive to changes in the lipid environment (section 2.2.2).

2.3 MEMBRANE TRANSPORT

There are many comprehensive reviews on this topic (Christensen, 1975; Ellory & Lew, 1977; Bonting & de Pont, 1981; Ellory & Young, 1982; Stein, 1967, 1986, 1989, 1990; Jain, 1988; Ellory *et al.*, 1988; Gennis, 1989; Sacks & Fleischer, 1989; Klingenberg, 1989; Aronson, 1990). As already noted, the hydrophobic interior of the lipid bilayer forms an impermeant barrier to most polar species, such as glucose, ATP, amino acids, nucleosides and ions. The movement of these molecules across a membrane must therefore be facilitated by specific transport processes mediated by intrinsic membrane proteins. These membrane proteins are collectively known as *permeases* and each exhibit some form of substrate specificity.

Transport of a small molecule into a cell can be either passive (involving no energy input) or active (coupled either directly to chemical energy input (by ATPase activity; known as primary active transport) or indirectly to movement down its electrochemical gradient of a coupled substrate(s) (known as secondary active transport)).

2.3.1 Passive transport mechanisms

Passive transport relies on diffusion of the substance down its electrochemical gradient with no expenditure of energy. For certain small non-polar molecules which have an appreciable solubility in the lipid bilayer (eg ethanol), movement of the molecule across the membrane can be by simple passive diffusion. The rate of diffusion depends on the partition coefficient of the substance into the membrane (Stein, 1986). Polar substances cannot cross the membrane effectively by dissolving in the hydrophobic interior of the bilayer. However passive movement of a substance across the membrane is still possible if the membrane has a permease for that substance, by a process known as facilitated diffusion. Two types of transporter exist that mediate passive transport, the pore/channel and the carrier (Table 2.3).

(i) Pores and channels. Pores and channels consist of proteins that form a tunnel through the membrane, in which solute binding-sites are accessible from either side at the same time. Pores are generally defined as having poor selectivity, and tend to discriminate solutes primarily on the basis of their size. Channels, however, can be highly selective (not only on the bases of size and charge, but also on solute interaction energies with both water and the channel interior), and can mediate the movement of ions and organic substrates (Kirk *et al.*, 1994). Furthermore, channels have different states of operation, open or closed, controlled by gating mechanisms responsive to voltage (Labarca *et al.*, 1980), ligands (Loewenstein, 1984), membrane stretch (Guharay & Sachs, 1984), and other stimuli (Raftery *et al.*, 1983).

(ii) Carriers. Carriers form a distinct class of membrane transporter from channels/pores and primary active transporters. Simple carriers are facilitated diffusion systems. However, more complex carriers can also obligatorily couple the movement of different substrates, and hence function as secondary active transport systems (see section 2.3.2).

Carriers acting to facilitate diffusion (ie mediating a solely passive process) allow net flux of a substrate down its electrochemical gradient. One of the best studied of these systems is the glucose transporter in erythrocytes and other cells, termed GLUT1 (Holman

Channels	Carriers
tend not to exhibit saturation kinetics	tend to exhibit saturation kinetics (of Michaelis-Menten type)
do not exhibit *trans*-stimulation	may exhibit *trans*-stimulation under appropriate conditions
tend to have a higher turn-over number (c. 10^6 molecules substrate per second)	tend to have a lower turn-over number (10 - 2000 molecules substrate per second)
have only limited specificity	have a more precisely defined specificity
do not appear to undergo conformational change during the transport event	appear to undergo conformational change during the transport event

Table 2.3 Differentiating between carrier- and channel-mediated transport events. Numerical data are from Stein (1990).

et al., 1990; Gould *et al.*, 1991; Lienhard *et al.*, 1992). The glucose transporter exemplifies a family of related proteins, performing specialised roles at the cellular level. Five subtypes of the glucose transporter have been identified to date, each having kinetic characteristics suited to a particular function. Alterations in GLUT-mediated transport are believed to play a role in disease states associated with insulin resistance.

A simple kinetic model for a carrier is shown in Figure 2.12. Substrate is moved across the membrane from the *cis* to the *trans* compartment by the carrier C. Intrinsic to its property of facilitating diffusion is its ability to change binding-site orientation (by conformational change) from one side of the membrane to the other. The carrier C has two conformational states depending on whether the substrate binding-site is facing the *cis* or *trans* compartment. Carrier-mediated and channel/pore-mediated transport can often be distinguished on a kinetic basis (see Table 2.3).

2.3.2 Active transport mechanisms

Active transport is defined as movement of a substrate against its electrochemical gradient. Energy is required for the movement of solute by this method, which usually results in an accumulation of substrate on one side of the membrane.

(i) Primary active transporters. These are ion pumps, such as the Na^+/K^+ ATPase (Skou & Norby, 1979; Sweadner & Goldin, 1980; Stein, 1990), wherein translocation is directly coupled with an energy-yielding reaction. The action of such ion pumps generates or maintains electrochemical gradients upon which secondary active transporters depend. The movement of one (driving) solute down an electrochemical gradient established by primary active transport, results in the obligatorily coupled movement of the other (driven) solute(s) up its electrochemical gradient.

(ii) Secondary active transporters. These operate according to the principles of carrier action but obligatorily couple the movements of more than one substrate (see Figure 2.13). Such cotransporters, within the limitations imposed by the deviations from ideal behaviour, ie slippage (movement of substrate through the carrier by a non-ideal pathway) and leakage (movement of substrate by a pathway in parallel to the cotransporter), can therefore cause the net movement of one substrate down and another substrate up their electrochemical gradients (Stein, 1989). Models of this type of system are shown in Figure 2.13. Such systems may be either antiporters (countertransporters) or symporters (cotransporters), rather than only symporters (Figure 2.13), depending on the relative

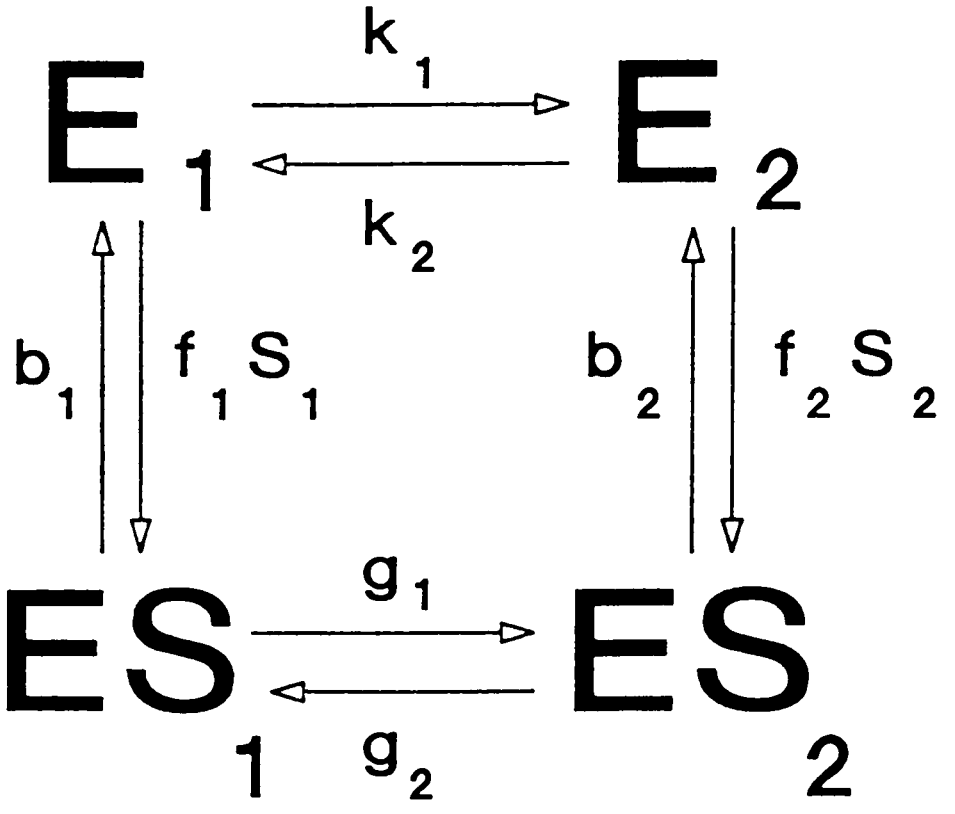


Figure 2.12 Kinetic model of carrier function. In its external position (E_1), the carrier (E) binds the substrate (S) forming the carrier-substrate complex (ES_1). The carrier then reorientates towards the inside (ES_2), where the substrate, S, is released, freeing the carrier. The subscripts *1* and *2* refer to sides 1 and 2 of the membrane. The letters *f* and *b* are the rate constants for formation and breakdown of the ES complex, *k* for the transformations of the carrier that allow access of substrate to its binding-sites at sides 1 and 2, whilst *g* describes that for the conformational changes of ES between the ES_1 and ES_2 forms. After Stein (1990).

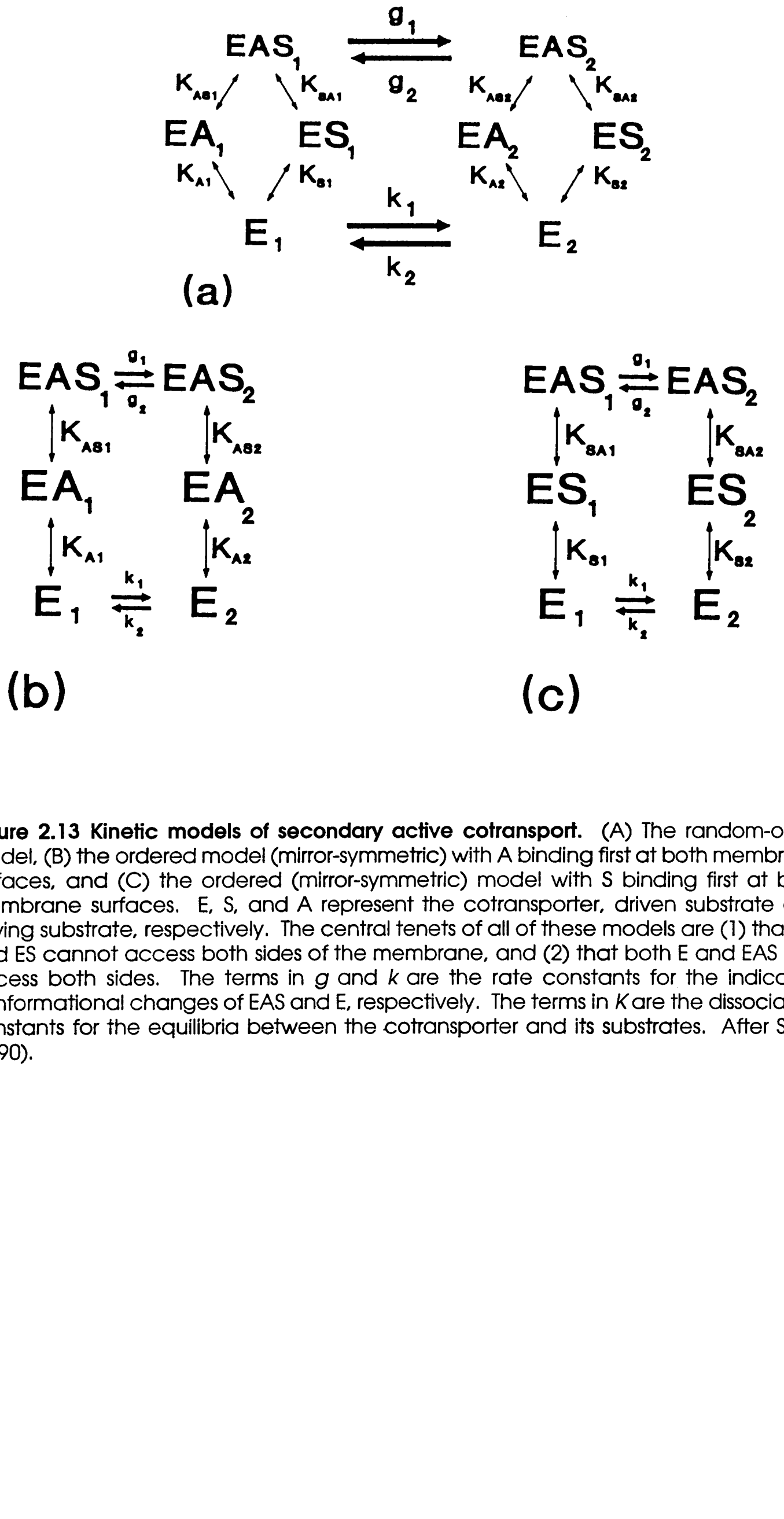


Figure 2.13 Kinetic models of secondary active cotransport. (A) The random-order model, (B) the ordered model (mirror-symmetric) with A binding first at both membrane surfaces, and (C) the ordered (mirror-symmetric) model with S binding first at both membrane surfaces. E, S, and A represent the cotransporter, driven substrate and driving substrate, respectively. The central tenets of all of these models are (1) that EA and ES cannot access both sides of the membrane, and (2) that both E and EAS can access both sides. The terms in *g* and *k* are the rate constants for the indicated conformational changes of EAS and E, respectively. The terms in *K* are the dissociation constants for the equilibria between the cotransporter and its substrates. After Stein (1990).

directions of the driving and driven solutes.

2.3.3 Membrane receptors

Membrane receptors are macromolecules that have the dual function of recognising a chemical signal and initiating a biological response (de Robertis & de Robertis, 1987). In many cases, a ligand binds to the receptor site to initiate a response. These receptors may be:

(i) Protein receptors. Transmembrane proteins are well adapted to a receptor role as they can combine well-defined three dimensional extracellular structures, possession of intracellular sites, and capability of undergoing conformational changes on binding of a substrate. Furthermore, such protein receptors may be up- or down-regulated in response to genetic control (Stryer, 1988).

(ii) Ganglioside receptors. Ganglioside structures have been defined briefly above (section 2.2.1). They are synthesized in the Golgi apparatus and are located in the plasma membrane with their ceramide moiety inserted into the lipid bilayer and their hydrophilic, ionogenic group faced predominantly towards the outer environment of cells (Singer, 1974; Fishman & Brady, 1976; Miller-Podraza *et al.*, 1982). The arrangement of gangliosides within membranes and observations concerning ganglioside interactions suggest that gangliosides are involved in receptor-mediation of bioactive factors (Fishman, 1982; Hakomori, 1984, 1986; Thompson & Tillack, 1985; Bock *et al.*, 1988). Many data have accrued pertaining to the role of gangliosides in toxin-cell interaction (Table 2.4).

However, although many such interactions are documented, it is rare that the documentation is so complete that the specific ganglioside can be unreservedly proposed as the distinct single receptor entity with the exceptions of cholera toxin (G_{M1}) (Moss & Vaughan, 1988a,b), and *C. perfringens* δ toxin (G_{M2}) (Jolivet-Reynaud *et al.*, 1993).

Gangliosides interact with a variety of other proteins eg serum albumin (Venerando *et al.*, 1982). There are several possibilities for ganglioside-protein interaction (after Hakomori, 1984): (a) gangliosides themselves are receptor entities (as for cholera toxin), (b) gangliosides are secondary auxiliary receptors; primary receptors could be either "ganglioproteins" or other high affinity proteins; eg *E. coli*-derived LT-1 has (in addition to ganglioside binding-sites) a second higher affinity binding-site that is comprised of galactoprotein (Holmgren *et al.*, 1982, 1985; Zemelman *et al.*, 1989; Griffiths & Critchley, 1991), (c) gangliosides act as co-factors of protein receptors eg G_{M3} for Paul-Bunnell

bacterial toxin	interactant claimed	references
Vibrio cholerae cholera toxin	G_{M1}	van Heyningen *et al.*, 1971 Holmgren *et al.*, 1973
Aeromonas sobria cytotonic enterotoxin	G_{M1}	Gosling *et al.*, 1993
Clostridium tetani tetanus toxin	G_{T1b}, G_{Q1b}, G_{D1b}	van Heyningen, 1963 Ledley *et al.*, 1977 Holmgren *et al.*, 1980 Lazarovici & Yavin, 1985 Critchley *et al.*, 1986 Schiavo *et al.*, 1991 Schengrund *et al.*, 1991
Clostridium botulinum botulinus toxin	G_{T1b}	Simpson & Rapport, 1971 Kitamura *et al.*, 1980 Schengrund *et al.*, 1991
Staphylococcus aureus α-toxin	sialosylparagloboside	Kato & Naiki, 1976 Linder & Bernheimer, 1991
Vibrio parahaemolyticus thermostable direct haemolysin	G_{D1}, G_{T1a}	Takeda *et al.*, 1975, 1976
Shigella dysenteriae Shiga toxin	G_{b3}	Jaciewicz *et al.*, 1986 Lindberg *et al.*, 1987
Escherichia coli Verotoxins 1, 2 (VT1, VT2)	G_{b3}	Lingwood *et al.*, 1987 Cohen *et al.*, 1987 Waddell *et al.*, 1988, 1990
Escherichia coli heat-labile toxin 1 (LT-1)	G_{M1}	Fishman, 1982 Holmgren *et al.*, 1982, 1985
Escherichia coli heat labile toxin 2a	G_{M1}, G_{D1a}, G_{D1b}, G_{D2} G_{T1b}, G_{Q1b}	Holmes *et al.*, 1986 Fukuta *et al.*, 1988
Escherichia coli heat labile toxin 2b	G_{D1a}, G_{T1b}	Fukuta *et al.*, 1988
Bordetella pertussis AC toxin	neuraminidase-sensitive ganglioside	Gable *et al.*, 1985 Gordon *et al.*, 1989
Clostridium perfringens δ toxin	G_{M2}	Alouf & Jolivet-Reynaud, 1981 Jolivet-Reynaud & Alouf, 1983 Cavaillon *et al.*, 1986 Jolivet-Reynaud *et al.*, 1988, 1989, 1993

Table 2.4 Interaction of bacterial toxins with glycolipids. Refer to Table 2.2 and Figures 2.5 and 2.6 for description of ganglioside structure.

antigen (Watanabe *et al.*, 1979), (d) gangliosides act as modulators of protein receptors, on the assumption that receptors are allosteric and have gangliophilic sites (Bremer & Hakomori, 1984), and (e) ganglioside interactions with bioactive factors are non-specific, incidental phenomena.

Evidence suggests that gangliosides may be associated with cytoskeletal residues (Streuli *et al.*, 1981; Hagmann & Fishman, 1982). For example, Sahyoun *et al.* (1981) have reported that choleragen bound to rat erythrocytes was not readily extracted by non-ionic detergents and remained associated with a cytoskeletal residue.

Neuraminidase, an enzyme secreted by a variety of bacteria (eg *V. cholerae* (Burnet & Stone, 1947; Ada *et al.*, 1961; Dzeniek *et al.*, 1972; Kabir *et al.*, 1984), *C. perfringens* (Burton, 1963; Cassidy *et al.*, 1965), and *Aeromonas hydrophila* (Kabir *et al.*, 1984)), acts to cleave terminal neuraminic acid residues from glycoproteins, gangliosides and other glycolipids. Purified preparations of this enzyme have been used as an experimental tool to modify membrane structure (Lauf *et al.*, 1971; Derevitskaya *et al.*, 1978; Watanabe *et al.*, 1990; Stein *et al.*, 1987). Using this approach, the nature of some bacterial toxin receptors have been investigated, for example: (a) *C. perfringens* δ toxin (Jolivet-Reynaud & Alouf, 1983) and *C. tetani* tetanus toxin (Lazarovici & Yavin, 1985) where the target cell was treated, and (b) *E. coli* heat-labile toxins where gangliosides themselves were treated (Fukuta *et al.*, 1988).

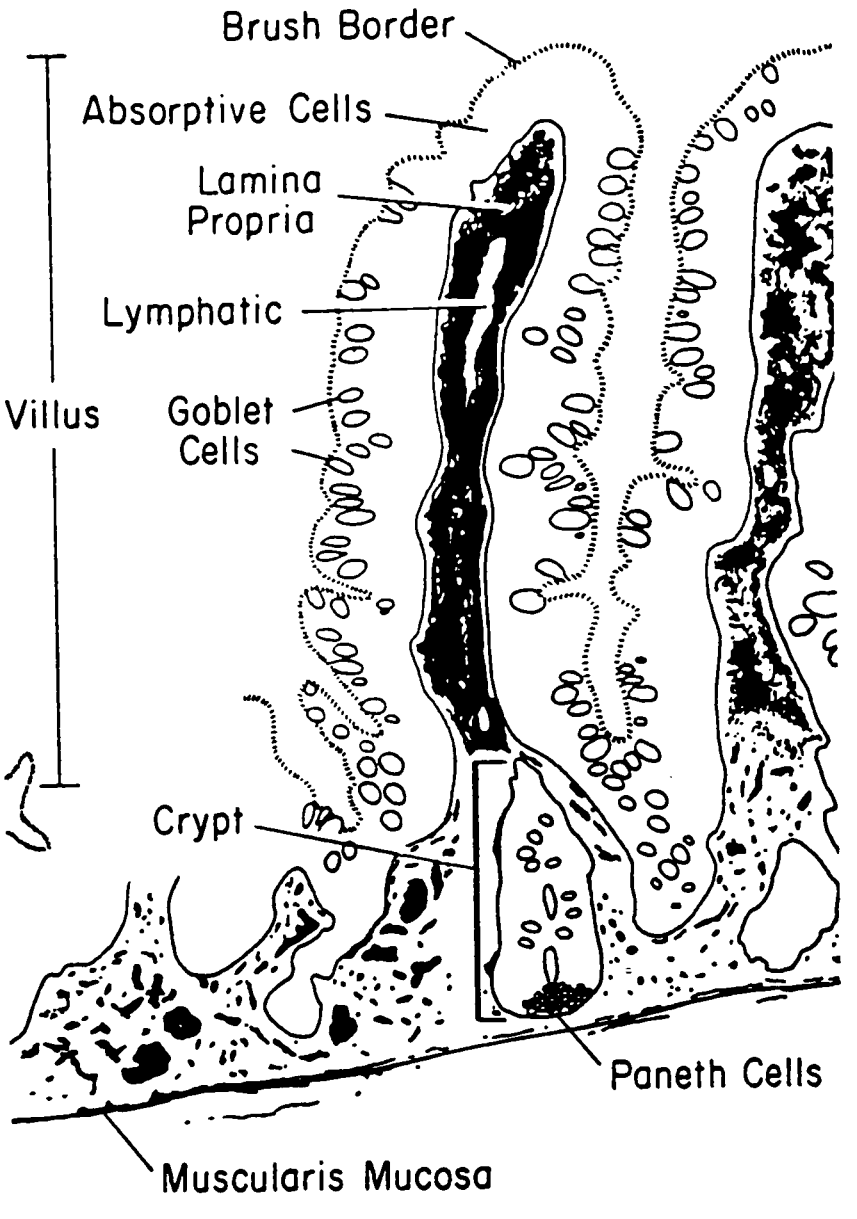
2.4 INTESTINAL STRUCTURE AND FUNCTION

In order to understand pathophysiological change, a grounding in normal structure and function is needed. Here, an overview of the seminal features of fluid and electrolyte absorption is presented (Johnson, 1985; Schultz, 1991), particularly with regard to providing the physiological framework for the action of several bacterial toxins discussed later (section 2.5). The structure of the small intestine is well-treated elsewhere (Ham & Cormack, 1987; Madara, 1991) and is briefly outlined in Figure 2.14. Before considering the processes underlying electrolyte and water transport in the small intestine, it is useful to examine the processes underlying ion transport in other, model epithelia.

2.4.1 Models of epithelial ion transport

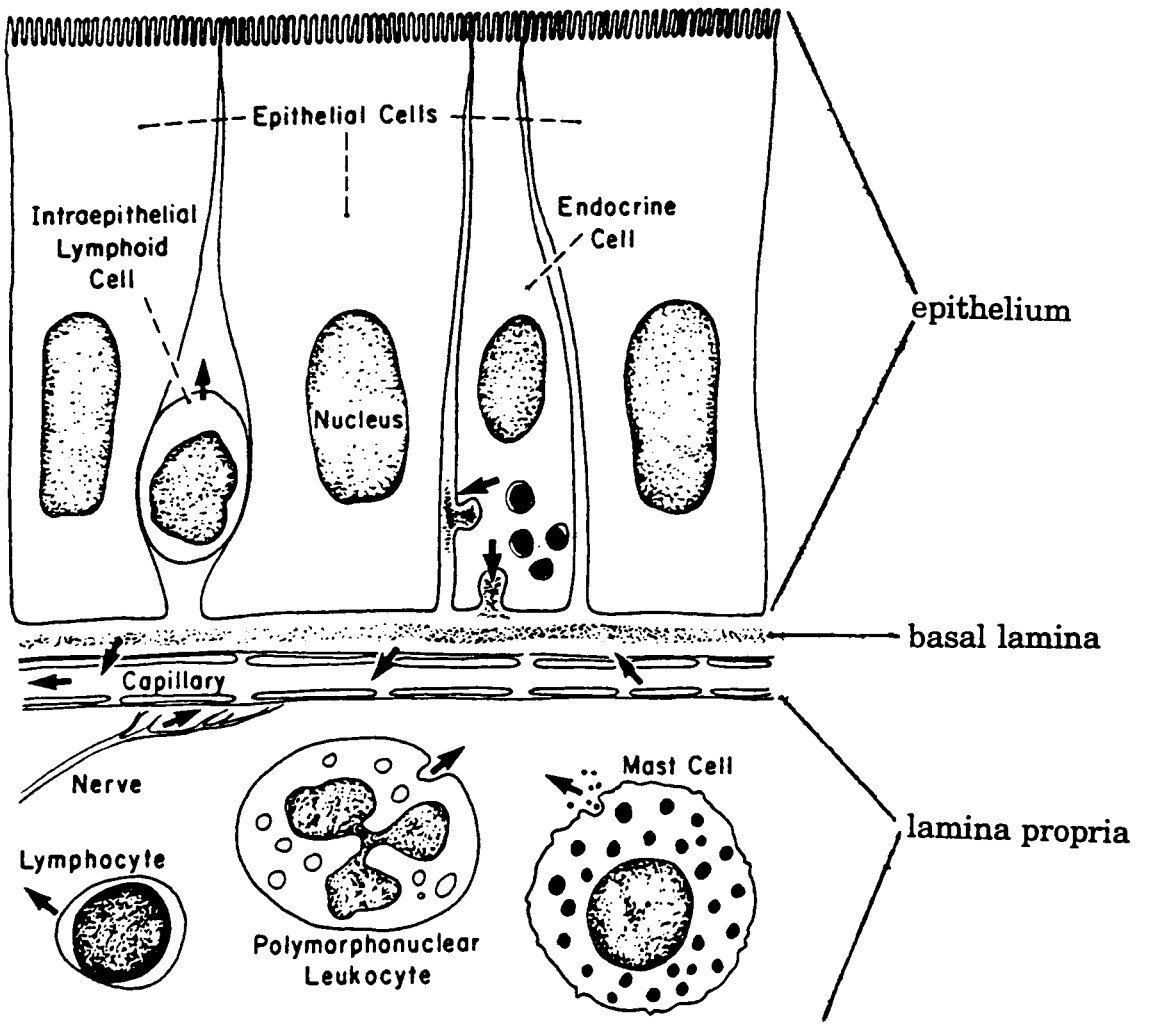
Working physiological models of epithelial ion transport have been developed from data produced over the last half-century (Ussing & Zerahn, 1951; Koefoed-Johnson & Ussing,

A



B

lumen: contains dietary intake and alimentary secretions



From Madara (1991)

See over for legend

previous page:

Figure 2.14 Structural features of the small intestine.

(A) Structure of the small intestinal mucosa. The epithelium of the small intestine rests on a layer of connective tissue, termed the *lamina propria*, which is itself supported by a thin smooth muscle layer, the *muscularis mucosa*. Together the epithelium, *lamina propria* and *muscularis mucosa* form the mucosa and are underlaid by a deeper layer of connective tissue known as the *submucosa*. The surface of the *lamina propria* forms many projections which increase the small intestinal surface area by about 10 x. The depressions between villi are termed crypts, also lined by confluent epithelial cells. Aside from mechanically supporting the epithelial cells, the *lamina propria* also contains the blood and nerve supply to the villus and epithelium. Each villus also contains a centrally located lymphatic lacteal with a highly permeable endothelial lining for drainage of escaped plasma proteins and uptake of particulate matter such as chylomicrons. Covering the surface of the *lamina propria* is an amorphous sheet, the basal lamina, on which the epithelium rests directly.

(B) Schematic illustration of epithelial cells and associated microenvironment. Epithelial cells form a confluent layer at the surface of the small intestinal mucosa. It is the properties of these cells that confer the special ion-motive properties on the small intestine. These cells are connected to each other by junctional complexes of three parts: (i) the *macula adherens*, (ii) the *zonula adherens*, and (iii) the *zonula occludens* (tight junction). The *zonula occludens* serves to (a) limit transport by the paracellular pathway (see Madara (1991) for in depth review), and (b) define the apical and basolateral membrane domains which have different membrane transport properties. It is this asymmetry that underlies the vectorial transport of solutes and electrolytes (see Figures 2.15 and 2.17) by the small intestinal mucosa. Absorptive cells display a striking brush border composed of uniform microvilli measuring 1 μm in height at the luminal surface, expanding the apical surface area by 1 - 1.5 orders of magnitude. Undifferentiated crypt cells also have microvilli but these are shorter and less dense than those of the apical membrane of the absorptive villus cells.

1958; Field, 1981; Diamond, 1982; Taylor & Palmer, 1982; Harvey *et al.*, 1988; Taylor, 1989; Schultz & Hudson, 1991; Turnheim, 1991). Models seek to explain (i) the arrangement of membrane transporters (section 2.3) in distinct apical and basolateral membranes, (ii) the function of tight junctions and the paracellular route, (iii) the function of the epithelium in response to electrochemical gradients, (iv) intrinsic feedback and feedforward stimuli, and (v) second messenger control.

The paradigm system for transepithelial Na^+ movement is described by the Koefoed-Johnsen and Ussing (KJU) model (Koefoed-Johnsen & Ussing, 1958). It is couched in terms of three membrane transport processes and their restriction to one or other face of the epithelium (Figure 2.15A): (i) passive movement of Na^+ into the cell across the apical cell membrane, (ii) passive exit of K^+ from the cell across the basolateral cell membrane, and (iii) active Na^+/K^+ exchange over the basolateral cell membrane. The appeal of this model lies in its simplicity, since it postulated no new processes beyond those membrane transport events already known to exist. The original model, though substantially correct, has nonetheless evolved considerably in recent years (Figure 2.15B,C,D; Diamond, 1982; Taylor & Palmer, 1982; Turnheim, 1991).

A minor modification of the model came from the discovery that the basolaterally sited Na^+/K^+ ATPase does not swap 1 Na^+ for 1 K^+ as originally assumed, but rather exchanges 3 Na^+ for 2 K^+ (Nielson, 1979; Figure 2.15B), as is also true for non-epithelial cells (Eaton *et al.*, 1982).

The KJU model ignores the possible importance of the paracellular pathway, anatomically equivalent to the tight junctions between cells. This route of water and ion transport is not important in tight epithelia (such as frog skin, mammalian and amphibian urinary bladders, and stomach), where the tight junctions are tight and essentially impermeant to ions, which can therefore only traverse the epithelium via the cellular pathway. However, in leaky epithelia (such as renal proximal tubule, small intestine and gall-bladder), the tight junctions are leaky and are the major route for passive transepithelial ion movement (Figure 2.15C). Tight and leaky epithelia also differ in the way in which Na^+ enters the epithelial cell across the apical membrane. In tight epithelia, Na^+ enters by an ion channel (Fuchs *et al.*, 1977; Li *et al.*, 1982); in contrast, in leaky epithelia, Na^+ entry is coupled to the (symport) entry of a partner (Cl^- or a non-electrolyte such as glucose or an amino acid) or to (antiport) exit of H^+ in parallel with Cl^-/HCO_3

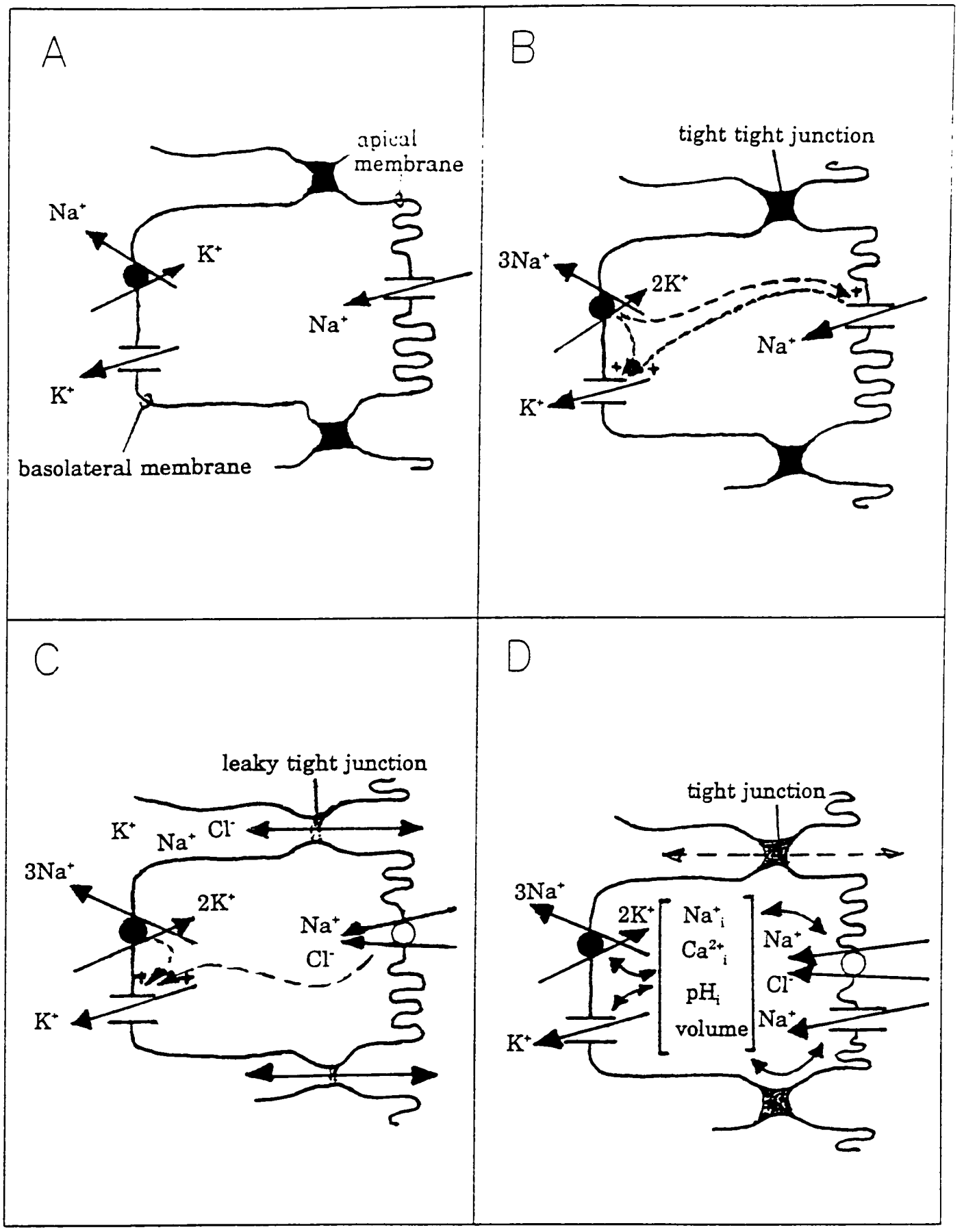


Figure 2.15 Evolution of the Koefoed-Johnsen Ussing model of epithelial transport. (A) As originally postulated in 1958. (B) Present form of the model as applied to a tight epithelium. (C) Present form of the model as applied to a leaky epithelium. (D) Present form of the model as applied to an intermediate epithelium with candidate intrinsic regulators.

exchange (Schultz, 1986).

Another development of the KJU model arose from consideration of the dynamics of the process of transepithelial Na^+ movement, in particular the mechanisms responsible for coordination of events occurring at the apical and basolateral membranes (Figure 2.15D). The first indication of 'cross-talk' between membranes came from a study (MacRobbie & Ussing, 1961) in which cell-swelling was used as an indirect measurement of ion entry in the frog skin; inhibition of the basolateral Na^+/K^+ ATPase reduced the ionic permeability of both basolateral and apical membranes. The converse type of cross-talk (regulation of basolateral membrane function by events at the apical membrane) has also been demonstrated. For example, in frog and toad urinary bladders (examples of tight epithelia), amiloride blockade of apical Na^+ channels caused a decreased basolateral K^+ conductance as rapidly as apical Na^+ conductance (Davis & Finn, 1982). In the leaky epithelium of *Necturus maculosus* small intestine (Gunter-Smith *et al.*, 1982), addition of arginine or glucose (solutes cotransported with Na^+) caused a rapid increase in apical conductance and then a slow increase in basolateral conductance. Furthermore, basolateral K^+ conductance appears to be regulated by the activity of the basolateral Na^+/K^+ ATPase (Higgins, 1977; Helman *et al.*, 1979; Schultz, 1981; Gunter-Smith *et al.*, 1982). Several factors are implicated in <u>intrinsic</u> control of transcellular Na^+ movement by 'communication events' (both feedback and feedforward) between the apical and basolateral membranes, and between separate transporters within the basolateral membrane. These include (i) $[Na^+]_i$, (ii) $[Ca^{2+}]_i$, (iii) $[H^+]_i$, and (iv) cell volume (Figure 15B), and are discussed in more detail below. The second messengers cAMP and cGMP are also implicated in control events (Field, 1981), but their action tends to be viewed as the intracellular response to <u>extrinsic</u> control <u>eg</u> neural and hormonal factors (Taylor & Palmer, 1982) or bacterial toxins (section 2.5).

A model based on the KJU paradigm has been developed for cellular Cl^- secretion (Figure 2.15D; Welsh, 1987; Liedtke, 1989; Field, 1993; Simmons, 1993) in which the pump-leak combination (Na^+/K^+ ATPase + K^+ channel) is sited basolaterally, along with an electroneutral, loop diuretic-sensitive, $Na^+/K^+/2Cl^-$ cotransporter. A Cl^- channel is the predominant functional feature of the apical membrane (Field, 1993; Simmons, 1993), through which Cl^- diffuses from the cell down its electrochemical gradient.

(i) Intracellular Na^+. Assuming that the sodium pump (Na^+/K^+ ATPase) is uninhibited

and unsaturated, then $[Na^+]_i$ in the physiological range can be an important determinant of Na^+/K^+ ATPase activity (de Weer, 1985). Raising $[Na^+]_i$ by permeabilising the apical membrane causes the expected increase in pump rate or net transcellular Na^+ flux (Graf & Giebisch, 1979; Halm & Dawson, 1983; Lewis & Wills, 1983). However, pump rate is not uniquely determined by $[Na^+]_i$ and $[K^+]_s$ but is also influenced by a number of other factors (Hildman *et al.*, 1982; Schultz & Hudson, 1991) including $[K^+]_i$ (Garrahan & Glynn, 1967; Hoffman & Forbrush, 1983), $[Ca^{2+}]_i$ (Hoffman *et al.*, 1984; Yingst, 1988), $[H^+]_i$ (Eaton *et al.*, 1984; Hoffman, 1985), the intracellular ADP/ATP ratio (Hoffman *et al.*, 1981; Kennedy *et al.*, 1986), and the electrical potential difference across the membrane (de Weer *et al.*, 1988).

Studies on tight epithelia (Essig & Leaf, 1963; Biber, 1971; Erlij & Smith, 1973; Finn, 1975; Lewis *et al.*, 1976; Turnheim *et al.*, 1978) indicate that inhibition of the sodium pump results in a decreased apical membrane sodium permeability. Because this effect was dependent on the mucosal Na^+ concentration (and hence presumably $[Na^+]_i$), a negative feedback influence from basolateral events to the apical membrane was proposed in which changes to $[Na^+]_i$ secondary to altered pump rates were implicated as the effector (Lewis *et al.*, 1976). However, subsequent investigations including electrophysiological measurements in rabbit and toad urinary bladders (Eaton, 1981; Palmer, 1985) and studies of isotopic Na^+ influx into toad bladder apical membrane vesicles (Chase & Al-Awqati, 1983; Garty *et al.*, 1987) suggest that changes in $[Na^+]_i$ *per se* have little effect on apical membrane Na^+ permeability (Figure 15B).

(ii) Intracellular Ca^+. Many studies indicate that sodium absorption is partially inhibited under experimental conditions in which cytosolic Ca^{2+} is raised (reviewed in Windhager & Taylor, 1983; Taylor & Windhager, 1985; for the role of Ca^{2+} in volume regulatory changes in epithelia, see below). For example, increasing serosal Ca^{2+} caused a decrease in the frog skin apical membrane amiloride-sensitive Na^+ permeability. Furthermore, the calcium ionophores A23187, X357A and ionomycin decreased net transport across the toad urinary bladder (Taylor, 1975; Wiesmann *et al.*, 1977; Ludens, 1978; Taylor *et al.*, 1987), frog skin (Erlij *et al.*, 1986), and isolated perfused rabbit kidney collecting tubules (Friedman *et al.*, 1981; Frindt & Windhager, 1983, 1986). Such findings suggest that cytosolic Ca^{2+} can influence transcellular Na^+ transport in both tight and leaky epithelia. In apical membrane vesicles of the tight epithelium of the toad bladder, amiloride-

sensitive $^{22}Na^+$ influx (via apical membrane sodium channels) decreased in the presence of submicromolar free Ca^{2+} concentrations ($K_{1/2}$ c. 500 nM) (Chase & Al-Awqati, 1983). These findings are consistent with results of electrophysiological and vesicle studies on the toad bladder (Garty & Asher, 1985; Palmer, 1985), and patch clamp studies on the apical membranes of rat cortical collecting tubule cells (Palmer & Frindt, 1987), which suggest indirect modulation of sodium channel activity by cytosolic Ca^{2+}. However, there is also evidence for a direct, pH-sensitive, Ca^{2+}-channel interaction (Garty *et al.*, 1987).

Evidence of a role for $[Ca^{2+}]_i$ in the regulation of basolateral K^+ permeability is rather more advanced (see below, also). The Ca^{2+}-activated K^+ channel was first identified in human erythrocytes (Gardos, 1958) and has now been identified in a variety of cell-types (Schwarz & Passow, 1983; Petersen & Maruyama, 1984). Boyd and Walley (1984) produced evidence for the existence of a Ca^{2+}-activated K^+ channel in the basolateral membrane of the vascularly perfused frog small intestine, by measuring $^{86}Rb^+$ efflux into both luminal and basolateral compartments in response to Ca^{2+}, using serosal A23187 to manipulate $[Ca^{2+}]_i$. Evidence also indicates that the increase in basolateral K^+ permeability that underlies Cl^- secretion is a result of increased activity of Ca^{2+}-activated K^+ channels (Peterson & Maruyama, 1984; Welsh & McCann, 1985). In secretory epithelia, Ca^{2+} appears to play a role in the activation of both apical Cl^- channels and basolateral K^+ channels (Schultz & Hudson, 1991).

Four pathways appear to regulate $[Ca^{2+}]_i$ in most eukaryotic cells (including epithelial cells) (Carafoli, 1987): (a) the Ca^{2+}-calmodulin-activated, Ca^{2+}-extruding ATPase - these pumps have been identified in the basolateral membranes of small intestinal cells (Nellans & Popovitch, 1981; Hildman *et al.*, 1982), (b) the Na^+-Ca^{2+} exchanger - identified in the basolateral membranes of intestinal and renal cells (Blaustein, 1974; Hildman *et al.*, 1982; Ghijsen *et al.*, 1983; Heeswijk *et al.*, 1984; Lorenzen *et al.*, 1984), (c) Ca^{2+}-selective channels - suggested initially by the existence of verapamil-sensitive diffusional entry in the basolateral membranes of rabbit ileum and rat descending colon (Donowitz & Welsh, 1986; verapamil is a well-established blocker of voltage-dependent Ca^{2+} channels in a variety of non-epithelial cells), and (d) intracellular Ca^{2+} pools - Ca^{2+} is sequestered in intracellular organelles, primarily the endoplasmic reticulum and mitochondria. The exchange of pooled Ca^{2+} with cytosolic Ca^{2+} occurs via a similar set of transporters to those outlined in (i) to (iii) above. In enterocytes, extrinsic agonists may cause an

increase in $[Ca^{2+}]_i$ by causing: (i) increased plasma membrane Ca^{2+} permeability (Donowitz *et al.*, 1982; Donowitz, 1983; Chang *et al.*, 1986; de Jonge & Vaandrager, 1987; Homaidan *et al.*, 1989), (ii) release of Ca^{2+} from non-mitochondrial stores by either inositol 1,4,5-triphosphate (IP_3; produced in response to muscarinic agonists and serotonin) after the action of phospholipase C on the membrane phospholipid phosphatidylinositol 4,5-biphosphate (PIP_2) (Sachs & Muallem, 1989; Chang & Musch, 1990), or cyclic nucleotides (Homaidan *et al.*, 1989; Semrad & Chang, 1990).

As discussed by Carafoli (1987), the interactions between these systems that determine the steady-state, and transient changes in, $[Ca^{2+}]_i$ are complex and unresolved. There are conflicting reports concerning the relative importance of the Ca^{2+} ATPase and the Na^+/Ca^{2+} exchanger as determinants of cytosolic Ca^{2+} levels. Heeswijk *et al.* (1984) examined the kinetic properties of these two membrane transporters in basolateral membrane vesicles from rat kidney cortex, concluding that the Ca^{2+} pump has a four to five times greater capacity than the exchanger, so that the latter is unlikely to play a significant role in Ca^{2+} homeostasis. Furthermore, it is likely that the exchanger is swamped by the ATPase, and hence has little effect on $[Ca^{2+}]_i$. In contrast, the study of Lorenzen *et al.* (1984) on *Necturus maculosus* renal proximal tubules suggested that the basolateral Na^+/Ca^{2+} exchanger has an important role in determining $[Ca^{2+}]_i$. The study of Yang *et al.* (1988) produced a compelling body of evidence that changes in the magnitude of the electrochemical gradient for Na^+ across the basolateral membrane of *N. maculosus* renal proximal tubule cells causes rapid (within minutes) changes in $[Ca^{2+}]_i$ consistent with Na^+/Ca^{2+} exchanger operation. The likelihood of a role for the Na^+-Ca^{2+} exchanger in control of $[Ca^{2+}]_i$ appears to vary between epithelia, dependent on the relative preponderance of other pathways contributing to $[Ca^{2+}]_i$.

Although the mechanisms by which cytosolic Ca^{2+} modifies apical membrane sodium permeability in either tight or leaky epithelia remain unclear, and vary between types of epithelia, cytosolic Ca^{2+} is implicated as an important modulator of the rate of apical Na^+ entry, Na^+/K^+ ATPase activity, basolateral membrane K^+ permeability and anion secretion (in secretory epithelia).

(iii) Intracellular pH. Intracellular pH is tightly regulated in animal cells and much information exists on the mechanisms that maintain pH_i (Roos & Boron, 1981; Thomas, 1984). Garty *et al.* (1985) found that pH_i changes had no effect on amiloride-sensitive

$^{22}Na^+$ influx into apical membrane vesicles of toad urinary bladder epithelium. In contrast, in the seminal microelectrode studies on the frog skin (Harvey *et al.*, 1988; Harvey & Ehrenfeld, 1988), manoevures causing intracellular acidification (within the physiological range ($7.1 < pH_i < 7.4$)) caused a decrease in both $^{22}Na^+$ influx across the apical membrane and the basolateral K^+ conductance. There are several possible explanations for these differences: (a) response to pH_i varies according to the epithelium studied, (b) procedures for preparation of vesicles may modify the sensitivity of apical Na^+ channels to pH_i, and (c) H^+ ions may not act directly but via a cytoplasmic mediator. This latter possibility appears unlikely given the extreme rapidity of the response of apical membrane Na^+ permeability to changes in pH_i in the frog skin (Harvey *et al.*, 1988) and patch-clamped areas (both whole cell and cell-detached patches) of the rabbit kidney cortical collecting tubule (Palmer & Frindt, 1987). Because of the high sensitivity of Na^+ (apical) and K^+ (basolateral) conductances to pH_i, pH_i has been proposed as an important intrinsic regulator of transepithelial ion transport. Furthermore, Harvey *et al.* (1988) characterised the dynamics of change in pH_i, $[Na^+]_i$ and apical Na^+ conductance. At the onset of an acid load in the frog skin, $[Na^+]_i$ decreased, possibly as a result of decreased apical membrane Na^+ permeability. Subsequently $[Na^+]_i$ increased; this may have been due to inhibition of the basolateral Na^+/K^+ ATPase though an increase in $[Na^+]_i$ could also occur from activated basolateral Na^+/H^+ exchange (Ehrenfeld *et al.*, 1987; Harvey & Ehrenfeld, 1988; Harvey *et al.*, 1988). Results from this and other studies (Harvey & Kernan, 1984; Harvey & Ehrenfeld, 1988) suggest that the Na^+/K^+ ATPase is inhibited at acidic pH_i and that the gain in $[Na^+]_i$ is due to stimulated Na^+/H^+ exchange. Increases in $[H^+]_i$ have been shown to decrease Na^+/K^+ ATPase activity (Eaton *et al.*, 1984; Homareda & Matsui, 1985) which could lead to a raised $[Na^+]_i$ if it continued unchecked. The sensitivity of the apical membrane Na^+ permeability to pH_i may serve to limit such an increase in $[Na^+]_i$. These effects are important in light of previous studies that have shown near constancy of $[Na^+]_i$ with increased overall transport rate (Wills & Lewis, 1980; Thomas *et al.*, 1983; Turnheim *et al.*, 1983). The stimulation of transepithelial Na^+ movement by aldosterone has also been attributed to a change in intracellular pH. Alkalinisation of the cytosol, caused by aldosterone-induced activation of Na^+/H^+ exchange (Oberleithner *et al.*, 1987) may be responsible for the increased transepithelial Na^+ movement, observed in the presence of this mineralocorticoid (Rick *et al.*, 1988).

(iv) Cell volume. The membranes surrounding most animal cells are fragile and highly permeable to water. Such cells tend to have an ability to regulate their volume in a homeostatic fashion, after cell volume changes eg by exposure to anisotonic media (Siebens, 1985). An increase in the rate of Na^+ entry across the apical membrane by any of the mechanisms illustrated in Figure 15 results in an immediate increase in the intracellular osmolyte content (this being especially true for Na^+-coupled influx involving sugars, amino acids and Cl^-) that causes an increase in cell volume and total water content (Schultz *et al.*, 1966; Czaky & Esposito, 1969; Armstrong *et al.*, 1970). Volume changes in epithelial cells may therefore underly intrinsic regulatory changes. Swelling epithelial cells by exposure to hypotonic media appears to activate K^+ (and perhaps Cl^-) conductive pathways across the basolateral membrane, permitting loss of KCl and water, and thereby causing shrinkage towards their original volume. Ussing (1982) showed that swelling the cells of frog skin epithelium activated a basolateral Cl^- conductance. Since the basolateral K^+ conductance is large in this tissue, activation of Cl^- channels allows KCl loss from the cell. Studies on the *Necturus maculosus* intestinal epithelium showed that exposure to 6 or 12 % hypotonic solutions, or mucosal sugars or amino acids, caused (a) hyperpolarisation of the basolateral membrane (Lau *et al.*, 1984), and (b) an increase in basolateral K^+ permeability which could be abolished when cell swelling was prevented using hypertonic perfusate (Lau *et al.*, 1986). Similarly, in frog urinary bladders, regulatory volume decreases instigated a rise in basolateral K^+ permeability (Davis & Finn, 1985). In rabbit urinary bladders a decreased epithelial cellular volume caused a decrease in basolateral K^+ permeability (attributable to highly specific Ba^{2+}-sensitive K^+ channels) together with a decrease in pump function (Lewis *et al.*, 1985). When isolated toad urinary bladder cells were exposed to a hypotonic medium, there was rapid cell swelling followed by a regulatory volume decrease (Wong & Chase, 1986). Cell swelling was associated with an increase in the membrane permeability to Ca^{2+} and an increase in $[Ca^{2+}]_i$. Preventing the increase in $[Ca^{2+}]_i$, by decreasing $[Ca^{2+}]_o$ or loading the cells with an excess of quin2, abolished the volume regulatory decrease response.

Cultured human intestinal cells (Intestine 407) have been used to study the role of volume regulatory systems in (a model) intestinal function by Hazama and Okada (1988). These workers found: (a) exposure of these cells to a hypotonic solution activated both K^+ and Cl^- conductances, (b) deprivation of extracellular Ca^{2+}, or chelating of $[Ca^{2+}]_i$ by

loading the cells with excess quin2, suppressed the volume regulatory response, and (c) microinjection of Ca^{2+} into these cells caused an increase in quinine-sensitive K^+ conductance but failed to increase Cl^- conductance. This was interpreted to mean that volume regulatory decrease in these cells involves activation of both K^+ and Cl^- channels, but that only the former is activated by Ca^{2+}.

The proposal that the increase in basolateral K^+ permeability is in essence a component of a regulatory volume decrease mechanism is consistent with the finding that Na^+-coupled sugar and amino acid transport by the small intestinal epithelium is accompanied by a decrease in $[K^+]_i$ (Grasset *et al.*, 1983; Brown & Sepulveda, 1985a,b) rather than the increase that might be expected by a three to four fold stimulation of pump rate at the basolateral membrane. The increase in basolateral K^+ permeability appears to allow the loss of cell $[K^+]_i$ (accompanied by Cl^- and/or other anions) to compensate for the accumulation of osmolytes or amino acids minimising cell swelling). As noted above, an increase in basolateral Na^+/K^+ ATPase activity need not be the direct result of an increase in $[Na^+]_i$. Schultz and Hudson (1991) raise the intriguing possibility that basolateral sodium pump activity may be stimulated by cell swelling, even though such a swelling causes a decrease in $[Na^+]_i$. Lewis *et al.* (1985) had previously suggested that a decrease in the volume of rabbit urinary bladder epithelial cells may be accompanied by a decrease in the number of Na^+ pump sites. The mechanisms responsible for regulatory volume decrease in epithelial cells can be activated by very small increases in (or challenges to) cell volume (Schultz & Hudson, 1991). This exquisite sensitivity means that it is unlikely that regulatory volume decrease occurs in response to dilution of an essential intracellular solute (messenger). It is more probable that such mechanisms are activated by mechanical consequences of volume alterations, either by changes in membrane stress or by an increase in the tension exerted on cytoskeletal elements that are anchored to the cell membrane. One candidate sensor is the stretch-activated channel (Sachs, 1986a,b), first identified by Guharay and Sachs (1984). The presence of stretch-activated Ca^{2+} channels in the basolateral membranes of choroid plexus epithelial cells has been documented (Christensen, 1987), and these channels can be activated by suction pressures as small as -5 mmHg. Stretch-activated channels appear to be activated by tensions corresponding to osmotic pressure differences generated by only a few milliosmoles, mediating a Ca^{2+} influx with the subsequent activation of basolateral K^+ channels. However the importance

of this process in other epithelia remains speculative.

(v) Control of transepithelial ion movement in response to extrinsic factors. Extrinsic factors such as neurotransmitters and hormones may alter the rate of transepithelial movement, by affecting factors already implicated in intrinsic regulation. For example, atrial natriuretic factor inhibited of Na^+/H^+ exchange in avian intestine (Semrad & Chang, 1990). Extrinsic factors may affect intracellular factors not involved in intrinsic control, such as the cyclic nucleotides cAMP and cGMP. A more detailed treatment of intracellular mediation of extrinsic control is given below for the special case of the small intestine (section 2.4.2).

2.4.2 Ion transport in the small intestine

(i) Fluid and electrolyte transport in the small intestine. The small intestine is an heterogeneous epithelium (see Figure 2.14), containing several cell-types with different roles. Most water absorption occurs in the small intestine (Castro, 1985). Whilst c. 7 - 10 litres of water (including that provided by endogenous secretions) enter the small intestine per day, only c. 600 ml reaches the colon (actually capable of absorbing c. 2 - 3 $l.(day)^{-1}$). However, if there is malabsorption in the small intestine colonic absorptive capacity may be exceeded, resulting in diarrhoea. Although it is possible for water to be added to faeces from the colonic mucosa, the predominant source of water and electrolytes in a diarrhoeic stool is usually the small intestine (Castro, 1985).

In health, the volume moving from lumen to blood (by absorption) is usually greater than that moving from blood to lumen (by secretion), resulting in net absorption. In the absence of food, ions are the most important contributor to osmotic pressure in the intestine, rates of secretion and absorption being ultimately dependent on both osmotic and hydrostatic pressures across the epithelium. Though the ionic composition of the luminal contents varies along the length of the intestine and is different from that of faeces, the luminal fluid is generally maintained isotonic with plasma. The relative impermeability of the colonic mucosa to water allows stool fluid to be (usually) hypertonic, c. 350 - 400 $mOsm.(l)^{-1}$.

The values for the ionic content of luminal fluid, given in Figure 2.16, indicate that the overall absorption of Na^+ becomes increasingly prevalent moving from proximal to distal intestine, partly indicating the increasing tendency to tightness of the alimentary epithelium. Furthermore, in the distal ileum and colon, the Cl^- is conserved by

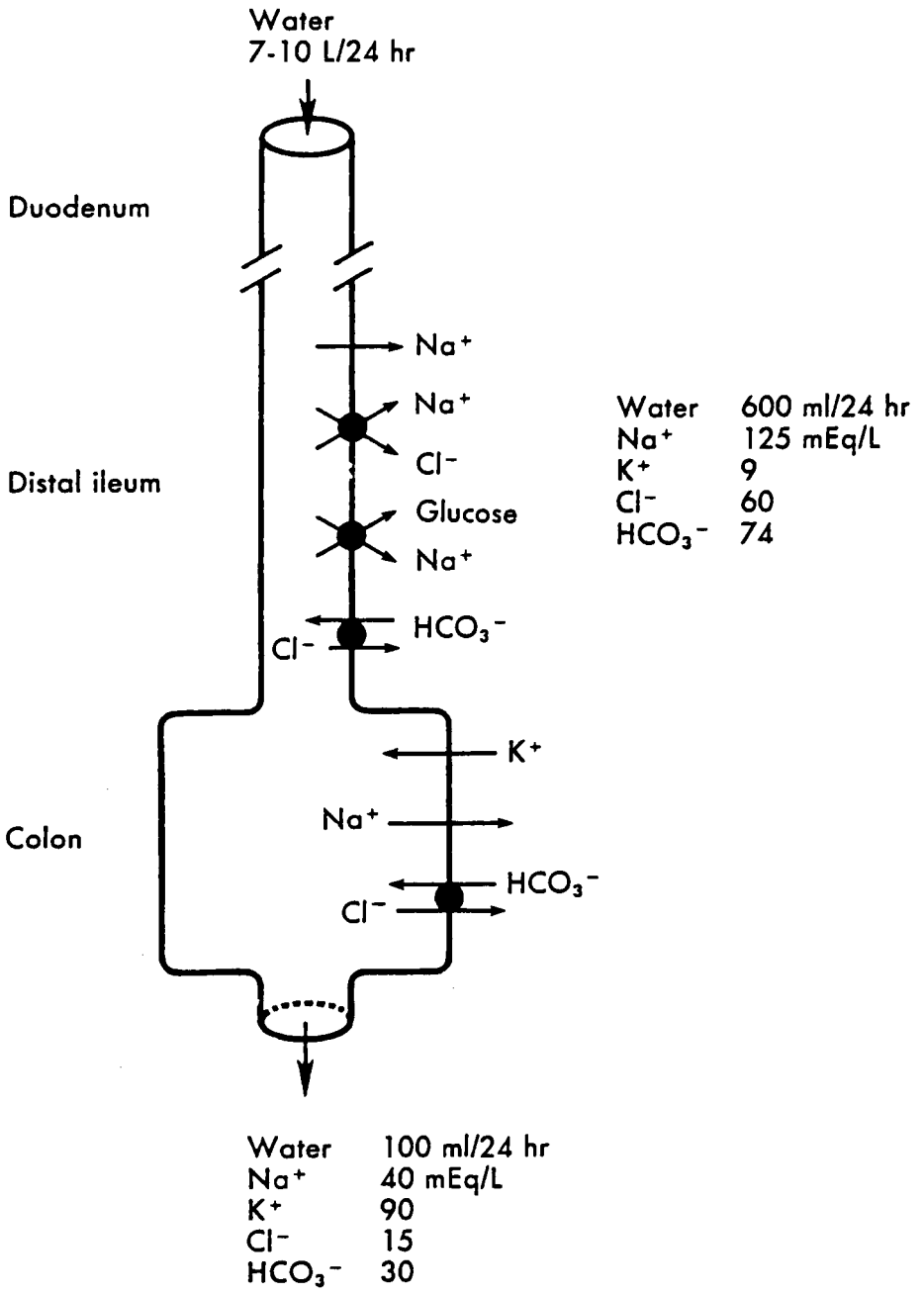


Figure 2.16 Volume and composition of luminal fluid in the human intestine. After Castro (1985).

mechanisms performing Cl^- exchange for metabolically-derived HCO_3. The K^+ concentration remains approximately equal to that of serum (4 - 5 $mEq.(l)^{-1}$) throughout the small intestine, and the volume of intestinal contents decreases during passage. In the colon, however, the luminal K^+ concentration rises dramatically due to net K^+ secretion. A prolonged diarrhoea results in a hypokalaemic metabolic acidosis because of poor small intestinal K^+ absorption, colonic K^+ secretion and Cl^-/HCO_3 exchange.

(ii) Membrane physiology of the small intestine. Many of the important features of small intestinal function reflect the general principles outlined by the KJU model and its modern embroidery (section 2.4.1): (a) the ultimate driving force for intestinal active transport is the basolaterally-sited Na^+/K^+ ATPase, (b) as suggested by the ubiquity of the ATPase, Na^+ is actively absorbed throughout the length of the intestine, (c) transport of other solutes against their electrochemical gradients is linked either directly or indirectly to Na^+ transport (section 2.3.3), (d) the small intestine is a leaky epithelium in which most of the ion conductance is via the paracellular shunt pathway (Frizzell & Schultz, 1972). The permiselectivity and overall permeability of the paracellular pathway vary from proximal to distal intestine, as shown for human intestine (Davis *et al.*, 1982) - the jejunum is most permeable and displays a slight cation preference (important because it allows an electrical potential to be built up across this junction), while the ileum is less permeable (causing increased restriction of passive solute transport and more effective exertion of an osmotic pressure) and more cation selective, (e) although the capacity to absorb and secrete electrolytes appears to be present throughout the length of the small intestine, evidence points to an anatomical separation of the sites of secretory and absorptive processes; villus cells are the principal site of absorption, whereas crypt cells appear to be the main secretory sites (Roggin *et al.*, 1972; de Jonge, 1975; Frizzell *et al.*, 1975; Field *et al.*, 1980; Welsh *et al.*, 1982a,b), (f) K^+ absorption by the mammalian small intestine appears to be strictly diffusional (Gilman *et al.*, 1963; Turnberg, 1971), (g) Na^+ enters small intestinal absorptive cells by either cotransport with nutrients such as glucose and amino acids, a neutral mechanism that involves cotransport with Cl^-, or a channel (Sellin *et al.*, 1989; Sullivan & Field, 1991). In the ileal region, Cl^- enters the enterocyte in neutral exchange for HCO_3.

Membrane vesicle studies have helped to elucidate the topography of ion transporters from the small intestine, according to apical/basolateral membrane position, crypt/villus

location, and position along the length of the intestine. Results from these and other experiments (on isolated enterocytes or epithelial layers) indicate that the Na^+/H^+ exchanger is present in both the brush-border (Murer *et al.*, 1976; Gunther & Wright, 1983; Knickelbein *et al.*, 1983; Cassano *et al.*, 1984) and basolateral (Barros *et al.*, 1986; Knickelbein *et al.*, 1988; Tosco *et al.*, 1988) membranes throughout the small intestine. When crypt and villus cells were separated by sequential isolation prior to vesicle preparation, Na^+/H^+ exchangers were found in basolateral membranes of both cell types and in brush borders from villus (but not crypt) cells (Knickelbein *et al.*, 1988). However, whilst Cl^-/HCO_3 exchangers appear to exist in mammalian ileal (but not jejunal) brush border membranes (Knickelbein *et al.*, 1988), they have not been found in small intestinal basolateral membranes (Sullivan & Field, 1991). The model for intestinal active chloride secretion is similar to that outlined above (section 2.4.1) as the paradigm Cl^- secretory apparatus (Field *et al.*, 1989; Field, 1993; Simmons, 1993).

Membrane vesicle studies, however, do not always reflect the transport activities of the intact tissues from which they are derived. A transport process manifest in the intact epithelium may be inactivated in the preparation of membrane vesicles, or it may require cytosolic regulators not present in the intra- or extra-vesicular solutions used. Similarly, transport processes readily demonstratable in vesicles may be latent in the epithelium under most circumstances. With this reservation, but with the broad support of studies on isolated cells and the intact epithelium, the seminal membrane transport features of villus and crypt cells are given in Figure 2.17.

(iii) Second messenger control of intestinal function. A number of intestinal hormones and neurotransmitters have been identified which can alter intestinal transport both *in vivo* and *in vitro* (Donowitz & Welsh, 1987; Powell, 1987). As shown in Table 2.5, these agents can be grouped broadly into those that stimulate net secretion (secretagogues), either by inhibiting active absorption or or by stimulating active secretion, and those that have the opposite effects (proabsorptive agents). Some of these agents have a direct effect on enterocytes; others activate systemic, submucosal, or subepithelial processes that secondarily alter intestinal electrolyte transport. Secretagogues (Table 2.5) appear to stimulate electrolyte (and secondarily, fluid) secretion by increasing the epithelial cellular levels of second messengers such as cyclic nucleotides and free cytosolic Ca^{2+}, and activating certain protein kinases (de Jonge & Rao, 1990; Rao & de Jonge, 1990). Net

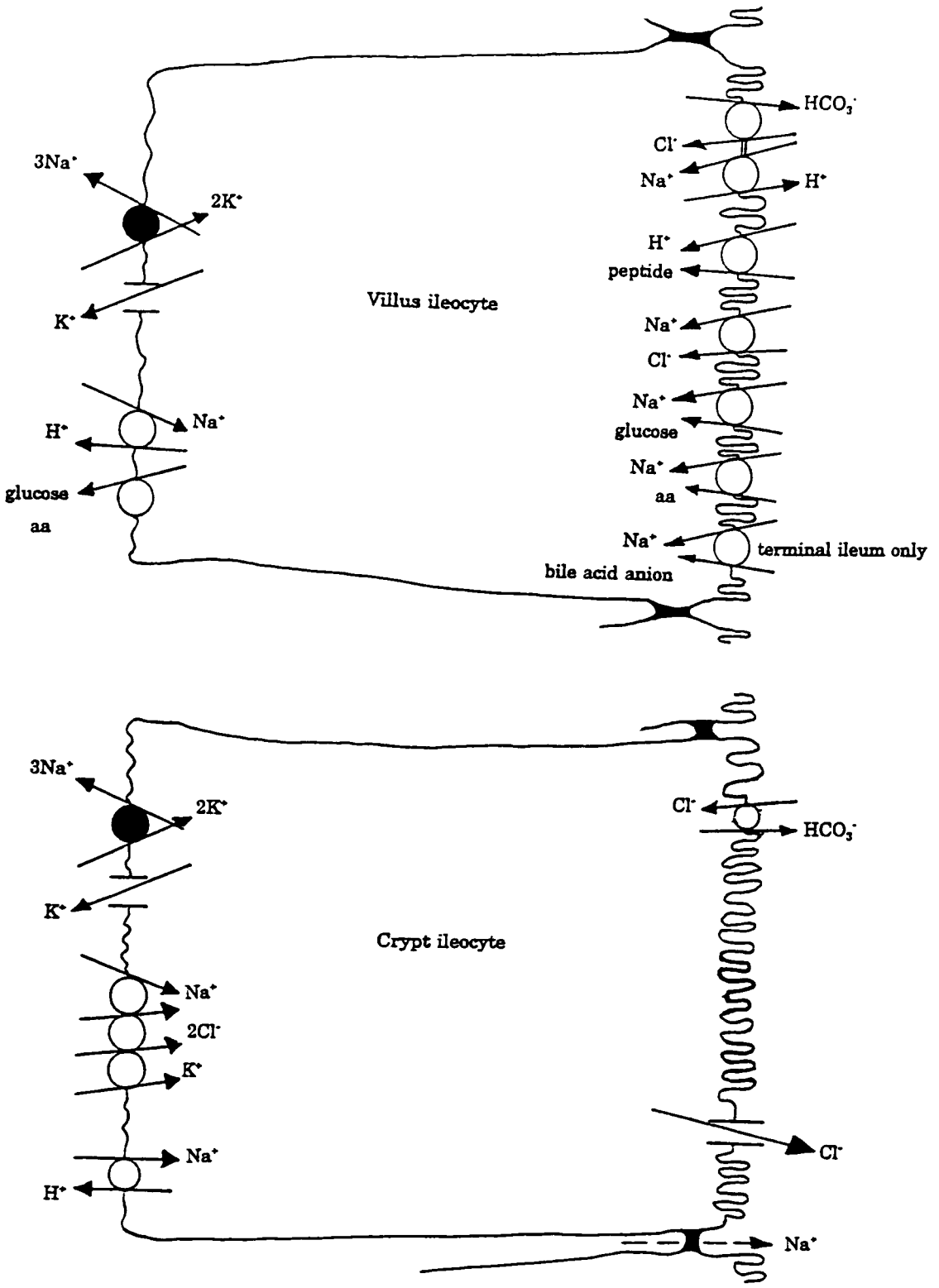


Figure 2.17 Models of membrane transport in villus and crypt enterocytes. Derived from data as given in text. Villus and crypt enterocytes appear to be adapted for rather different transport processes; villus cells tend to be absorptive, and crypt cells tend to be secretory in nature.

	agent	intracellular mediator	selected references
a)	Vasoactive intestinal peptide	cAMP	Shwartz *et al.*, 1977 Binder *et al.*, 1980
	Adenosine		Chang & Rao, 1991
	Histamine		Chang & Rao, 1991
	Acetylcholine	protein kinase C and/or Ca^{2+}	Zimmerman & Binder, 1982 Dharmsathaphorn *et al.*, 1984
	Substance P		Chang *et al.*, 1986
	Serotonin		Hirose & Chang, 1988 Hansen & Jaffe, 1993
	Prostaglandins	cAMP and protein kinase C/Ca^{2+}	Racusen & Binder, 1980 Field *et al.*, 1981
	Bradykinin		Musch *et al.*, 1983 Hojvat *et al.*, 1983
	Atrial natriuretic peptide	cGMP→↑Ca^{2+}_i→↓Na^+/H^+exchange→↓pH_i	Semrad *et al.*, 1990 Catto-Smith *et al.*, 1991
	Guanylin	cGMP	Forte *et al.*, 1993
	Vasopressin	unknown	Chang & Rao, 1991
b)	α_2-Adrenergic agonists: epinephrine norepinephrine	?G_i ⇒inhibition of cAMP-mediated effects	Hubel, 1976 Chang *et al.*, 1982, 1983, 1984
	Somatostatin	G_i (and ? other) ⇒inhibition of cAMP- and Ca^{2+}-mediated effects	Dharmsathaphorn *et al.*, 1980 Guandalini *et al.*, 1980
	Neuropeptide Y	mediation via α_2-Adrenergic receptors	Hubel, 1985 Brown *et al.*, 1990 Anthone *et al.*, 1991a,b
	Enkephalins	nerve cell δ receptors... ?	Turnberg *et al.*, 1991
	Adrenocorticoids: Aldosterone Glucocorticoids	nuclear receptors... ?	Marusic *et al.*, 1981 Sellin & Field, 1981 Binder *et al.*, 1986

Table 2.5 Extrinsic factors that (a) stimulate secretion or inhibit absorption, or (b) stimulate absorption or inhibit secretion. Data are collated from listed sources.

secretion may result from a stimulation of anion secretion and/or inhibition of active absorption in intestinal epithelial cells. Such secretory and absorptive processes are distinct and may be regulated independently of each other (Waldman *et al.*, 1977). The differential absorptive and secretory effects of particular agonists may reflect differences in receptor expression, or in the responsiveness of different cell types (Roberts *et al.*, 1988; Welsh *et al.*, 1982). Some intestinal secretagogues (Table 2.6) may (a) activate basolaterally membrane-bound adenylate cyclase causing an incease in cellular cAMP (Walling *et al.*, 1978), (b) activate guanylate cyclase to generate an increase in cGMP (Mittal & Murad, 1982), or (c) cause an increase in $[Ca^{2+}]_i$. Increases in $[Ca^{2+}]_i$ in the small and large intestine are associated with the activation of anion secretion (Rao & Field, 1983), while that in the ileum also involves the inhibition of neutral Na^+ and Cl^- absorption (Donowitz & Welsh, 1986). In enterocytes, $[Ca^{2+}]_i$ is in the sub-micromolar range (c. 100 - 250 nM; Semrad & Chang, 1987, 1990). Mechanisms by which $[Ca^{2+}]_i$ changes occur have been reviewed above (section 2.4.1). Increases in $[Ca^{2+}]_i$ activate a number of cellular biochemical processes, some involving the stimulation of protein kinase C (a phospholipid and Ca^{2+}-dependent enzyme) or other kinases (Rao & de Jonge, 1990; Chang & Rao, 1991; Chaudhuri *et al.*, 1993), and others the stimulation of calmodulin (a Ca^{2+}-dependent regulatory protein). The cyclic nucleotides act predominantly via intracellular protein kinases, enzymes that catalyse the transfer of a terminal phosphate group of ATP to the hydroxyl group of a serine, threonine, or tyrosine residue of a protein to form a phosphomonoester bond (Edelman *et al.*, 1987; Rao & de Jonge, 1990), although they can act via kinase-independent routes (Chang & Rao, 1991).

As revealed in the preceding discussion, there is an extensive range of regulatory systems involved in the control of ion movement by the small intestinal epithelium. The relative importances of, and the interactions between, these different systems remain poorly understood (Turnheim, 1991; Schultz & Hudson, 1991). The diversity of regulatory systems of the small intestine and other epithelia may ensure a high degree of flexibility and plasticity in response to environmental change. However, given incomplete understanding of the interactions between controlling factors, and the fact that there is a diversity of control parameters that can be affected by any toxin action, analysis of the mechanisms underlying perturbation of epithelial function by bacterial toxins is likely to

be rather complicated (Turnberg, 1991) and the use of model systems is advocated (sections 2.8.1 - 2.8.3).

2.5 BACTERIAL TOXINS

Recent research achievements in the field of bacterial protein toxins reflect the extensive and productive blending of disciplines such as molecular genetics, protein chemistry, crystallography, immunology, neurobiology, pharmacology and biophysics. Space does not permit a comprehensive coverage of all membrane-disrupting toxins produced by bacteria (see: Bernheimer & Rudy, 1986; Bhakdi & Tranum-Jensen, 1986; Freer, 1986; Harris, 1986; Stephen & Pietrowski, 1986; Hardegee & Tu, 1988; Iglewski & Clark, 1990; Alouf & Freer, 1991; Braun & Focareta, 1991; Li, 1992; Read & Stein, 1993, and references therein for exhaustive detail). Here, the role of toxins is considered and a working classification of bacterial toxins expounded.

2.5.1 Role of toxins in the context of bacterial pathogenesis

From an evolutionary perspective, a pathogen is a highly adapted micro-organism that has the capacity to cause disease (ie overt damage to its host) because its survival-reproduction strategy is benefitted by the pathogenic process (Falkow, 1990). Disease itself is an inadvertent outcome of the microbial infection. The determinants of pathogenicity are diverse but often include a group of co-regulated complementary genetic properties suited for the interaction of a particular micro-organism with its host niche. In particular, biochemical lesions, with or without accompanying structural damage, underly pathogenicity induced by bacteria. There are two predominant mechanisms by which such damage is caused (Stephen & Pietrowski, 1986): (i) activation by microbial substances (often inherently non-toxic) of immune system components that act to the disadvantage of the host (immunopathological damage), and (ii) the production of toxin(s) that interacts directly with appropriate targets.

The definition of the term 'toxin' is more complicated than might be thought. The most eloquent of recent attempts was that of Bonventre (1970) who defined toxins as a 'special class' of poisons that differ from, for example, cyanide or mercury by virtue of their: (i) microbial origin, (ii) protein structure, (iii) high M_w, and (iv) antigenicity. This view is inadequate because such a classification (i) includes proteins of doubtful significance in disease (moreover modes of action of many toxins are not understood eg *Clostridium*

difficile toxin A (Fiorentini & Thelestam, 1991)), and (ii) excludes non-protein toxic complexes such as endotoxin (Wicken & Knox, 1980).

The importance of the cell membrane in toxin-induced disease has already been noted (Chapter 1). With the exception of toxins that attack extracellular components (section 2.5.2), the cell membrane must either be crossed or attacked directly. This dissertation is concerned with the characterisation of the primary action of the class of *Vibrio* toxins on cell membranes, and the pathophysiological sequelae of membrane function. As discussed previously (Table 1.1 and section 2.5.2), disruption of cellular processes by toxins may be the critical event in the pathogenesis of infectious disease.

2.5.2 Toxin mechanisms

Bacterial toxins are an extremely heterogeneous class of molecule, varying in site of action, mechanism of action, molecular weight and physicochemical properties. They are 'smart' weapons in the biological warfare waged between species (Read & Stein, 1993) because many have the capability to be targetted precisely to specific molecules, cells and tissues (Ehrlich, 1900). In the first instance, they may be classified into three separate groups on the basis of their site of action (see Figure 2.18 and Table 2.6), namely extracellular components, intracellular components, or the cell membrane itself. Table 2.6 is by no means exhaustive but serves to indicate the broad sweep of bacterio-toxicological research and allow the identification of mechanistic trends eg the existence of a whole group of thiol-activated cytolysins isolated from different species and genera of bacteria. A number of differences and similarities of sites and mechanisms of action allow definition of further classes.

(i) Toxins acting on extracellular components. Bacterial proteases have been recognised for many years and are especially important in the case of Clostridial pathogens which destroy body tissues, and other organisms which cause pneumonias eg *Legionella pneumoniae* and *Pseudomonas aeruginosa* (Stephen & Pietrowski, 1986). The recent identification of a toxin, ZOT (Fasano *et al.*, 1991), elaborated by *V. cholerae*, which attacks tight junctions, furthers the notion that extracellular sites can be important targets of toxin action.

(ii) Toxins acting at intracellular sites. Evidently, there are a number of intracellular sites at which toxins may act once they or their active part have been internalised (Table 2.6; Alouf & Freer, 1991). One identifiable class of such toxins is that of the ADP-

C

Membrane

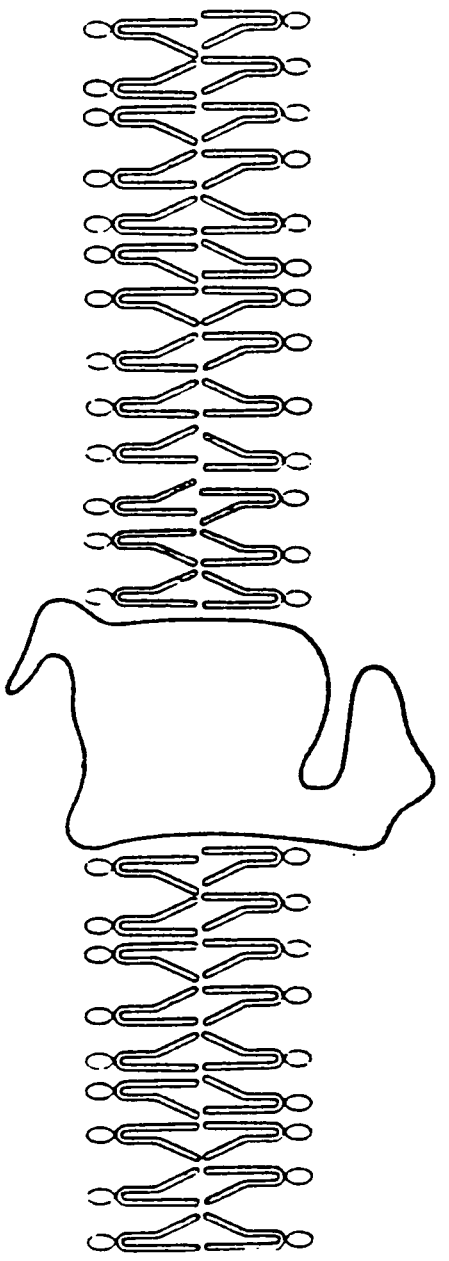
eg phospholipases and pore-formers

A

Extracellular environment

eg collagenases

and *zonula occludens* toxin



B

Intracellular environment

eg ADP-ribosylating toxins

and protein synthesis inhibitors

Figure 2.18 Possible sites of bacterial toxin action. A primary classification of bacterial toxins may be made according to their site of action (see Table 2.6), which may be (A) extracellular, (B) intracellular (which necessitates a mechanism for crossing the membrane), or (C) the cell membrane itself.

gross site of action	particular site of action	toxin	organism	molecular weight	membrane receptor (if applies)	biochemical action	pathological effect	selected references
act on extracellular components	connective tissue	collagenase	*Pseudomonas aeruginosa*	33 kd	--	cleavage of elastin and collagen chains types I, III, and IV.	structural damage and necrosis at site of infection; implicated in pneumonial disease	Wellisch *et al.*, 1984 Heck *et al.*, 1986
		protease	*Legionella pneumophilia*	40 kd	--	breakdown of extracellular structural proteins	alveolar haemorrhage, vesiculation and necrosis of type I alveolar epithelium; implicated in Legionnaires disease	Baskerville *et al.*, 1986 Conlan *et al.*, 1986
		hyaluronidase	Steptococcal sp.	(i) 55 kd (ii) 40 kd	--	breakdown of extracellular cement substances	structural damage to connective tissue allows spread of bacterium	Benchetrit *et al.*, 1984, 1987 Hynes & Ferretti, 1989 Schaufuss *et al.*, 1989 Sting *et al.*, 1990 Paton *et al.*, 1993
		exfoliatin	*Staphylococcus aureus*	32.5 kd	--	intraepidermal cleavage either between the granulosa and the spinous layers or at the granulosa layer of the epidermis	areas of epidermis are lifted away by an accumulation of serous fluid causing large areas of the body to be denuded; cause of staphylococcal scalded skin syndrome (SSSS)	Kaplan *et al.*, 1986 Arco *et al.*, 1991
	tight junctions	*zonula occludens* toxin	*Vibrio cholerae*	10 - 30 kd	--	alters ileal *zonula occludens* morphology by decreasing strand complexity	causes a rapid increase in ileal tissue conductance; implicated as cause of diarrhoea	Fasano *et al.*, 1991
cross cell membranes and have intracellular sites of action	intracellular targets by ADP-ribosylation	cholera toxin	*Vibrio cholerae*	AB_5; A: 27 kd B: 11.6 kd	G_{M1}	ADP-ribosylation of $G\alpha_s$ causing prolonged activation of adenylate cyclase	inceased levels cAMP that turn on Cl^- secretory processes in intestine; implicated as cause of diarrhoea	Moss & Vaughan 1986, 1988a,b, 1989 Cassuto *et al.*, 1982, 1983 Jodal *et al.*, 1993
		heat-labile enterotoxin	entero-toxigenic *Escherichia coli*	as above	terminal galactose residue of G_{M1} and glycoproteins (85-150 kd)	as above	as above	Moss & Vaughan 1988b, 1989 Zemelman *et al.*, 1989
		diphtheria toxin	*Corynbacterium diphtheriae* infected with β-tox phage	58.3 kd in 2 fragments, A: 21 kd B:37 kd	Vero cells: 14.5 kd binding protein in complex with a 27 kd protein	ADP-ribosylation of EF-2	inhibition of protein synthesis	Williams *et al.*, 1990 Mekada *et al.*, 1991 Chloe *et al.*, 1992

	exotoxin A	*Pseudomonas aeruginosa*	66.6 kd	LM fibroblasts: 300 kd glycoprotein with N-linked carbohydrate	as above	as above	Ogata *et al.*, 1990 Thompson *et al.*, 1991 Chaudry *et al.*, 1992 Nicholls & Youle, 1992 London *et al.*, 1993 Zdanovsky *et al.*, 1993
	pertussis toxin	*Bordetella pertussis*	A (S1)-B_5(S2S3S4$_2$S5); S1: 28 kd S2: 23 kd S3: 22 kd S4: 11.7 kd S5: 9.3 kd	Chinese Hamster Ovary (CHO) cells: 165 kd glycoprotein	ADP-ribosylation of $G\alpha_i$	uncouples receptors from G_i; inhibition of deactivation of adenylate cyclase; whooping cough	Moss & Vaughan, 1988b Witvliet *et al.*, 1989 Simon *et al.*, 1991
intracellular targets by mechanisms other than ADP-ribosylation	verotoxin	entero-haemorrhagic *Escherichia coli*	AB_5; A: 32 kd B: 7.7 kd	G_{b3}, G_{b4}	N-glycosidation excising adenine 4324 from 28S rRNA	inhibition of protein synthesis; damage to vascular endothelium in colon and kidney	Endo *et al.*, 1988 Tesh *et al.*, 1991
	anthrax toxins:	*Bacillus anthracis*	PA: 83 kd (activated by protease activity (?furin)) EF: 89 kd LF: 93 kd	most eukayotic cells have receptor (appears to be protein) for PA, which subsequently mediated the binding of EF and LF to the cell surface, so that they will be internalised by endocytosis.	PA:role in receptor linkage and membrane translocation of EF and LF EF: Ca^{2+}-calmodulin-dependent-adenylcyclase LF:unknown	PA+EF: edema toxin PA+LF: lethal toxin	Robertson *et al.*, 1988 Bragg & Robertson, 1989 Koehler & Collier, 1991 Leppla, 1991 Pezard *et al.*, 1991 Stepanov, 1991 Klimpel *et al.*, 1992
	botulinum toxins	*Clostridium botulinum*	LH; L: 50 kd H: 100 kd	sialosyl and protein residues on peripheral presynaptic membrane	after internalisation, the active toxin form acts as a Zn^{2+}-dependent protease to cleave VAMP/synaptobrevin (an integral membrane of synaptic vesicles upon which synaptic vesicular movement depends) at a single site	inhibition of Ca^{2+}-dependent acetylcholine release at nerve-muscle junctions; other neurotransmitter release; paralysis	Schantz & Johnson, 1992 Stevens *et al.*, 1991 Schengrund *et al.*, 1991 Huttner, 1993 Montecucco & Schiavo, 1993

		tetanus toxin	*Clostridium tetani*	LH; L: 50 kd H: 95 kd	as above	as above	blocks transmitter release at inhibitory synapses in central nervous system, causing spasm of voluntary muscles	Ahnert-Hilger *et al.*, 1990 Schengrund *et al.*, 1991 Link *et al.*, 1992 Schantz & Johnson, 1992 Facchiano *et al.*, 1993 Halperne & Loftus, 1993 Hohne-Zell *et al.*, 1993 Huttner, 1993 Montecucco & Schiavo, 1993
attack cell membranes directly	membrane lipids: phospholipases	phospholipase C	*Pseudomonas aeruginosa*	78 kd	phospholipase C: sphingomyelin, phosphatidylcholine, phospatidylethanolamine, phosphatidylserine, lysophosphatidylcholines and ethanolamines are enzyme substrates	enzymatically splits substrate (see following figure for site of action)	disruption of membrane integrity; ? contribute to invasiveness of pathogen	Liu, 1974, 1979 Berka & Vasil, 1982 Ostroff *et al.*, 1989
		damselysin	*Vibrio damsela*	69 kd	phospholipase D: sphingomyelin, phosphatidylcholine and phosphatidylethanolamine are enzyme substrates	enzymatically splits substrate (see following figure for site of action)	disruption of membrane integrity; ? important in cellular destruction during septacaemia	Morris *et al.*, 1982 Kreger, 1984 Kothary & Kreger, 1985 Kreger *et al.*, 1987 Daniel *et al.*, 1988 Kreger *et al.*, 1988
	pore-formers	streptolysin O	*Streptococcus pyogenes*	60.2 kd	cholesterol	susceptible cells undergo colloid osmotic lysis after toxins have acted in a two-step process; toxin binds to membrane triggering a conformational change leading to membrane disruption of a multi-hit nature.	disruption of cell membrane integrity; ? cell death by colloid osmosis	Badin & Denne, 1978 Fisher *et al.*, 1981 Bkakdi & Tranum-Jensen, 1985, 1988 Niedermeyer, 1985 Hugo *et al.*, 1986 Alouf & Geoffroy, 1991
		pneumolysin	*Staphylococcus pneumoniae*	52.8 kd	cholesterol	as above	as above	Kalin *et al.*, 1987 Kanclerski & Mollby, 1987 Kanclerski *et al.*, 1987 Boulnois *et al.*, 1991
		cereolysin O	*Bacillus cereus*	55.6 kd	cholesterol	as above	as above	Cowell *et al.*, 1978 Turnbull, 1981 Alouf & Geoffroy, 1991
		alveolysin	*Bacillus alvei*	51.8 kd	cholesterol	as above	as above	Launay & Alouf, 1979 Thelestam *et al.*, 1981 Geoffroy *et al.*, 1990 Alouf & Geoffroy, 1991

perfringolysin O	*Clostridium perfringens*	52.5 kd	cholesterol	as above	as above	Mitsui *et al.*, 1979a,b Ohno-Iwashita *et al.*, 1986, 1988 Iwamoto *et al.*, 1987 Alouf & Geoffroy, 1991
tetanolysin	*Clostridium tetani*	45 kd	cholesterol	as above	as above	Rottem *et al.*, 1990 Alouf & Geoffroy, 1991 Bhakdi *et al.*, 1993
listeriolysin O	*Listeria monoctyogenes*	58 kd	cholesterol	as above	as above	Datta *et al.*, 1990 Alouf & Geoffroy, 1991 Low *et al.*, 1992 Yoshikawa *et al.*, 1993
thermostable direct haemolysin	*Vibrio parahaemolyticus*	dimer of 2 subunits each of 20.1 kd	G_{D1a}, G_{T1}	binds to receptor; forms fixed size (c. 2 nm diameter) pores.	membrane disruption leading to colloid osmosis; implicated in cardiotoxic and gastrointestinal pathologies	Takeda, 1983, 1988 Honda *et al.*, 1992 Honda & Iida, 1993
El Tor haemolysin	*Vibrio cholerae*	successively cleaved protein:	?ganglioside	binds to receptor and forms lesion	membrane disruption leading to lysis by an as yet undefined mechanism; implicated in gastrointestinal pathologies	Honda & Finkelstein, 1979 Yamamoto *et al.*, 1984, 1986, 1990a Ichinose *et al.*, 1987 Hall & Drasar, 1990
aerolysin	*Aeromonas hydrophila* *Aeromonas sobria*	49.5 kd from 54 kd protoxin form (activated by extracellular protease)	mammalian erythrocytes: glycophorin	oligomerises and then binds and inserts a transmembrane heptameric pore	membrane disruption leading to colloid osmosis; implicated as a cause diarrhoeal disease	Chakraborty *et al.*, 1990 Wilmsen *et al.*, 1990 Buckley, 1991 van der Goot *et al* ., 1992 Parker *et al.*, 1994
α-toxin	*Staphylococcus aureus*	33.4 kd	? sialosylparagloboside but high affinity sites, 1200-1700 per rabbit erythrocyte	binds and then oligomerises to form a transmembrane hexameric pore	membrane disruption leading to colloid osmosis; has been implicated in fulminant endocarditis	Bernheimer, 1974 Kato & Naiki, 1976 Harshman, 1979 Fussel *et al.*, 1981 Gray & Kehoe, 1984 Vann & Proctor, 1988 Belmonte *et al.*, 1987 Ikigai & Nakae, 1987a,b Chakraborty *et al.*, 1990 Bkakdi & Tranum-Jensen, 1991 Cescatti *et al.*, 1991 Hildebrand *et al.*, 1991
δ-toxin	*Clostridium perfringens*	40 kd	G_{M2}	binds to G_{M2}, with subsequent pore formation, and lysis by an as yet undetermined mechanism	membrane disruption leading to lysis; ? role in enteritis	Alouf & Jolivet-Reynaud, 1981 Jolivet-Reynaud *et al.*, 1982, 1988, 1989, 1993 Jolivet-Reynaud & Alouf, 1983 Cavaillon *et al.*, 1986

gramicidin A	*Bacillus brevis*	1.8 kd	? lipid bilayer - especially of Gram positive bacteria	molecule dimerises in head to head fashion to form a transmembrane pore.	membrane disruption of sensitive bacteria leads to collapse of transmembrane proton gradient, inhibiting active transport; bacterial death	Hotchkiss, 1944 Urry, 1971 Veatch & Stryer, 1977 Andersen, 1984 Wallace & Ravikumar, 1988
gramicidin S	*Bacillus brevis*	1.8 kd	as above	peptide molecules anchor in the vicinity of the polar heads of lipids deforming the lipid packing; an accumulation of peptide liberates membrane fragments causing a transient increase in permeability before re-annealing	as above	Katsu *et al.*, 1989
colicin A	*Escherichia coli*	63 kd; C-terminal pore-forming domain is 23 kd	*E. coli* outer membrane: BtuB (vit B12 receptor), OmpF porin.	binds and then oligomerises to form a transmembrane pore	as above	Parker *et al.*, 1989 Bourdineaud *et al.*, 1990 Pattus *et al.*, 1990 Benedetti *et al.*, 1991 Li, 1992

Table 2.6 Classification and details of bacterial toxins.: Data collated from indicated references and reviews (Bernheimer & Rudy, 1986; Stephen & Pietrowski, 1986; Alouf & Freer, 1991; Li, 1992).

ribosylating toxins (Rappuoli & Pizza, 1991). These toxins are usually composed of two functionally distinct domains, designated A (the toxic moiety) and B (the vector, responsible for binding to the surface receptor and delivering A across the plasma membrane). All of the A domains have a common mechanism of action (and a common structure of the catalytic site): mono-ADP-ribosylation (ie the transfer of an ADP-ribose group to a nitrogen atom of the side-chain of an amino acid (arginine, diphthamide, asparagine or cysteine)) of a target protein that controls crucial aspects of cell physiology.

(iii) Toxins attacking the membrane directly. There are several mechanisms of membrane attack:

(a) enzymic alteration of membrane structure affecting either (a) lipid components of the membrane eg phospholipases (Table 2.6; sites of phospholipase action are given in Figure 2.19; Avigad, 1976), or (b) intrinsic membrane proteins (eg neuraminidase (see section 2.3.3)).

(b) aggregation of endogenous membrane components has been proposed to explain melittin action on erythrocytes, in particular by the aggregation of Band III tetramers (Clague & Cherry, 1989).

(c) formation of pore structure by toxin *per se* (eg streptolysin O and staphylococcal α-toxin (Bhakdi & Tranum-Jensen, 1985; Bhakdi *et al.*, 1985a,b)). Pore-forming toxins may form either large pores through which intracellular contents leak out directly, or small pores, lysis occurring only secondary to swelling after influx of extracellular osmolytes as a result of the increase in permeability. Colloid osmotic lysis may be inhibited by adding large impermeant molecules to the extracellular solution. Such a manoevure serves to balance the osmotic pressure exerted by those cellular components too large to pass through the membrane pores (Levitt, 1990). An estimate of the size of the pore has been obtained for several pore-forming agents by examining the degree of osmotic protection afforded by various extracellular osmolytes (eg a pore diameter of c. 3 nm for α-toxin of *Staphylococcus aureus*; Fussle *et al.*, 1981). This group of exotoxins require part of their structure to be capable of membrane penetration. This is of exceptional interest in thermodynamic terms because it requires a transition between aqueous and lipid-surrounded forms of the peptide (Figure 2.20). For some pore-forming toxins (eg alamethicin and staphylococcal δ-toxin), α-helical wheel analysis of primary strucures (Figure 2.20; Ojcius & Young, 1991) indicates that by adopting α-helical conformation

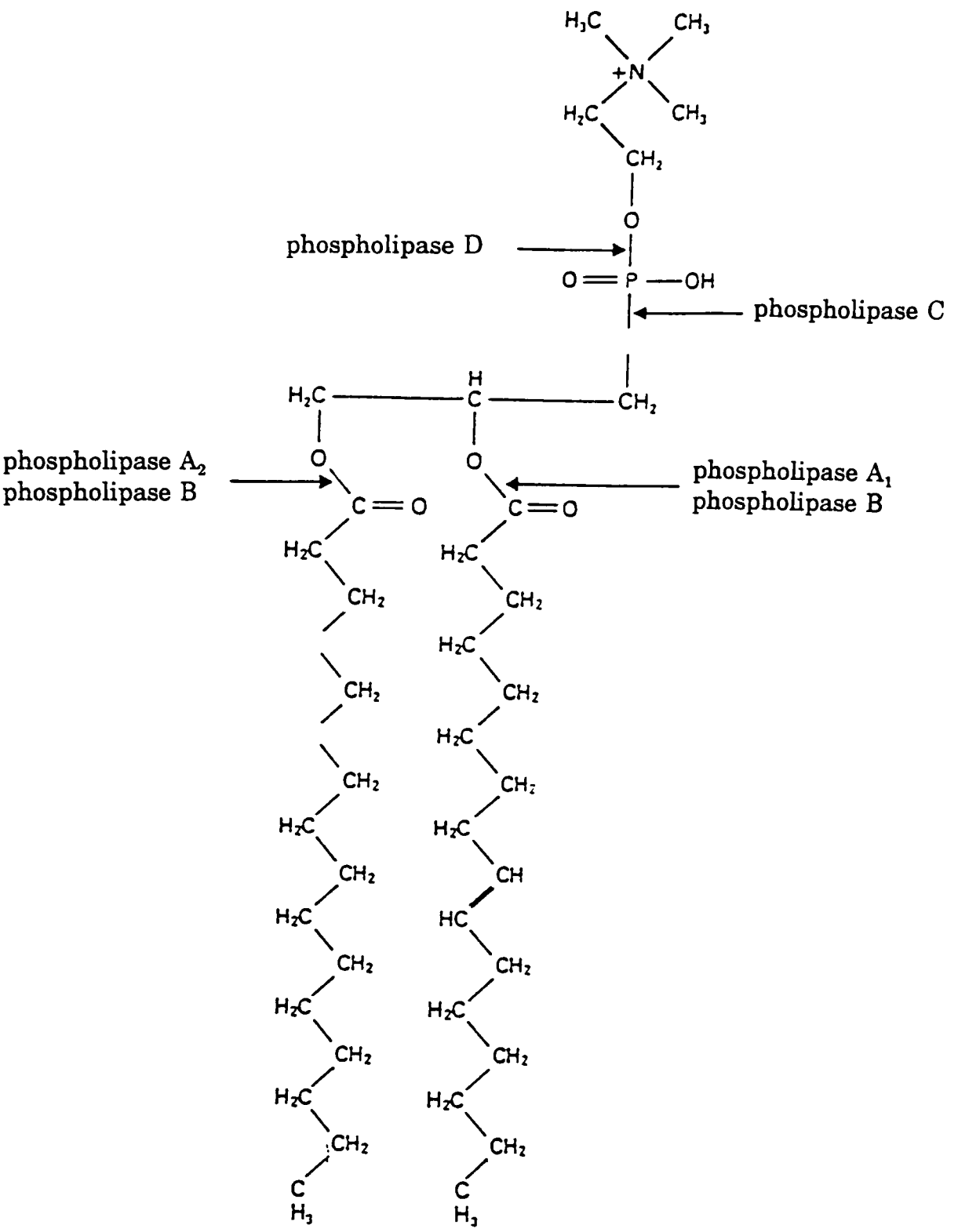
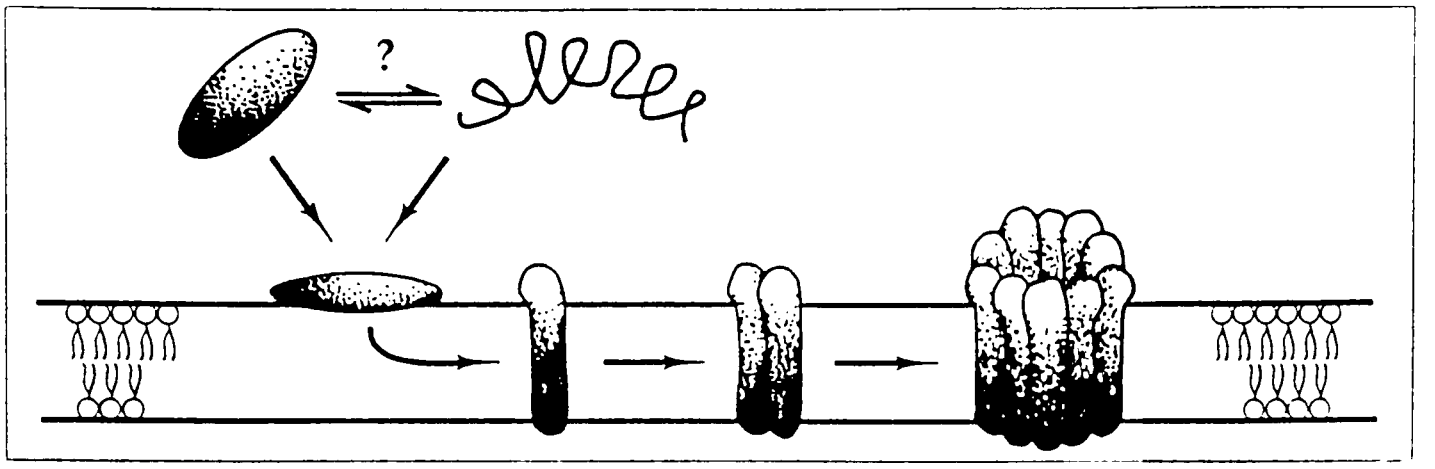


Figure 2.19 Sites of phospholipase action. After Stephen and Pietrowski (1986).

A



B

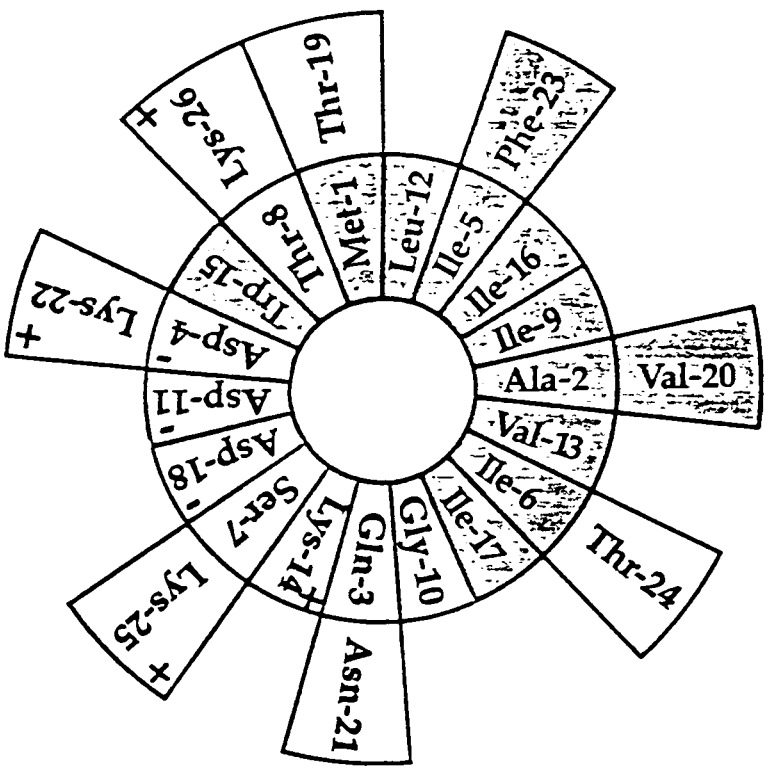


Figure 2.20 Analysis of primary structure by the α-helical wheel. A number of pore-forming proteins create channels through a barrel-stave mechanism of action (Ehrenstein & Lecar, 1977), in which three discrete steps are identified (see (A), after Ojcius & Young, 1991): (i) water soluble monomers bind to the membrane, (ii) these insert into the membrane, and (iii) they aggregate (like barrel-staves) to form an oligomeric pore. If a toxin has such a mechanism of action and its transmembrane region is α-helical then the helix is likely to be amphipathic, ie to fall into discrete hydrophobic and hydrophilic (such indices being given in Stryer, 1988) regions when viewed down the long axis of the helix (eg for staphylococcal δ-toxin, (B) the hydrophobic residues being shaded and predominantly on one side; after Ojcius & Young, 1991). An amphiphilic structure is predicted if the hydrophobicity of the structure varies with a periodicity of 3.6 residues. The presence of such a structure may be predictive of the mechanism of action.

these could span the membrane, with a laterally exhibited amphipathicity. This would be a thermodynamically favoured structure if their hydrophilic segment lined a multimeric pore and their hydrophobic segment was in contact with the lipid bilayer. Whilst the existence of such a structure is highly suggestive of mechanism of action (particularly for lower M_w toxins), its absence by no means excludes such an array being formed but with either internally shielded hydrophilic residues in a globular region within the bilayer, or the transmembrane sequence assuming some other motif (eg β-sheet) or structure.

(d) crenation-fragmentation of the membrane as suggested for gramicidin S and melittin (Katsu *et al.*, 1989). Peptide molecules accumulate predominantly in the outer leaflet of the bilayer deforming the target cell into crenature. A large accumulation causes the membrane structure to become unstable. With aggregature of the toxin molecules, fragments of the membrane are released.

Despite advances made over the last two decades, there still remain considerable areas of ignorance about the precise mechanisms by which bacterial toxins disrupt membrane function. The putative pathophysiological actions of several paradigm diarrhoeal toxins are detailed in Figure 2.21. Cytolytic toxins may have subtle effects on cellular physiology at concentrations below those required for lytic effects. Current knowledge of the mechanisms of TDH and ETH and their roles in pathogenesis are considered below (sections 2.6.4 and 2.7.4).

2.6 PATHOGENICITY OF *V. PARAHAEMOLYTICUS*

2.6.1 History and importance of *V. parahaemolyticus* (Fujino, 1974)

On October 21st, 1950, an outbreak of food-poisoning occurred in southern Osaka, Japan. There were 272 hospital admissions, symptoms being those of acute gastroenteritis, and 20 (7.4 %) died subsequently. The incriminated food-source was a small half-dried sardine, known as 'shirasu' (*Engraulis japonica*). The main autopsy findings were recorded (Fujino *et al.*, 1951) and are documented with clinical symptoms in Table 2.7.

Microscopic examination of the shirasu showed that it was heavily contaminated with bacteria. The supernatant of centrifuged shirasu was innoculated onto various culture media and incubated at 37 °C under both aerobic and anaerobic conditions. No

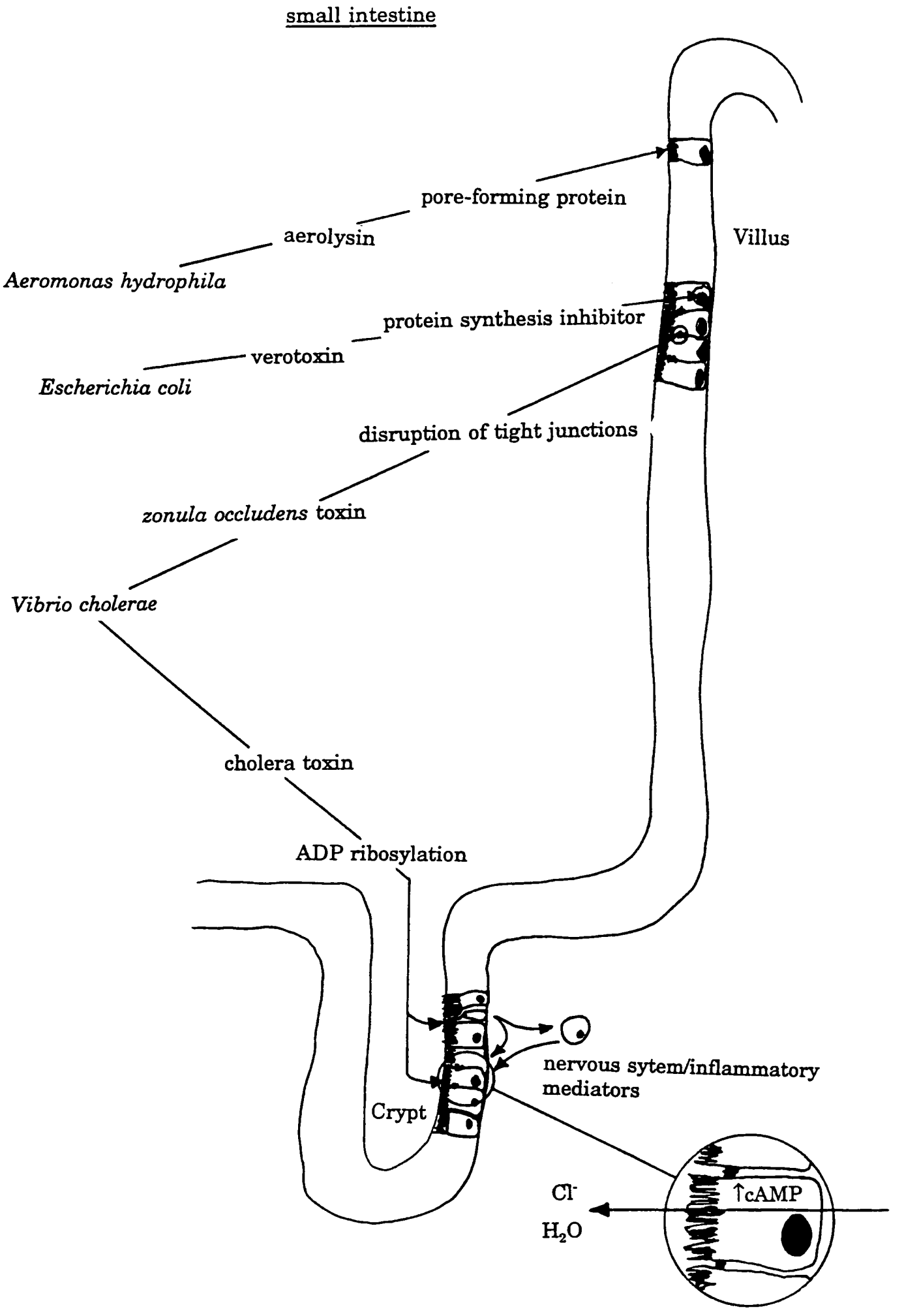


Figure 2.21 Sites of action of some paradigm diarrheogenic bacterial toxins. The putative sites of action of several bacterial toxins are given. For original studies, see Table 2.6.

(a) Clinical manifestation of gastroenteritis associated with *Vibrio parahaemolyticus*	Mizoguchi, 1963	Barker, 1974	Shaw *et al.*, 1976, *in* Joseph *et al.*, 1982
symptom	study 1: % patients (n = 2416)	study 2: % patients (n = 1211)	study 3: % patients (n = 87)
diarrhoea	94.9	98	100
abdominal pain	85.8	82	86
nausea	-	71	-
headache	39.8	42	-
vomiting	35.6	52	26
fever	35.1	27	23
lassitude	29.4		
chill	22.8	24	-
tenesmus	17.7	-	-

(b) autopsy findings from human fatalities (Fujino, 1974)
catarrh of the stomach
hyperaemia of intestinal mesentery
erosion of jejunum and ileum
congestion of various organs (eg adrenal gland)
interlobal haemorrhage in lungs

Table 2.7 Features of (a) gastroenteritis associated with *Vibrio parahaemolyticus*, and (b) autopsies of fatalities ensuing. Data are taken from the indicated references.

Salmonella, *Shigella*, *Proteus* or *Clostridium* were found. Two kinds of Gram-negative bacteria were isolated. Attempts to separate these by transfer to new agar were unsuccessful. Fujino *et al.* (1953) used an animal passage technique with subsequent innoculation onto blood agar plates to isolate a previously unidentified bacterium that was extremely pathogenic to mice. This bacterium had a non-curved long axis and was actively motile with a single polar flagellum; its movement resembled that of *V. cholerae.*

Following Skerman's key (Skerman, 1949) for the generic identification of bacteria, the bacterium was assigned to the genus *Pasteurella.* Because the characteristics of the bacterium resembled those of *Pasteurella haemolytica*, it was named *Pasteurella parahaemolytica* (Fujino *et al.*, 1951). A bacterium with identical characteristics was isolated as a result of bacteriological investigations into the cause of another outbreak of food poisoning (Takizawa, 1956). On the basis of subsequent examination, Sakazaki *et al.* concluded that the pathogenic halophilic bacteria reported by Takikawa and Fujino *et al.* were the same and that this organism should be classified in the genus *Vibrio.* Thus the organism was named *Vibrio parahaemolyticus* (Sakazaki *et al.*,1963).

2.6.2 Clinical significance of *V. parahaemolyticus*

In Japan and Taiwan, *V. parahaemolyticus* remains an extremely important diarrhoeogenic agent (Spriggs & Sack, 1990), causing 50 - 70 % of all cases of food-borne gastro-enteritis (Sakazaki & Balows, 1981). This prevalence has been confirmed in another study in Japan which found that the isolation frequency from cases of food-poisoning was higher for *V. parahaemolyticus* than any other organism (Kudoh *et al.*, 1988). All these outbreaks are associated, either directly or indirectly, with contaminated sea-food (Doyle, 1990), and it is especially common in Japan, largely due to the custom of eating raw fish.

However the importance of *V. parahaemolyticus* is not restricted to Japan and Taiwan (Barker, 1974; Ota, 1990). There are reports of outbreaks in the West Coast of USA and Mexico (Abbott *et al.*, 1989), Haikou City, China (Xu-Hy, 1989), Florida (Klontz, 1990), Barcelona, Spain (Marne & Aznar, 1988), Manila (Adkins *et al.*, 1987), and Chesapeake Bay, Maryland (Hoge *et al.*, 1989). Environmental isolation of *V. parahaemolyticus* from estuarine waters and sea-food has been achieved many times (Chan *et al.*, 1986; Kaysner *et al.*, 1990).

The role of *V. parahaemolyticus* in extra-intestinal infection is controversial, mainly

because many early reports may have incorrectly identified *V. vulnificus* as *V. parahaemolyticus* (Farmer *et al.*, 1985). *V. vulnificus* produces a potent cytolysin, known as vulnificolysin (Kreger *et al.*, 1988) that appears to be a unique cholesterol-binding, but non-thiol-activated cytolysin (Miyoshi *et al.*, 1985; Shinoda *et al.*, 1985), and is strongly associated with both primary septicaemia and wound infection (Blake *et al.*, 1980). Several more recent and well-documented case studies indicate that *V. parahaemolyticus* has also instigated extra-intestinal pathogenic episodes, for example eye infection (Tison & Kelly, 1984), wound infection (dual with *V. vulnificus*) (Plotkin *et al.*, 1990), post-operative septicaemia (Ahsan *et al.*, 1988), and reactive arthritis (Tamura *et al.*, 1993). However, extra-intestinal *V. parahaemolyticus* infection remains a rare occurrence (Farmer *et al*, 1985).

The main symptoms of gastro-enteritis caused by *V. parahaemolyticus* are diarrhoea, nausea, vomiting, abdominal pain, low-grade fever and chills (Table 2.7; Farmer *et al.*, 1985). The frequency of diarrhoea is usually less than ten times per day, though in some cases it is more than twenty times (Honda & Iida, 1993). Diarrhoea is usually watery, though it can be mucoid, bloody, or mucoid and bloody (Honda & Iida, 1993). Similarly, the disease is normally mild, although sometimes fatal. Rehydration is usually the only treatment required, although antimicrobial therapy may also be beneficial with tetracycline as the drug of choice (Farmer *et al.*, 1985).

In the absence of histopathological studies on the effect of TDH on cardiac tissues, it remains unclear whether the fatalities accorded to *V. parahaemolyticus* infection have been due to a direct cardiotoxic action (there have been ECG abnormalities in patients suffering from *V. parahaemolyticus*-associated gastroenteritis (Honda *et al.*, 1976c; Bhattacharya *et al.*, 1984)) or hypovolaemic shock, secondary to fluid loss. It is conceivable that hypokalaemia (secondary to fluid loss) (Castro, 1985), or hyperkalaemia (secondary to permeabilisation of systemic erythrocytes), has a role in the lethal pathophysiology of *V. parahaemolyticus*. Cardiovascular abnormalities have also been noted in cholera patients, where they have been attributed to dehydration, acidosis and electrolyte imbalance (Pierce & Mondal, 1974).

2.6.3 Virulence factors of *V. parahaemolyticus*

Much important evidence has been gathered concerning the role of particular virulence factors in *V. parahaemolyticus*-associated disease. The Kanagawa reaction measures the

ability of an isolate of *V. parahaemolyticus* to cause lysis of rabbit erythrocytes, manifest as a clear zone around the bacterial colony on a specially developed blood agar, Wagatsuma agar (Wagatsuma, 1968; Miwatani & Takeda, 1976). Haemolysis indicates a positive Kanagawa phenomenon (ie KP^+); a KP^- result is one in which there is no haemolysis. A positive Kanagawa reaction was found in > 96 % of pathogenic isolates, and < 1 % of environmental isolates (Miyamoto *et al.*, 1969). Strains isolated from vehicles of food poisoning also tended to be non-haemolytic (Kato *et al.*, 1965, 1966; Miyamoto *et al.*, 1969). Sakazaki *et al.* (1968) also reported that haemolytic ability on Wagatsuma agar was closely associated with the pathogenicity of *V. parahaemolyticus*, *in vivo*. Symptoms of gastroenteritis developed after accidental ingestion of 3×10^5 viable cells of a KP^+ *V. parahaemolyticus* strain (Sanyal *et al.*, 1973); in contrast, ingestion of in excess of 10^9 KP^- cells by human volunteers did not cause any symptoms (Sakazaki *et al.*, 1968). Human volunteer experiments have failed to produce diarrhoea after challenge with $(1 - 2) \times 10^{10}$ KP^- *V. parahaemolyticus* cells, whilst demonstrating diarrhoea after ingestion of only 2×10^5 to 6×10^7 KP^+ cells (Sanyal & Sen, 1974). The ligated rabbit ileal loop test has been used by several groups to test the enteropathogenicity of *V. parahaemolyticus* (Twedt & Brown, 1974; Ghosh *et al.*, 1974; Sakazaki *et al.*, 1974a,b; Zen-Yoji *et al.*, 1974; Brown *et al.*, 1977). A positive ileal loop test correlated well with KP (Takeda, 1983), although reservations have been expressed about false positive results (Johnson & Calia, 1976).

Although there is a possibility that the correlation between KP and pathogenicity is dependent upon virulence factors other than the haemolysin(s), it is likely that the haemolysin(s) has a pathogenic role. Several haemolytic factors have been isolated from cultures of *V. parahaemolyticus*, including the thermostable direct haemolysin (TDH) (Obara, 1971; Zen-Yoji *et al.*, 1971, 1974, 1975; Sakurai *et al.*, 1973; Takeda, 1983; Janda *et al.*, 1988; Honda *et al.*, 1992; section 2.6.4), the related haemolysin (TRH) (Honda *et al.*, 1988), the lecithin-dependent thermolabile haemolysin (LDH) (Taniguchi *et al.*, 1986), another distinct heat-stable haemolysin (δ VPH (Taniguchi *et al.*, 1990)), a phospholipase A (Yanagase *et al.*, 1970), a lysophospholipase (Yanagase *et al.*, 1970), and other less well characterised cytotoxic or cytotonic entities (Table 2.8). All such agents have a potential role in the pathogenesis of human *V. parahaemolyticus*-associated diarrhoea. The possible importance of factors other than TDH has been demonstrated by

Class of virulence factor	Virulence factor	Selected references
Toxins	Thermostable direct haemolysin (TDH)	Takeda, 1983 Honda *et al.*, 1992 Honda & Iida, 1993
	TDH-related haemolysin (TRH)	Honda *et al.*, 1988
	Phospholipase B	Yanagase *et al.*, 1970
	Phospholipase A & C	Yanagase *et al.*, 1970
	CHO cell elongation factor	Honda *et al.*, 1976b
	Heat-stable toxin	Taniguchi *et al.*, 1990
	Heat-labile toxin	Taniguchi *et al.*, 1986
	Cholera toxin-like factor	Kothary *et al.*, 1993 *in* Honda & Iida, 1993
	Protease	Honda & Iida, 1993
Colonisation/ invasion factors	Pili	Nakasone & Iwanaga, 1990, 1991, 1992 Yamamoto *et al.*, 1990a
	Adhesive factors	Yamamoto & Yokota, 1989
	Haemaglutinins	Yamamoto & Yokota, 1989 Eko *et al.*, 1992 Nagayama *et al.*, 1993

Table 2.8 Virulence factors of *Vibrio parahaemolyticus*.

observing lethal toxicities of *V. parahaemolyticus*-derived, TDH-negative, crude haemolysin preparations in 4 - 6 week old mice (Sarkar *et al.*, 1987).

In addition to putative toxic entities, factors conferring an ability to colonise a site have also been proposed as important virulence factors (Finlay & Falkow, 1989). For *V. parahaemolyticus*, such factors include pili (Nakasone & Iwanaga, 1990, 1991, 1992; Yamamoto *et al.*, 1990a), haemagglutinins (Yamamoto & Yokota, 1989; Eko *et al.*, 1992; Nagayama *et al.*, 1993) and other factors increasing adherence (Yamamoto & Yokota, 1989) (Table 2.8).

2.6.4 Role of the thermostable direct haemolysin (TDH)

This subject has been addressed by several reviews (Takeda, 1983; Ljungh & Wadstrom, 1983; Janda *et al.*, 1988; Takeda, 1988; Honda & Iida, 1993). The thermostable direct haemolysin (TDH) was established as the major KP^+-associated haemolysin (hence favoured as the primary virulence factor) by the work of Sakurai *et al.* (1973, 1975, 1976), where a sensitive immunodiffusion technique with antiserum to TDH was used to demonstrate that TDH was produced only by KP^+ strains. The gene (tdh) for this toxin has been cloned (Kaper *et al.*, 1985) and sequenced (Nishibuchi & Kaper, 1985). This sequence is in good agreement with the amino-acid sequence obtained by chemical means (Tsunasawa *et al.*, 1987).

(i) Purification. Purification of TDH from the culture supernatants of KP^+ *V. parahaemolyticus* has been achieved, after selective ammonium sulphate precipitation, by a variety of column chromatographic procedures (Obara, 1971; Zen-Yoji *et al.*, 1971, 1974, 1975; Nikkawa *et al.*, 1972; Sakurai *et al.*, 1973; Honda *et al.*, 1976c; Miyamoto *et al.*, 1980; Takeda, 1983). Zen-Yoji *et al.* (1974) obtained two kinds of purified TDH which differed in their mobilities on polyacrylamide gel electrophoresis; however this anomaly has not been observed by other investigators. The purified toxin was found to be a protein free from lipid and carbohydrate, having a molecular weight of about 42 kd by gel filtration (Honda *et al.*, 1976c; Miyamoto *et al.*, 1980), suggesting a homodimer structure, with each subunit having a molecular weight of c. 21 kd, by SDS-PAGE (Honda *et al.*, 1976a; Takeda *et al.*, 1978; Miyamoto *et al.*, 1980). The purified toxin is designated thermostable because heating at 100 °C for ten minutes causes no decrease in subsequent biological activity of this molecule (Zen-Yoji *et al.*, 1971; Douet *et al.*, 1992; Honda & Iida, 1993). The amino acid sequence has been determined (Nishibuchi &

Kaper, 1985; Tsunasawa *et al.*, 1987) showing that the TDH subunit consists of 165 amino acid residues with one disulphide bridge near the C-terminus (Cys^{151}-Cys^{161}).

Purification of quantities of toxins that are viable for subsequent testing requires systems that produce a large amount of toxin, as free from contaminant proteins as possible. The work of Cherwonogrodzky and Clark (1981, 1982a,b, 1984) has been seminal to commercial (Sigma) TDH production in this respect. It was found that production of Kanagawa haemolysin was under the control of external pH (rather than directly on the type of carbohydrate present) and essentially independent of the culture growth phase. Using pH-stat methodology, they identified the optimal pH to be close to 6.2. Optimal growth conditions and purification protocol for haemolysin production have been defined (Cherwonogrodzky & Clark, 1982b) and used (Sigma Chemical Co., 1992); nonetheless, the purity of this preparation has been questioned recently (Douet *et al.*, 1992), since the commercially produced preparation yielded an extra band after silver staining on SDS-PAGE.

(ii) Biological activities. TDH has been shown to be (a) haemolytic, (b) lethal, (c) cytotoxic to a variety of cell types including FL cells, HeLa cells, and cardiac myocytes, and (d) enteropathogenic.

(a) TDH as an haemolysin. Haemolytic activity of TDH is not dependent on the addition of lecithin (Sakurai *et al.*, 1973), hence it is termed the thermostable direct haemolysin. TDH and related molecules (see later) have a range of haemolytic activities on erythrocytes from different species, but interestingly have no activity on horse erythrocytes (Honda *et al.*, 1976c; Miyamoto *et al.*, 1980; Yoh *et al.*, 1986a,b; Yoshida *et al.*, 1991; Honda *et al.*, 1990). The haemolytic activity of purified TDH can be destroyed by pepsin or α-chymotrypsin treatment, but not by trypsin treatment (Zen-Yoji *et al.*, 1976). The neuraminidase-sensitive gangliosides G_{D1a} and G_{T1}, have been implicated as receptor entities for TDH, in part because toxin pre-incubation with these gangliosides decreases subsequent TDH activity, and further because thin layer chromatographs of the lipid constituents of horse erythrocyte membranes reveal an absence of these molecules (Takeda *et al.*, 1975, 1976). However Honda & Iida (1993) reveal, somewhat vaguely and without giving corroborative data, that 'inhibition experiments with gangliosides could not explain the different specificity of the TDH/TRH family of toxins against erythrocytes from various animals'. Honda *et al.* (1992) have reported several aspects of the mechanism of

TDH-induced haemolysis. It is the end result of colloid osmosis (also supported by findings of Chapter 4), at least a two step process (binding and lesion formation - first proposed by Sakurai *et al.* (1975)). Honda *et al.* (1992) further produced micrographic evidence that TDH is a pore-forming protein, although they noted a difference between the recorded functional (c. 1 - 2 nm) and structural (c. 30 - 35 nm) pore diameters. Such a discrepancy has been reported previously for other pores, for example that formed by staphylococcal α-toxin (Fussle *et al.*, 1981).

(b) TDH as a lethal agent. The lethal toxicity of *V. parahaemolyticus* to mice and guinea-pigs was reported by Fujino *et al.* (1953) in the seminal publication on the isolation of this organism. Purified TDH preparations were demonstrated to have a potent lethal toxicity to various experimental animals (Obara *et al.*, 1974; Zen-Yoji *et al.*, 1974, 1975; Honda *et al.*, 1976c) including mice, rats and guinea-pigs. For example, an intra-venous injection of 5 μg TDH has been shown to kill mice within 1 minute (Honda *et al.*, 1976c). The rapid death of the animals is thought to be due to the cardiotoxicity of TDH (Honda *et al.*, 1976a,c,d; Goshima *et al.*, 1977). Honda *et al.* (1976c) purified the lethal toxin from culture supernatants of KP^+ *V. parahaemolyticus* and observed that when fractions from successive column chromatographic procedures were assayed for both haemolytic activity and lethal activity in mice, the final preparation was a single homogeneous band on SDS-PAGE and these two parameters did not dissociate during the purification procedure. It was therefore concluded that TDH was the lethal toxin produced by *V. parahaemolyticus*. Although clinical sources tend to view *V. parahaemolyticus* as an almost exclusively intestinal pathogen (Farmer *et al.*, 1985), there is apparently no evidence excluding cardiotoxic action as being the cause of death in the fatalities recorded. Rats were injected intravenously with purified TDH and their electrocardiogram (ECG) and electroencephalogram (EEG) recorded simultaneously. Dramatic changes in ECG were documented well in advance of EEG changes (Honda *et al.*, 1976a,d). Lethal toxicity was lost (similarly to haemolytic activity) when TDH was preincubated with the gangliosides G_{D1a} and G_{T1}, whilst the gangliosides G_{M1} and G_{M2} had no such protective effect (Takeda *et al.*, 1976).

(c) TDH as a cytotoxin. TDH is cytotoxic to a range of cultured cells, including HeLa cells (Sakazaki *et al.*, 1974a), L cells (Sakazaki *et al.*, 1974a), FL cells derived from human amniotic membrane (Sakurai *et al.*, 1976), neuroblastoma cells (Honda & Miwatani

in Takeda, 1983), primary cultures of foetal mouse heart cells (Goshima *et al.*, 1978), and CCL-6 cells derived from human intestine (Takeda *et al.*, 1980). Studies on the effects of TDH on FL cells (Sakurai *et al.*, 1976) showed that when 6.6 x 10^5 cells$(ml)^{-1}$ were incubated at 37 °C with TDH (5 μg$(ml)^{-1}$), cell death (measured by ability to exclude Trypan Blue or release of alkaline phosphatase) started within 20 minutes, and was 95 % complete after 60 minutes. Prior to death, scanning electron microscopy of TDH-treated FL cells showed that there were marked changes in the morphology of the microvillous region of cells, the number and form of microvilli decreasing with time after the addition of toxin. The cardiotoxicity of TDH (described above, in (b) for whole animal experiments) was confirmed in cultured mouse heart cells (Honda *et al.*, 1976b) and in single isolated myocardial cells (Goshima *et al.*, 1977). In a further series of experiments, Goshima *et al.* (1978) found that the cessation of beating of mouse myocardial cells due to TDH toxicity was dependent on the presence of Ca^{2+} in the extracellular medium. Seyama *et al.* (1977) studied the effect of TDH on cells of the sino-atrial node and the right atrium of rabbits and showed that reduction in membrane resistance and depolarisation of the membrane, without any effect on the mechanism generating the action potential, were the major responses of both nodal and atrial cells to TDH. This depolarisation appeared to be the result of an increase in Na^+ conductance.

(d) TDH as an enterotoxin. Injection of 500 μg TDH can cause the development of turbid body fluid accumulation in ligated rabbit ileal loops (Zen-Yoji *et al.*, 1974). Histopathological analysis of such preparations reveals erosive lesions and desquamation of necrotic mucosa, concurrent with marked neutrophil infiltration into the intestinal wall (Zen-Yoji *et al.*, 1974; Takeda, 1983). Studies of suckling mice challenged orally with purified TDH (Obara *et al.*, 1974; Miyamoto *et al.*, 1980) demonstrated that administration of 2.0 - 12.5 μg TDH was sufficient to cause a transient diarrhoea, and death in some animals. Histopathological changes in the small intestine of animals experiencing diarrhoea after a relatively small TDH dose, were minimal. Light microscope examination showed the *lamina propria* to be odoematous and in ultrathin sections, intercellular junctions appeared to have been lost. With larger TDH doses more destructive changes were seen in the intestinal mucosa. This aspect of TDH action is consistent with the occasional finding of blood in the stools of some patients with gastro-enteritis of *V. parahaemolyticus* aetiology. The likely importance of TDH in *V.*

parahaemolyticus-associated disease has been confirmed by an elegant *in vitro* site-directed mutagenesis and Ussing Chamber study (Nishibuchi *et al.*, 1992). The culture filtrate of a parental TDH^+ strain caused a significant increase in short-circuit current (I_{SC}) across isolated rabbit ileal tissue compared with that induced by the culture filtrate of the derived TDH^- mutant. Return of the cloned *tdh-1* gene to the TDH^- mutant restored the ability of the mutant to increase I_{SC}. Furthermore, the capacity of a TDH-containing filtrate to increase I_{SC} was markedly inhibited by the addition of anti-TDH antiserum. Microscopic evaluation of the exposed rabbit ileal mucosa revealed no evidence of histological damage in response to TDH. It therefore appears that, at concentrations likely to be relevant pathophysiologically, TDH may act to perturb membrane (and epithelial) transport by mechanisms more subtle than cytolysis (Figure 2.22).

However, there is still no definitive understanding of the putative mechanism of TDH action (Bernheimer & Rudy, 1986) although recent morphological and haemolysis data (Honda *et al.*, 1992) are held to suggest that TDH is a pore-forming protein. The techniques of cellular physiology have been only scantily applied, and may greatly aid the definition of TDH-induced membrane transport modifications. Possible pathogenic roles for TDH are summarised in Figure 2.22.

(iii) Biology of TDH and V. parahaemolyticus. Further research by Clark & Cherwonogrodzky (1984) showed that certain KP^- isolates of *V. parahaemolyticus* from patients suffering acute gastro-enteritis could be 'switched' to a KP^+ phenotype, producing Kanagawa haemolysin, if they were 'induced' by D-Tryptophan. In addition, continued growth of KP^+ *V. parahaemolyticus* under optimal pH conditions for haemolysin resulted in a conversion of the KP^+ culture to a KP^- form, eventually testing negative for KP on Wagatsuma agar. The dependence of KP phenotype-switching on extracellular conditions helps explain why KP^+ isolates are obtained from patients' stools whilst the incriminated food-source yields only KP^- strains (Hoashi *et al.*, 1990). When a specific gene probe for the *tdh* gene was used on *V. parahaemolyticus*, the presence of the gene could be demonstrated in 16 % of apparently KP^- strains (Nishibuchi *et al.*, 1985). In light of these data, $KP^{-/+}$ switching was also been examined in *V. parahaemolyticus* strains passaged in mice (Karunasagar *et al.*, 1989). After two passages, some KP^- strains gave rise to haemolytic (KP^+) strains, where both heat-labile and heat-stable toxins could be induced, according to the strain examined. However, the heat-stable toxin so induced was

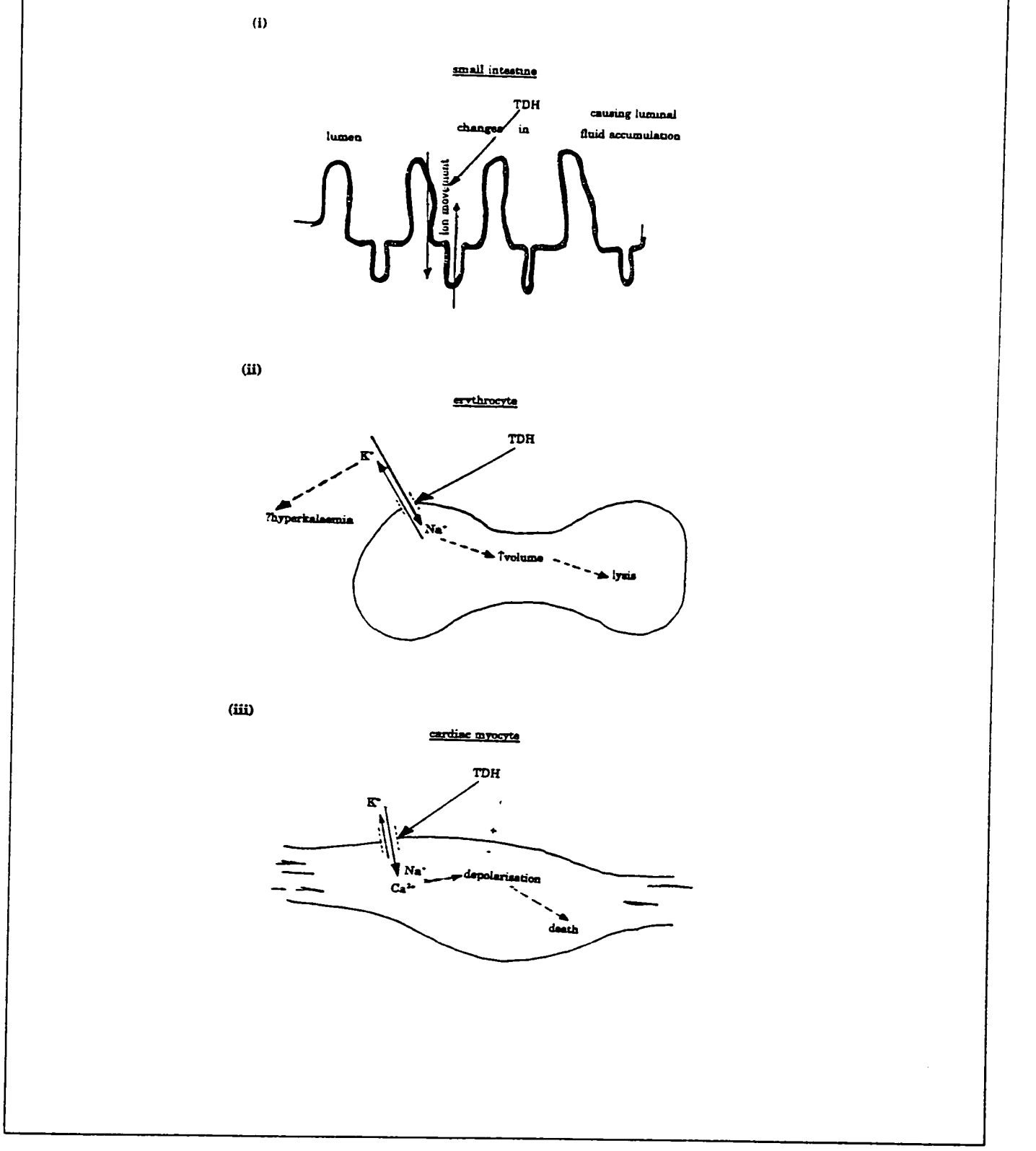


Figure 2.22 Possible actions of TDH in human disease. TDH has been shown to effect three targets: (i) small intestine, (ii) erythrocytes, and (iii) cardiac myocytes. See text for details. It is not clear which, if any, of these actions play a role in the pathogenesis of *V. parahaemolyticus* (see text).

immunologically distinct from the TDH produced by other KP^+ strains of *V. parahaemolyticus*.

Recently, the virulence of both TDH^+ and TDH^- strains of *V. parahaemolyticus* was examined in mice (Hoashi *et al.*, 1990), in conjunction with intra-peritoneal and orogastric challenge models. In mice killed by either type of challenge, pathological changes were virtually always observed, involving swelling, redness and fluid accumulation within the small intestine. Histologically, congestion, edema and vacuolation could be observed in mucosal and submucosal tissues, with ulceration at the tips of some villi. Lethality and pathological findings occurred similarly in both challenge models and irrespective of the TDH phenotype used. These investigators therefore suggested that enteropathogenesis caused by *V. parahaemolyticus* is attributable to a virulence factor other than TDH. For both types of challenge used, *V. parahaemolyticus* was isolated from the blood after death. Unfortunately, no reference is made to any testing of these *V. parahaemolyticus* isolates for KP, results of which would have been most interesting in light of the earlier work (see above; Karunasagar *et al.*, 1989) on KP phenotype switching. The abstraction of *V. parahaemolyticus* from the blood of mice that died after challenge is consistent with the hypothesis that a cardiotoxic action may be the pathophysiological mechanism by which *V. parahaemolyticus* causes death. Whilst factors other than TDH may be involved, TDH is not in any way excluded as a virulence factor by this work.

Much experimental data on *V. parahaemolyticus* and KP/TDH has been gathered in terms of genetic manipulation and molecular biology, including the cloning of the tdh gene (Kaper *et al.*, 1984a,b), determination of the nucleotide sequence of the tdh gene (Nishibuchi & Kaper, 1985), development of methods to detect the tdh gene and related DNA sequences by the DNA colony hybridisation technique (Nishibuchi *et al.*, 1985), and data on the duplication and variation of the tdh gene (Nishibuchi & Kaper, 1990). Such methodologies indicate that *tdh* can be transferred to other members of the *Vibrio* genus (Honda *et al.*, 1992; Honda & Iida, 1993), specifically *V. mimicus* (Terai *et al.*, 1990; Yoshida *et al.*, 1991), *V. hollisae* (Yoh *et al.*, 1986b; Yoh *et al.*, 1989; Yamasaki *et al.*, 1991) and *V. cholerae* non-O1 (Honda *et al.*, 1986a; Yoh *et al.*, 1986a; Baba *et al.*, 1991; Terai *et al.*,1991). Therefore TDH (and related peptides) potentially underly the diarrhoea induced by a range of *Vibrio* species.

2.7 PATHOGENICITY OF *V. CHOLERAE*

2.7.1 History of *V. cholerae*

Historically, cholera has had devastating effects on morbidity and mortality, particularly in areas lacking in hygiene and sanitation (Barua & Burrows, 1974; Feachem, 1981; Glass *et al.*, 1992; Tauxe & Blake, 1992). Until recently, only serogroup O1 of *V. cholerae* was associated with *cholera gravis*: profuse watery diarrhoea leading to life-threatening dehydration (Pierce & Mondal, 1974; Morris & Black, 1985; Tauxe & Blake, 1992). *V. cholerae* is taxonomically divided into two biotypes, El Tor and classical, and into two serogroups, Inaba and Ogawa. These bacteria achieved their pathogenic status by virtue of their capability to express the ToxR global regulation element, cholera toxin (CT) and toxin-coregulated pili (Herrington *et al.*, 1988). Recently, a *V. cholerae* non-O1 (*V. cholerae* O139) emerged to cause widespread *cholera gravis* in the Indian subcontinent, and is now recognised as the cause of the eighth cholera pandemic since 1817 (Albert *et al.*, 1993; Nair *et al.*, 1993; Ramamurthy *et al.*, 1993). This organism resembles O1 El Tor cholera strains from the seventh pandemic (as opposed to other non-O1 groups tested) in virulence genes, genomic background and expressed virulence factors (Hall *et al.*, 1993). The pathophysiology instigated by CT has been touched on already (Chapter 1; section 2.5; Cassuto *et al.*, 1982, 1983; Moss & Vaughan, 1986, 1988a,b, 1989; Jodal *et al.*, 1993). However CT-negative (*ctx*A$^-$) and non-O1, non-O139 strains are also associated with gastrointestinal illness (Blake *et al.*, 1980; Morris & Black, 1985).

2.7.2 Clinical significance of CT-negative *V. cholerae*

The relative frequency of non-O1 *V. cholerae* gastroenteritis is difficult to determine because most hospital laboratories do not use a stool-culture medium that is selective for members of the *Vibrio* genus (Wilson *et al.*, 1981; Morris & Black, 1985). Non O1 *V. cholerae* gastroenteritis shows a variety of symptoms that are different from those of cholera, especially abdominal cramps, fever, and mucous and bloody stools (Hughes, 1978; Blake, 1980; Morris & Black, 1985). This suggests that factors other than CT-like toxins are involved (Ichinose *et al.*, 1987). Gastroenteritis as a result of non-O1 *V. cholerae* is predominantly associated with either consumption of raw seafood (Morris *et al.*, 1981; Wilson *et al.*, 1981) or travel (Morris *et al.*, 1981), particularly with visits to Mexico (MMWR, 1969c). Patients with wound or ear infections usually have a history of sea-water exposure (Hughes *et al.*, 1978).

CT$^-$ strains engineered from pathogenic parent strains tend to retain a degree of pathogenicity. Attempts have been made to manufacture a live oral cholera vaccines with recombinant DNA technology (Kaper *et al.*, 1984a,b; Levine *et al.*, 1988a,b; Michalski *et al.*, 1993), in which generated mutants have precise deletions of genes encoding putative virulence factors, but other surface antigens and gene products are left intact. Volunteer studies involving ingestion of two candidate vaccine strains, JBK70 (El Tor, Inaba) and CVD101 (classical, Ogawa), derived from parent strains by deletion of the genes encoding CT (Levine *et al.*, 1988b) demonstrated that although a single oral dose of either vaccine was readily capable of colonising the human intestine and eliciting a potent vibriocidal response, even small doses of such mutants caused mild diarrhoea in approximately one half of the recipients. This degree of reactogenicity was deemed unnacceptable (Levine *et al.*, 1988b). By contrast, the classical Inaba vaccine candidate CVD103-HgR (*ctx*A and HgR cassette inserted into *hly*A locus) was well tolerated and conferred a high level of protective immunity against homologous challenge with its parent strain, 569B (Levine *et al.*, 1988a; Tacket *et al.*, 1992). Although CVD103-HgR gave 100 % protection against severe cholera-like diarrhoea in a heterologous challenge study using *V. cholerae* N16961 (El Tor, Inaba) and E7946 (El Tor, Ogawa), a reduced protective efficacy (62%) against any diarrhoea was observed (Tackett *et al.*, 1992; Lagos *et al.*, 1993), that is some vaccinees developed mild diarrhoea. Accordingly a new attenuated live vaccine candidate, of El Tor biotype, was developed (Michalski *et al.*, 1993) in which all known toxin (CT (Moss & Vaughan, 1988a,b), ZOT (Fasano *et al.*, 1991), ACE (Trucksis *et al.*, 1993), ETH (Hall & Drasar, 1990; Yamamoto *et al.*, 1990b)) genes were deleted. Whilst this strain engendered high antibody titres, it still caused diarrheoa and other symptoms, indicating that there are additional factors responsible for the adverse reactions of Δ*ctx* (ie *ctx*-deleted) *V. cholerae* strains (Michalski *et al.*, 1993), which may also be important in pathogenic isolates of CT$^-$ *V. cholerae*.

2.7.3 Plausible virulence factors of CT-negative *V. cholerae*

Most cholera toxin-negative strains of *V. cholerae* lack any cholera toxin-like activity (Morris *et al.*, 1981), and yet have other biological activities (Morris & Black, 1985). Furthermore (section 2.7.2) there are several other plausible accessory virulence factors in CT$^-$ *V. cholerae*. In particular, various *ctx*A strains can: (i) cause diarrhoea and death in infant rabbits and fluid accumulation in rabbit ileal loops (Spira *et al.*, 1978), and (ii)

colonise the small intestine and cause diarrhoea in the RITARD (removable intestinal tie-adult rabbit diarrhoea) model (Spira *et al.*, 1983). Such strains have also been shown to produce extracellular toxins, including (i) an enterotoxin also produced by *V. mimicus* and *V. fluvialis* (Nishibuchi & Seidler, 1983), (ii) a TDH-related haemolysin (Honda *et al.*, 1986a; Baba *et al.*, 1991; Terai *et al.*, 1991), and (iii) an haemolysin that is toxic to tissue-culture cells and lethal when injected into mice (Yamamoto *et al.*, 1984). This latter haemolysin is structurally and immunologically indistinguishable from the El Tor haemolysin (ETH) (Yamamoto *et al.*, 1984, 1986) and has enterotoxic activity (section 2.7.4; Ichinose *et al.*, 1987).

2.7.4 Role of ETH

(i) Purification and characterisation of ETH. The *V. cholerae* biotype El Tor was the cause of the seventh cholera pandemic (Honda & Finkelstein, 1979). This biotype was differentiated from classical *V. cholerae* on the basis of their ability to cause lysis of sheep and goat erythrocytes, in the Greig test (Greig, 1914; Finkelstein, 1973). However, during the course of the pandemic, most El Tor *Vibrio* isolates lost their capacity to express the hemolysin strongly (Finkelstein, 1966). Treatment of the haemolysin at 55 °C for 15 minutes almost completely destroyed haemolytic and lethal activities of the toxin (Honda & Finkelstein, 1979). Efforts to purify and characterise ETH produced biochemical data of an apparently contradictory nature; analyses of cloned haemolytic determinants indicated a M_w (80 - 84 kd) (Goldberg & Murphy, 1984, 1985; Manning *et al.*, 1984) rather greater than that found for the purified haemolysin (Hall *et al.*, 1985; Sutton *et al.*, 1985; Yamamoto *et al.*, 1986). Hall & Drasar (1990) clarified this situation, using survivor selection and amino-terminal microsequencing techniques to show that ETH is processed extracellularly by amino-terminal proteolysis; 83.2 kd, 68.6 kd, and 65.3 kd forms were identified. The structural gene for ETH has been sequenced (Yamamoto *et al.*, 1990b).

(ii) Action of ETH. Although the haemolytic properties of *V. cholerae* have been the subject of many investigations (Goto *et al.*, 1959; Watanabe & Seaman, 1962; Feeley & Pittman, 1963; Roy *et al.*, 1963; Ganguly *et al.*, 1966; Watanabe & Verwey, 1966; Kalsow & Newman, 1968a,b; Nath *et al.*, 1978), the pathogenic role of the El Tor haemolysin remains unclear. Honda and Finkelstein (1979) showed that purified ETH was rapidly lethal for mice, and cytotoxic for cultured Y-1 adrenal cells and beating rat heart

cells (Nath *et al.*,1978). Haemolytic activity was: (i) optimum at a pH near neutral, (ii) unaffected by the addition of divalent cations (Mg^{2+}, Ca^{2+} and Mn^{2+} at 5 mM), (iii) inhibited by mixed gangliosides (this inhibition being insensitive to the addition of choleragen, suggesting a factor other than G_{M1} is responsible for the inhibition), (iv) 'direct' in that addition of lecithin did not enhance its activity (Sakurai *et al.*, 1973), and (v) higher with rabbit, goat, mouse and sheep, and lower with chicken, horse and human erythrocytes, this spectrum differing from that of TDH of *V. parahaemolyticus* (Zen-Yoji *et al.*, 1971). On the basis of gel-filtration analysis, Honda and Finkelstein (1979) accorded ETH a M_w of 20 kd. This value contrasts markedly with other data indicating rather higher M_ws (Hall & Drasar, 1990). More recently, Ichinose *et al.* (1987) demonstrated ETH to be a potential enterotoxin because it was capable of causing fluid accumulation in ligated rabbit ileal loops (after De & Chatterjee, 1953), and the suckling mouse after peroral innoculation.

(iii) Biology of ETH and V. cholerae. Expression of the structural gene for ETH (*hly*A) does not correlate with disease symptoms, there being both virulent CT^+ *hly*A^- (classical biotype) strains and avirulent CT^- *hly*A^+ strains (eg strains 1074 and 1196; Levine *et al.*, 1982, 1988a,b). However, this by no means excludes the possibility that ETH plays a role in the pathogenesis of disease caused by haemolytic *V. cholerae* biotype El Tor and CT^- *V. cholerae*. It is noteworthy that the first pathogenic isolates of the El Tor biotype had produced dysenteric symptoms (Gotschlich, 1906). The structural gene for the potent cytotoxin vulnificolysin of *V. vulnificus* has considerable regions of homology with that encoding ETH (*hly*A) (Yamamoto *et al.*, 1990c). Further studies on the action of ETH may help resolve the enigma it represents.

2.8 MEMBRANE PHYSIOLOGY OF CELLS USED AS TARGET SYSTEMS

2.8.1 The erythrocyte as a model membrane system

The applicability of the erythrocyte to physiology is well summarised by Eric Ponder (1948): '...I tend to speak of the red cell as if it were a microcosm, and as if an understanding of its nature and properties would include an understanding of nearly everything else in the cellular world. To some extent this is true...'.

The erythrocyte is indeed a well characterised system for the investigation of membrane transport and its perturbations (Agar & Board, 1983; Ellory & Tucker, 1983; Harvey &

Ellory, 1989). It is an excellent model system because red cells: (i) are readily available, (ii) are easily manipulated by well established techniques, (iii) have well characterised membrane transport properties (Figure 2.23), (iv) have membrane transporters of similar characteristics to those identified in other tissues, (v) form an essentially homogeneous population, (vi) are a 'dead-end' cell-type, with relatively few metabolic events to complicate transport processes, and (vii) are enucleate and therefore do not exhibit complicated effects dependent on subsequent genetic expression.

The important aspects of human erythrocyte membrane transport (Ellory & Tucker, 1983) are shown in Figure 2.23. Red blood cells constitute an especially good system in which to investigate the action of TDH and ETH because (i) the segregation of haemolytic phenotype with pathogenic strains of the bacterium (see Kanagawa phenomenon, section 2.6.3), and (ii) results obtained from erythrocytes may provide a basis for speculation on plausible pathogenic roles of cytolysin in other cell types, with particular emphasis being accorded to cardiomyocytes and solute-transporting epithelial cells.

Erythrocytes from different species have rather different membrane properties (Ellory & Tucker, 1983). Accordingly, they are an interesting tool to probe aspects of membrane pathophysiology in comparative terms.

2.8.2 Chinese hamster ovary (CHO) cells

The cell physiology of these cells is not well understood, although they have long been used as a convenient assay-system for cholera toxin (CT) (Guerrant *et al.*, 1974; Hall *et al.*, 1993), CT-like toxins (Guerrant *et al.*, 1974) and other toxins (Hall & Drasar, 1990). CT and CT-like toxins stimulate cAMP production in these cell types which subsequently undergo a dramatic elongation (Guerrant *et al.*, 1974). Other toxins, operating by a distinct mechanism involving membrane attack, may cause lysis of these cells (Hall & Drasar, 1990). Recently, aspects of the physiology underlying CHO cell elongation have become clearer. Platelet activating factor (PAF) antagonists were inhibitory to CHO cell elongation induced by both CT and the membrane permeant cAMP analogue dibutyryl cAMP, and PAF itself stimulated cAMP-induced CHO cell elongation, leading to the proposal that increases in cAMP cause the production of PAF which then acts as an autacoid to stimulate cell elongation, possibly via prostaglandin systhesis (Thielman *et al.*, 1993).

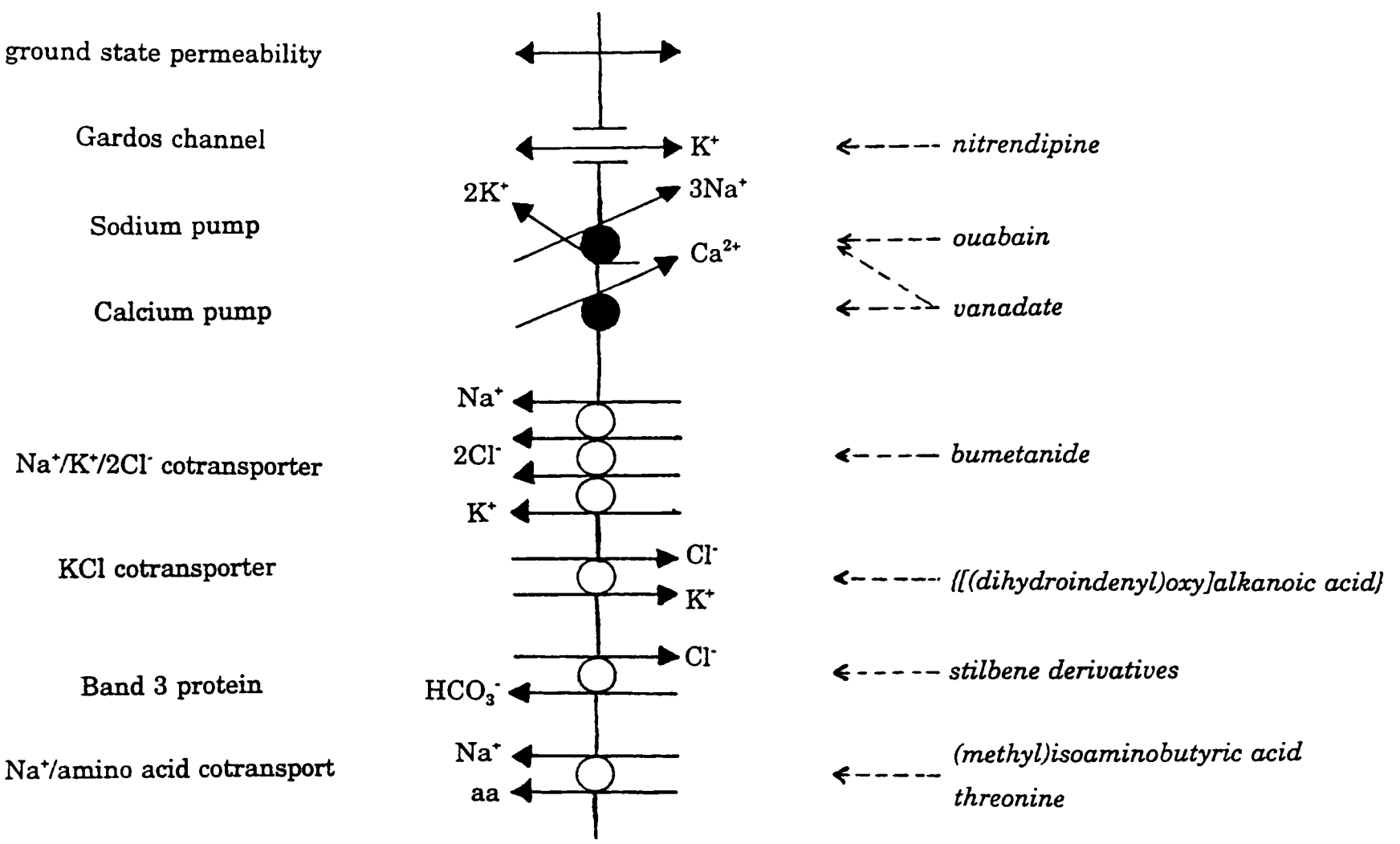


Figure 2.23 Membrane physiology of human erythrocytes. Derived from data in Agar & Board (1983). Open circles designate carriers, closed circles designate primary active transport systems; a channel is represented by two short parallel lines. Inhibitors of these systems are given.

2.8.3 Intestinal cell preparations

Enzymatically isolated cells (Kimmich, 1975; Ilundain, 1991) appear to maintain the membrane transport properties outlined above (Figure 2.17; Ilundain, 1991), although they lose the vectorial properties that characterise their epithelial structure and function.

This chapter has established the importance of, and reviewed the literature pertinent to, study of the effects of TDH and ETH (of *V. parahaemolyticus* and *V. cholerae*, respectively) on membrane function. The aims and methods so suggested are discussed in the following chapter.

CHAPTER 3

Aims, principles and methods

'"I need your clothes, your boots and your motorcycle..." (The Terminator)'
James Cameron, *in* Terminator II

3.1 AIMS

The case for study of the pathophysiological actions of diarrhetic bacterial toxins has been made in Chapter 1. The *Vibrio* cytolysins (in particular TDH) are assigned to an 'individualistic agents' category in the cytolytic peptide classification of Bernheimer and Rudy (1986), having no well defined mechanism of action. In an attempt to bridge this divide between established microbiology and clinical observation, the aims of this project were: (i) to obtain purified preparations of *Vibrio*-secreted exoproteins that are candidate virulence factors (Chapter 6), (ii) to characterise the salient biophysical features of the lesions induced in erythrocyte membranes by these factors (Chapters 4, 5, 7, 8), (iii) to identify perturbations of endogenous membrane function that might have pathogenic corollaries (Chapter 5, section 5.2.4), and (iv) to examine toxin action on an enterocyte target membrane (Chapter 9). In the following sections, materials and experimental procedures are described in detail.

3.2 GENERAL METHODS

3.2.1 Chemicals

Reagents used were of analytical grade and obtained from Sigma Chemicals Company (Poole, Dorset), unless otherwise stated. Hyaluronidase was type I-S, from ovine testis, and neuraminidase was type X, from *Clostridium perfringens*. Coomassie blue R-250 was obtained from Hoefer Scientific Instruments (San Francisco, California). Special classes of chemicals are considered below:

(i) Drugs. Nitrendipine (Bayer Pharmaceuticals), ouabain and bumetanide were made up as 10 mM stock solutions in dimethylsulphoxide (DMSO), MOPS-buffered saline (Table 3.1), and water with trace Tris base, respectively. 4,4'-Diisothiocyano-2,2' stilbene disulphonic acid (DIDS) and phloretin were made up as 25 mM stock solutions, in DMSO.

(ii) Radioisotopes. The radioisotopes $^{86}Rb^{+}$ and $^{45}Ca^{2+}$ (specific activities c. 8 - 10

Name: description	Constituents (mM exc. where stated)	pH (at room temperature)
MBS: MOPS buffered saline	NaCl (145), MOPS (10), glucose (5)	7.4 by addition NaOH (1 M)
MWS: $MgCl_2$ wash solution	$MgCl_2$ (107), MOPS (10)	7.4 by addition Tris base
ACD: Acid citrate dextrose	Na citrate (85), citric acid (71), glucose (111)	4.6
GCS: Gut cell solution	NaCl (80), $CaCl_2$ (1), D-mannose (10), K_2HPO_4 (3), $MgCl_2$ (1), Trizma-HCl (55), DL-β-hydroxybutyrate (0.5), L-glutamine (2.5), BSA (1 $mg(ml)^{-1}$)	7.4 by addition NaOH (1 M)

Table 3.1 Physiological solutions.

mCi(ml)$^{-1}$) were obtained (as cation chlorides) from NEN Research (Dupont (UK) Ltd., Stevenage, UK).

(iii) Kanagawa haemolysin. Kanagawa haemolysin (KH) was supplied by Sigma as a lyophilised powder containing approximately 50 % protein which was balanced with Tris/HCl buffer, EDTA, phenyl methyl sulphonyl fluoride and sodium azide. One haemolytic unit (HU) is defined by Sigma (Sigma Chemical Co., 1992) as that amount that will cause 50 % haemolysis of a 1 % erythrocyte suspension in phosphate buffered saline at pH 7.0 after 2 hours incubation at 37 °C followed by refrigeration for 12 - 24 hours at 4 °C. KH (1000 HU) was reconstituted into 10 ml of MBS (section 3.2.2; Table 3.1). Aliquots (0.5 ml) were stored at - 22 °C and thawed immediately prior to use.

3.2.2 Physiological solutions

Several solutions were commonly used and their compositions are listed in Table 3.1. Solution pH was set to the desired value by the addition of HCl, NaOH or Tris base, at 22 °C. Osmolarites were measured using either a Wescor 5100C vapour pressure or an automatic Camlab freezing-point osmometer (Roebling).

3.2.3 Incubations

Haemolysis and flux experiments were performed using a thermostatically controlled Camlab water bath at 37 ± 0.05 °C, or at other temperatures in a cooling water bath.

3.2.4 Centrifugation protocols

Various centrifugation procedures were used in the course of this project; details of each protocol are summarised in Table 3.2. Hereafter any centrifugation procedure (CP) is referred to by its given number.

3.3 CELL PREPARATIONS

This investigation employed erythrocytes (of several species), isolated rabbit intestinal cells, and Chinese hamster ovary (CHO) cells as targets for the action of putative diarrhetic toxins elaborated by members of the *Vibrio* genus. Cells were obtained as follows:

3.3.1 Erythrocytes

Human erythrocytes were either taken fresh from healthy adult donors (into either heparin (10 IU(ml)$^{-1}$) or acid citrate dextrose (ACD)) or obtained as outdated bank blood. Horse and hagfish (*Eptatretus stouti*) erythrocytes were obtained by venepuncture into

CP No.	centrifuge	rotor	force (g)	time (mins)
1	Sorvall	standard	3000	5
2	RT6000B		250	5
3	Eppendorf	standard	10000	0.167
4	microfuge			0.333
5	5412/5414			10
6	Sorvall RC-5B	GSA	4923	10
7	refrigerated		10415	20
8	superspeed	SS-34	4302	20
9	centrifuge		11951	4
10				20
11				90
12	Tomy	standard	10000	20
13				5
14	micro-Hct	standard	5000	3.5

Table 3.2 Centrifugation protocols used.

heparinised MBS or hagfish saline (NaCl (510 mM), MOPS (10 mM), glucose (5 mM), pH 7.4 by NaOH), respectively. Hagfish erythrocytes were kindly supplied by Dr. C. M. Harvey. After centrifugation the plasma/buffy coat was removed by aspiration. Erythrocytes were washed (5 x) by centrifugation (CP3), aspiration and resuspension in MBS (human/horse) or hagfish saline (hagfish). Procedures for haematocrit and microhaematocrit determinations are given below.

(i) Haematocrit (Hct) determination. The Drabkin's assay procedure (Dacie & Lewis, 1975) was used to determine the haematocrits of human red blood cell (HRBC) suspensions, by adding 100 µl suspension to 5 ml Drabkin's solution (from Sigma). This was left for at least 30 minutes at room temperature. Haematocrit values were derived by calculation from the sample absorption (A) at 540 nm in a Lambda-5 spectrophotometer (Perkin-Elmer). Over the range of interest, A_{540} and haematocrit are linearly related (Figure 3.1). The haematocrit (as a proportion, designated p) was calculated using the following equation:

$$\mathbf{Hct\ (p) = [A_{540} \times 51]/[247]} \quad ...(1)$$

where 51 is the dilution factor, and 247 is the accepted absorption of packed cells (Harvey & Ellory, 1989). The haematocrit was generally in the range 2 - 7 % for both haemolysis and flux experiments.

(ii) Microhaematocrit measurements. For experiments involving measurements of packed cell volumes (PCVs), plain capillary tubes were used to take up a sample of cell suspension (original PCV > 15 %). One end of the tube was sealed with a Cristaseal plug. The tubes were spun (CP14) starting at the time indicated; the PCV (as %) was then read on a microhaematocrit reader. All microhaematocrit equipment was obtained from Hawksley and Sons Ltd. (Lancing, Sussex). Verification of the Drabkin's analysis equation was performed (Figure 3.2), confirming 247 as the extrapolated value for packed cells prior to dilution.

3.3.2 Isolated rabbit enterocytes

Rabbit jejunal enterocytes were isolated using the protocol developed by Kimmich for chicken enterocytes (Kimmich, 1975, 1981; Ilundain, 1991), and previously adapted for rabbit enterocytes (Brown & Sepulveda, 1985a,b). White New Zealand rabbits (weighing 1.5 - 2.5 kg) were killed by either a blow to the neck or a thoracotomy under anaesthesia (halothane (ICI 2 - 2.5 %) in oxygen. A section of jejunum (c. 50 cm long), ie the

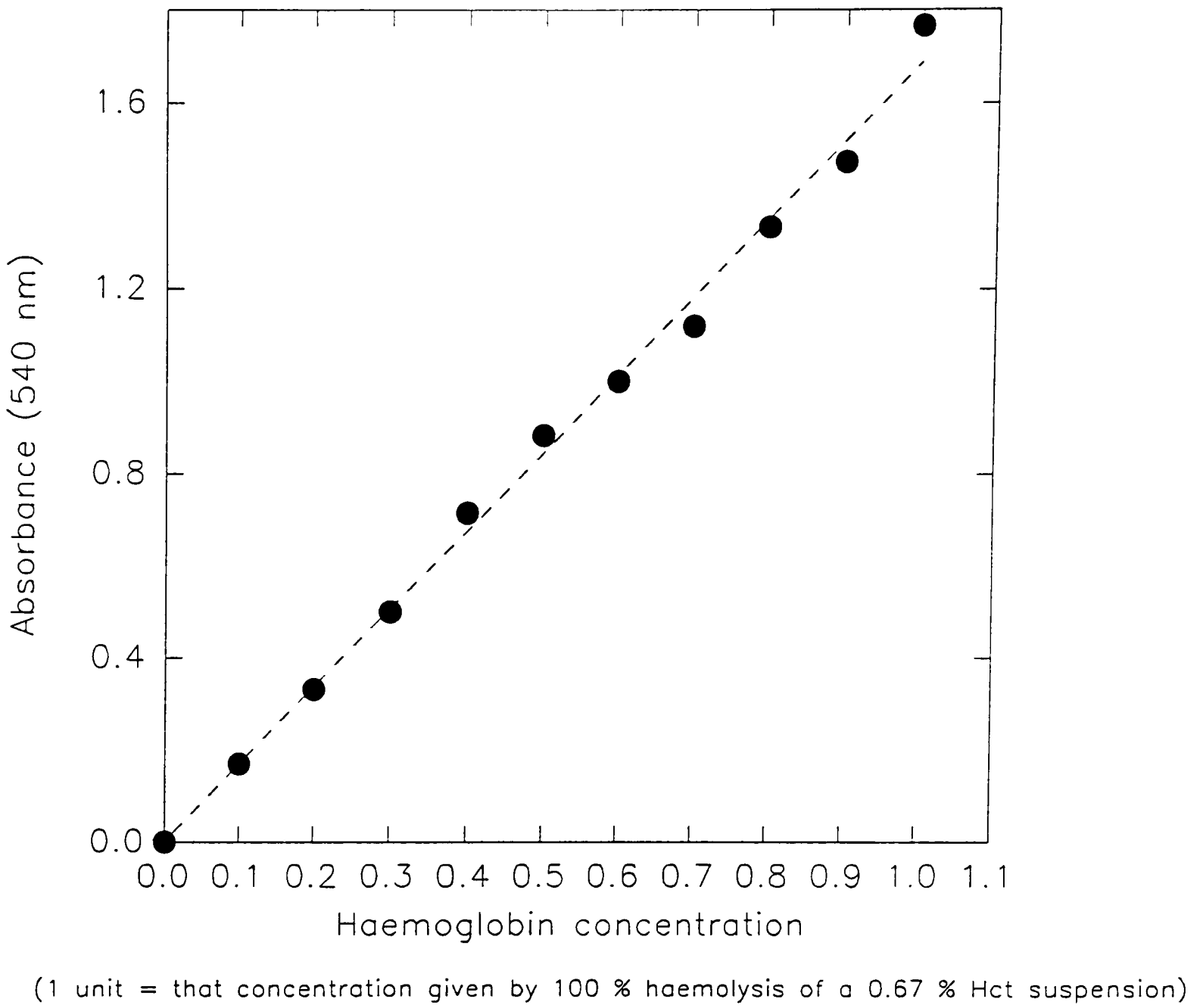


Figure 3.1 The relation between absorbance (540 nm) and haemoglobin concentration. Haemoglobin concentration is such that 1 unit as given is that which results from 100 % haemolysis of a 0.67 % Hct suspension.

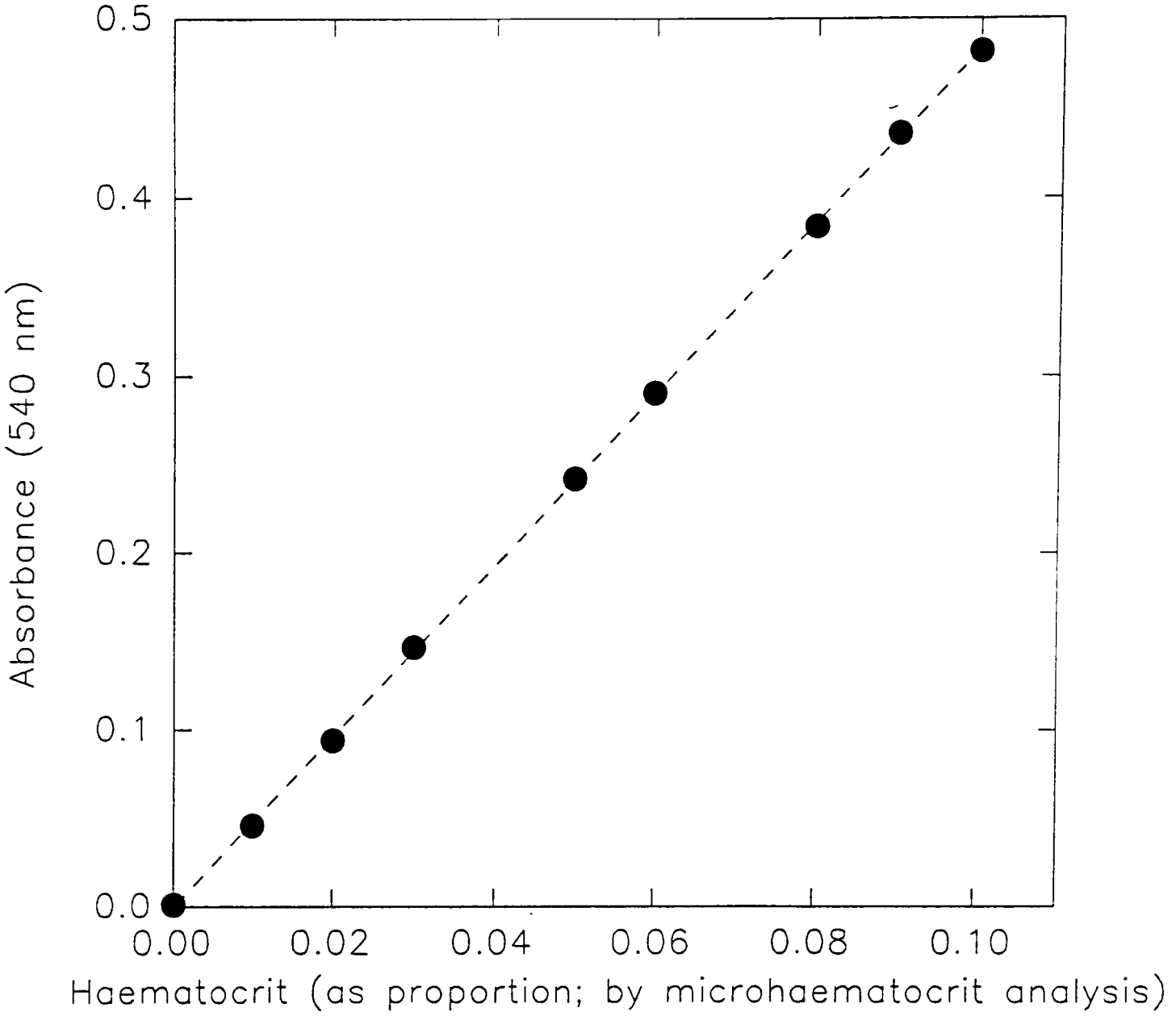


Figure 3.2 The relation between absorbance at 540 nm and microhaematocrit: verification of Drabkin's equation. A series of dilutions of an HRBC suspension (Hct = 10 % - measured using microhaematocrit equipment) was made and the absorbances gauged as described in text.

proximal portion of the mesenterial small intestine (Craigie, 1966), was rapidly dissected clear of the mesentery and other abdominal contents under near aseptic conditions, where working areas, containers and instruments were all liberally washed in 70 % ethanol. The tissue was rinsed clean (both serosally and luminally) using ice-cold GCS supplemented with DL-dithiothreitol (0.1 mM) to reduce the mucus content of the preparation (Kimmich, 1975), before being everted and tied off at both ends. The tissue was then transferred to GCS containing 1 $mg(ml)^{-1}$ hyaluronidase (Sigma; Type I-S from ovine testis) and incubated at 37 °C in a shaking water bath (70 cycles per minute) for 30 minutes.

After incubation, the tissue was transferred to hyaluronidase-free GCS where cells were released by gentle agitation with a plastic pipette tip. To remove remaining mucus and extracellular entities, cells were filtered through a tea strainer and then a 70 μm cell filter (Becton Dickinson, Oxford). The resulting filtrate was washed twice by centrifugation (CP2) and resuspension in GCS. Following the advice of Ilundain (1991), cells were exposed only to plastic containers and pipette tips.

Methods for cell quantification are considered below.

(i) Cell counting and cell viability. A 100 μl sample of the isolated cell suspension was added to an equal volume of Trypan Blue (0.4 % w/v in GCS). An aliquot of this mixture was placed on an improved Neubauer haemocytometer stage and examined by light microscopy (using an Olympus BH2 Microscope with attached Varimagni camera). The stage was graduated, being divided into 25 squares, each square itself being partitioned into 16 smaller divisions (Figure 3.3). A superimposed coverslip created a chamber with a volume of 0.1 μl (area of graduation = 1 mm^2, chamber depth = 0.1 mm; Johnstone & Thorpe, 1988). Four independent counts of cell number were made, and the number of cells per ml suspension was calculated by multiplication of the mean count by 2 (dilution factor) x 10^4 (volume conversion factor).

Isolated enterocytes lose their asymmetric function (Kimmich, 1975; Ilundain, 1991) although a degree of polarity with respect to the brush border (the salient morphological feature of intestinal epithelial cells) was observed for most cells (see Figure 3.4). Although cell-clumping was not such a major problem to cell-counting by haemocytometer (see above) as that reported by Kimmich (1975) for chicken intestinal cells, small clumps or groups of cells were still observed (Figure 3.5). The important goal

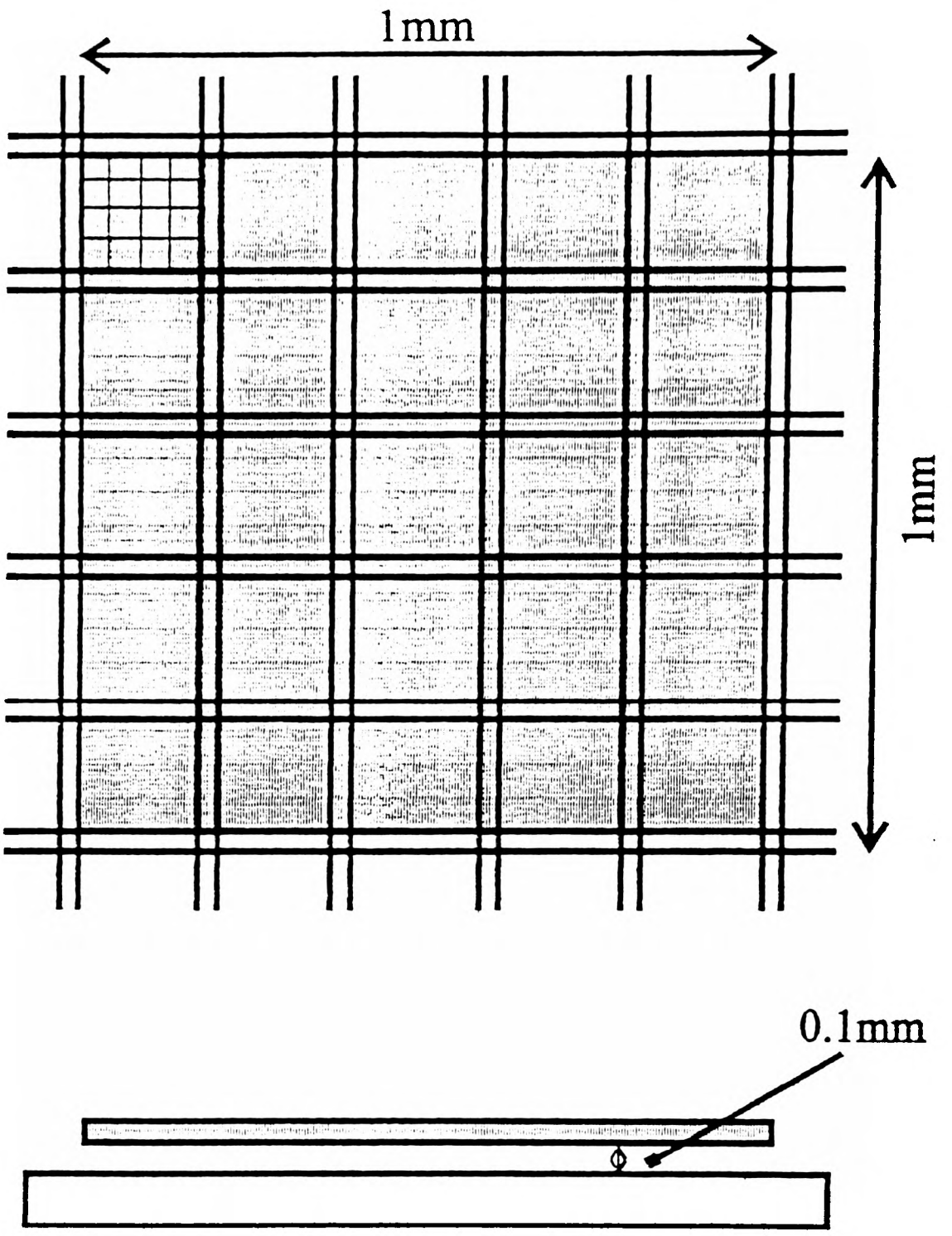


Figure 3.3 Counting chamber for quantification of cells. The Neubauer ruling consists of 25 large squares (enclosed by double lines; upper panel), each of which is divided into 16 smaller squares. The volume enclosed by the 25 large squares and the height of the coverslip above the ruled area (lower panel) is 1 x 1 x 0.1 (mm^3) = 0.1 mm^3.

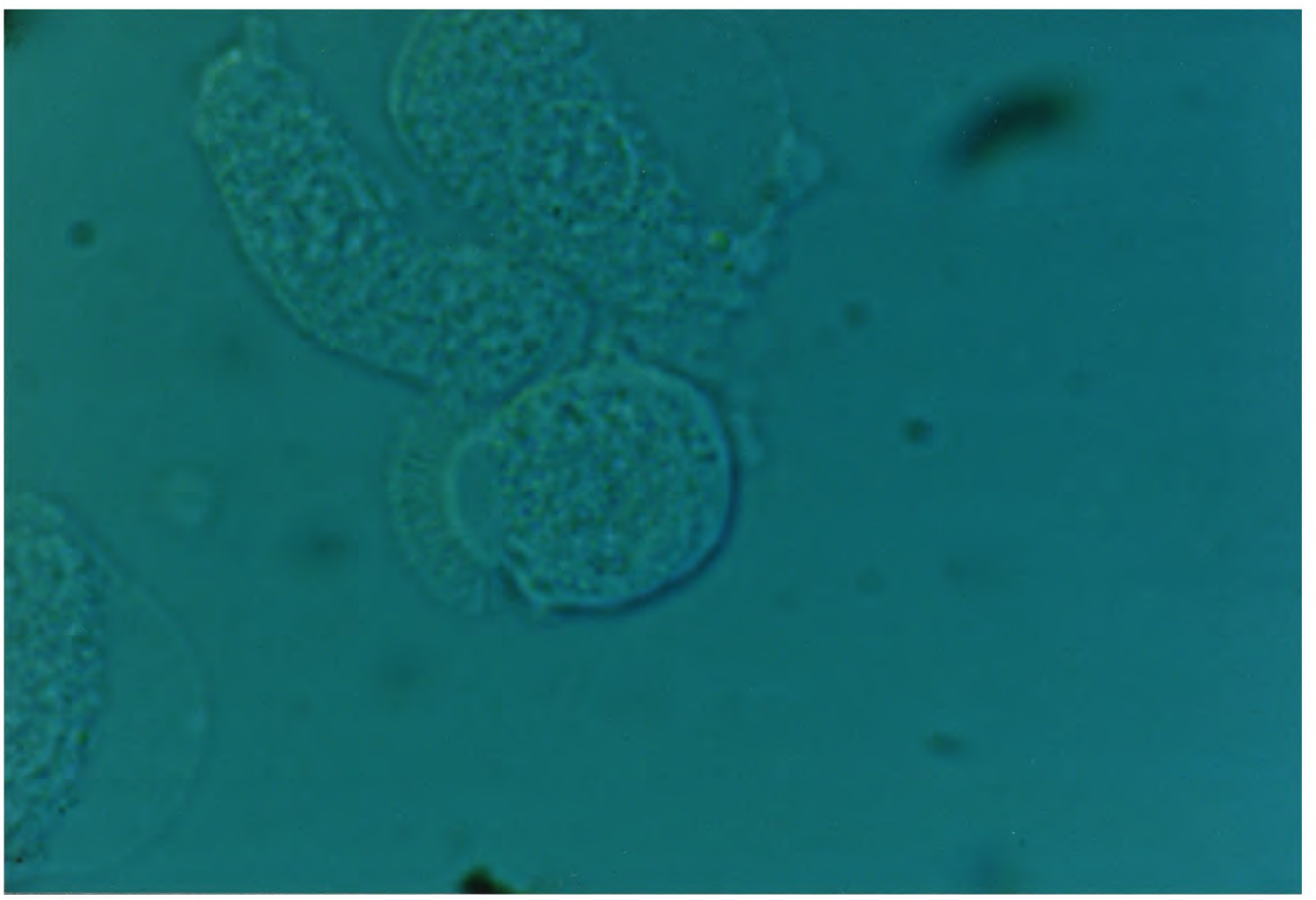


Figure 3.4 Typical isolated gut cell. For isolation procedure see text (section 3.3.2). Scale bar = 5 μm.

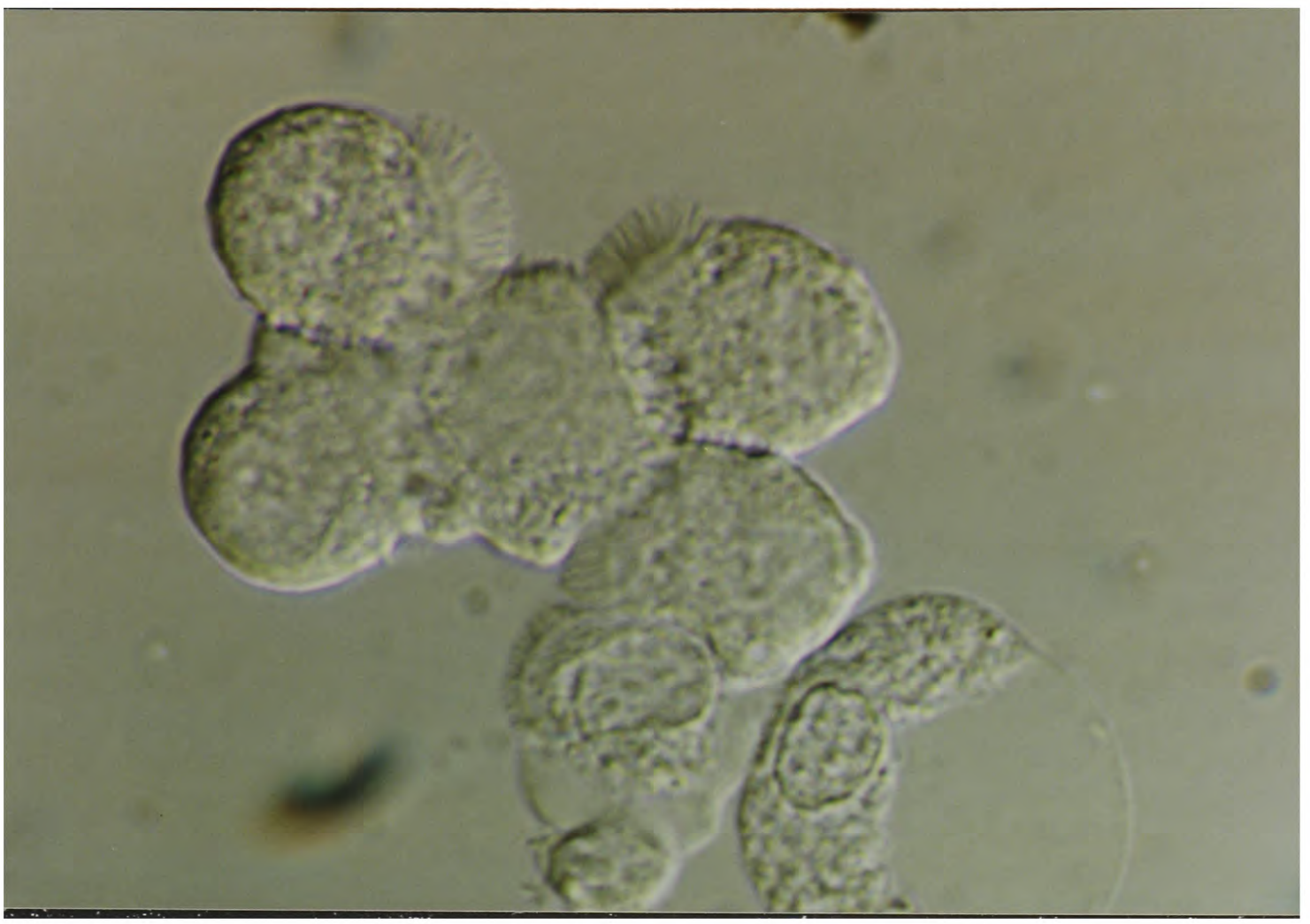


Figure 3.5 A clump of isolated gut cells. For isolation procedure see text (section 3.3.2). Scale bar = 5 µm.

of the preparation procedure was not to produce a suspension of individual cells as much as it was to free cells or groups of cells which retain their physiological capabilities and can be readily adapted to laboratory procedures.

Cell viability was assessed by determining the fraction of the cell population capable of excluding 0.2 % Trypan Blue (Girardi *et al.*, 1956). Trypan Blue is a strongly negatively charged dye which does not readily permeate the plasma membrane of intact cells because of the negative membrane potential. However cells in which there has been dissipation of this potential gradient will readily take up the dye (Drury & Wallington, 1967). Cell survival was assessed against time (Figure 3.6) for four isolations. Immediately after isolation the percentage of cells capable of excluding Trypan Blue was (mean ± S.E.M.) 68.2 ± 3.72 % (n = 4). Subsequent survival is greatly promoted by keeping the cells on ice. Accordingly, experiments were performed immediately after isolation and samples for counting were kept on ice until the end of the experiment.

(ii) Protein assay. The BCA (bicinchoninic acid) protein assay reagent (Pierce & Warriner) was used for the spectrophotometric determination of protein concentration (Smith *et al.*, 1985), because its convenient assay procedure eliminates the need for precisely timed reagent additions and vortexing inherent with the Lowry method (Lowry *et al.*, 1951). This assay combines the reaction of protein with Cu^{2+} in an alkaline medium (yielding Cu^{1+}) with a highly selective and sensitive detection reagent for Cu^{1+} (BCA). Two molecules of aqueous BCA readily interact with a single Cu^{1+} (Figure 3.7) to form a purple-coloured and water-soluble complex (Wiechelman *et al.*, 1988) that exhibits a strong absorbance at 562 nm, allowing the spectrophotometric quantitation of protein in aqueous solutions.

The standard protocol used involved the generation of a standard curve in which absorbance at 562 nm was plotted against sample protein (BSA) concentration. Appropriately diluted sample or standard (50 µl) was added to 1ml working reagent (freshly prepared by adding Reagent A (containing BCA in 0.2 M NaOH) to Reagent B (4 % w/v copper sulphate solution) and mixing), and the resultant mixture was incubated at 37 °C for 30 minutes. After cooling to room temperature, the samples were read at A_{562}, sample protein concentrations being calculated from the standard curve, a typical example of which is given in Figure 3.8.

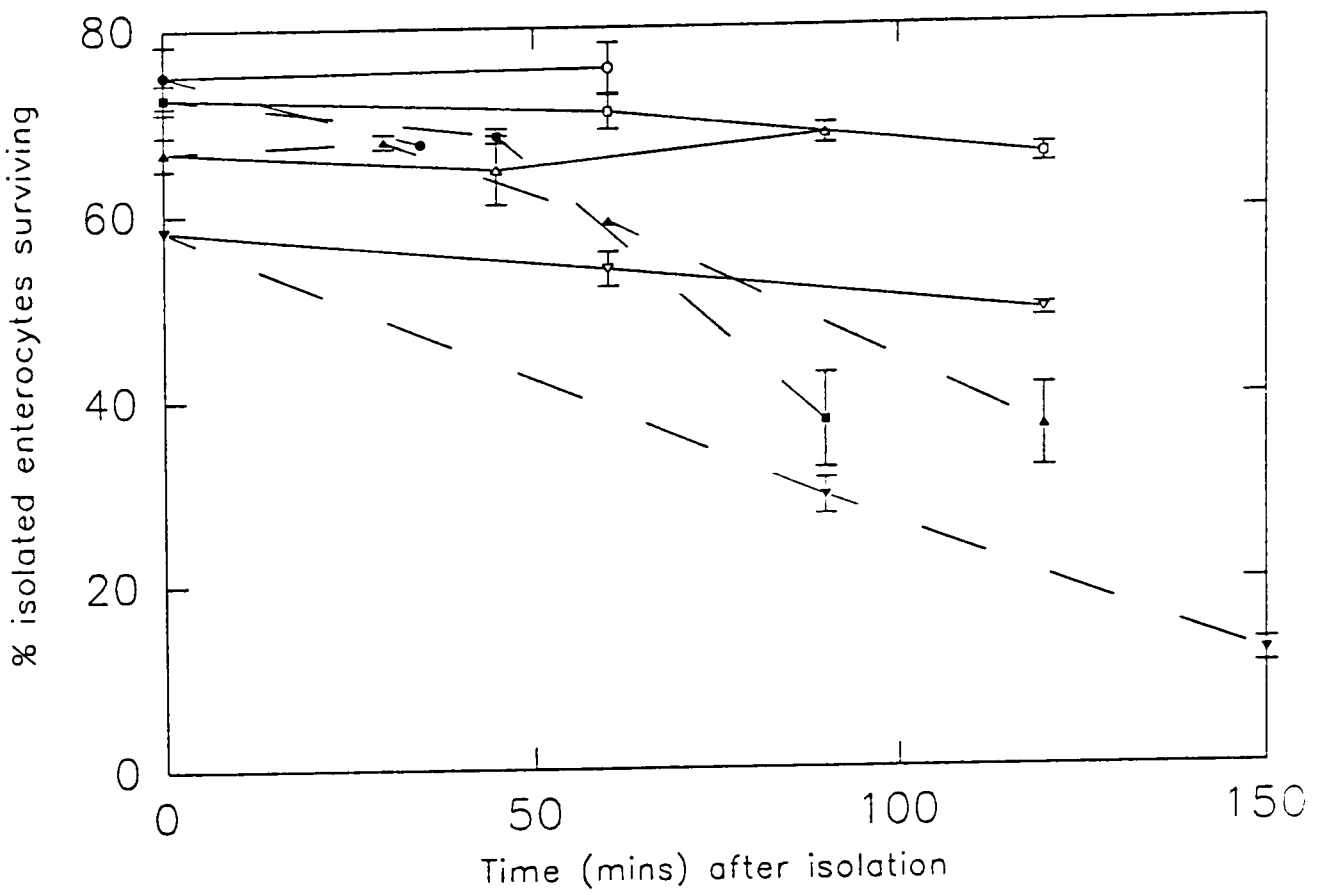


Figure 3.6 Survival of isolated enterocytes. Isolated enterocytes were re-suspended in GCS (approximate density = 6 x 10^6 cells$(ml)^{-1}$) and kept either on ice (solid lines) or at 37 °C (dashed lines), % survival being assessed by Trypan Blue exclusion (section 3.3.2). Each symbol type represents a different isolation.

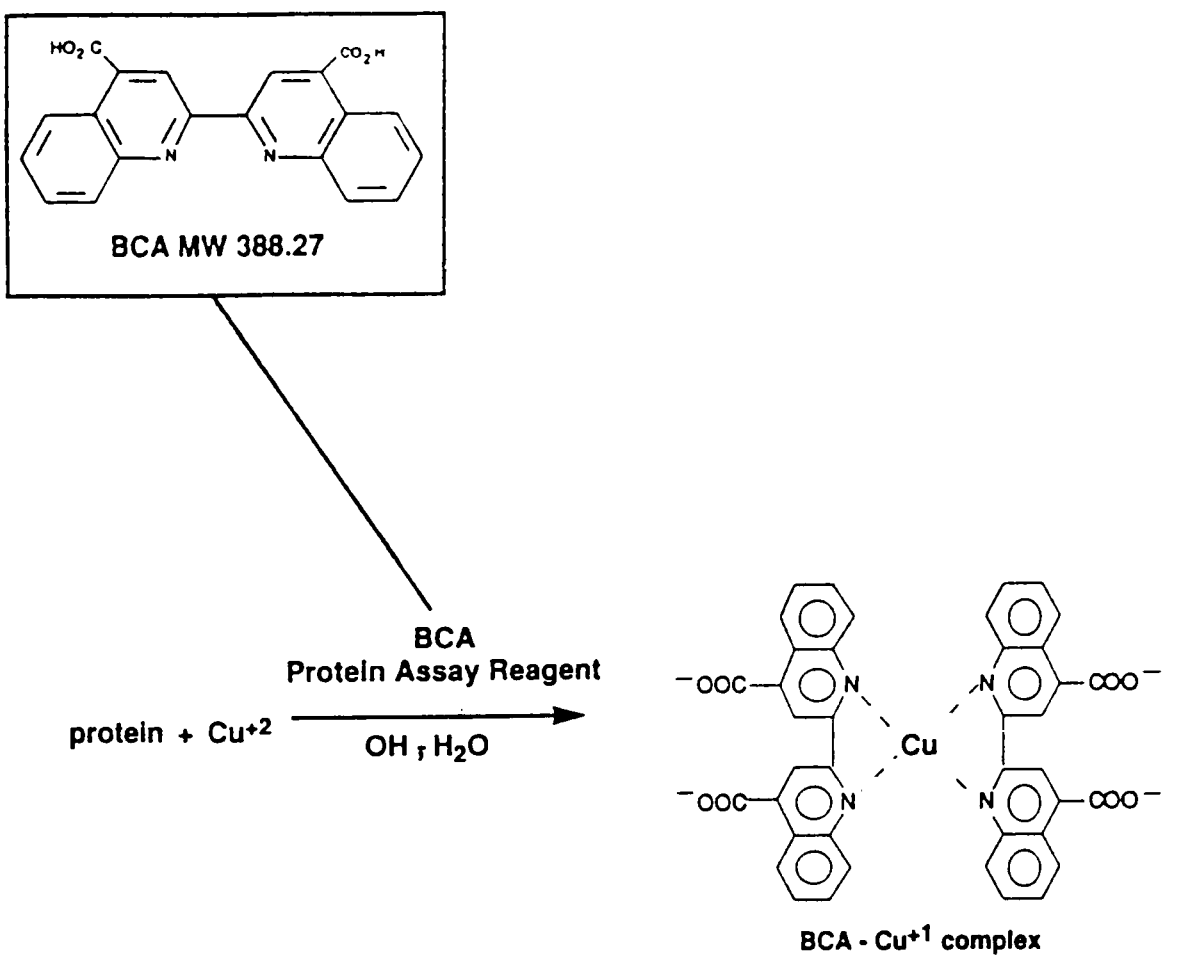


Figure 3.7 Reaction scheme for the BCA protein assay. This reaction combines the Biuret reaction (protein reacting with Cu^{2+} in an alkaline medium to produce Cu^{1+}) with the complexing reaction of Cu^{1+} with BCA. The purple reaction product, formed by the interaction of two molecules of BCA with one cuprous ion (Cu^{1+}), is water soluble and exhibits a strong absorbance at 562 nm. This allows spectrophotometric quantitation of protein in aqueous solutions. After Pierce, 1993.

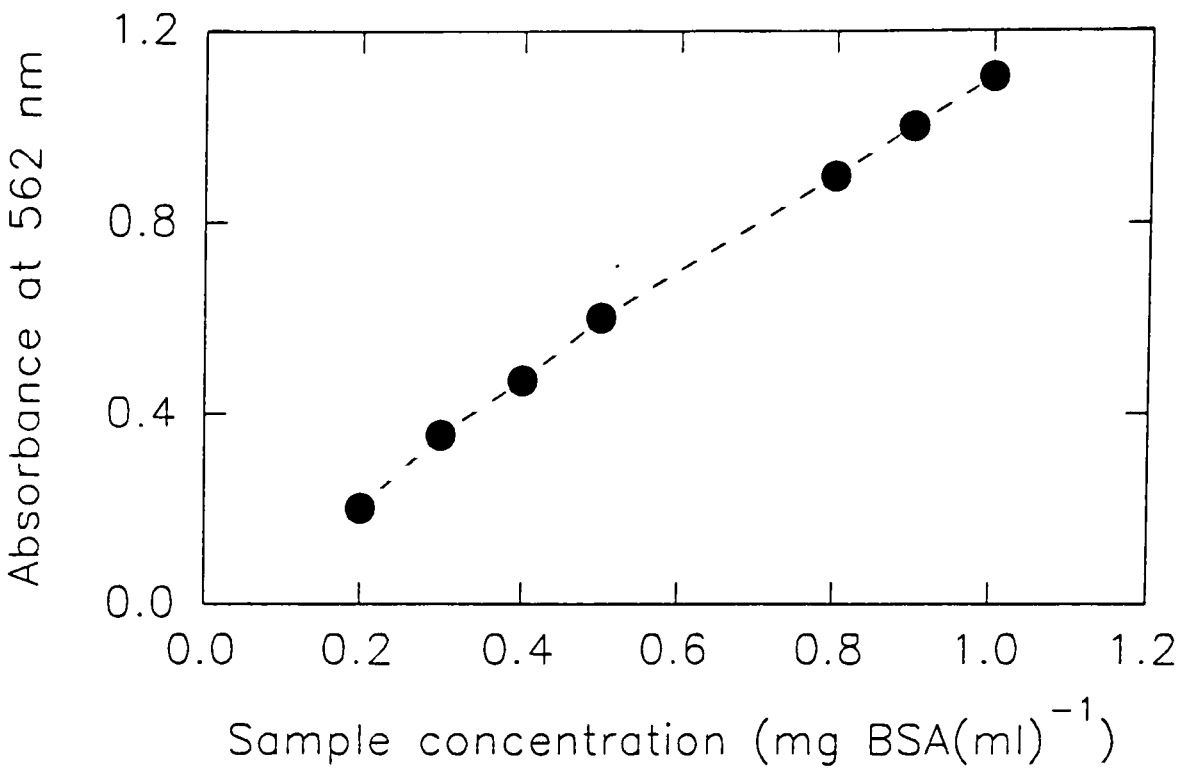


Figure 3.8 Typical calibration curve for the BCA protein assay. After addition of the working reagent to samples of standard (BSA), tubes were incubated at 37 °C for 30 minutes. Absorbance of samples was measured at 562 nm.

3.3.3 Chinese hamster ovary (CHO) cells

The CHO assay has long been used to test for two types of exoprotein activity. Such activities are assessed by a method (Lockwood *et al.*, 1982; Kothary, 1994) modified from that described earlier by Guerrant *et al.* (1974). Stock cultures of CHO cells were grown in Eagle's minimum essential salts medium (EMEM) supplemented with foetal calf serum (FCS) (10 % v/v), tryptose phosphate broth (TPB) (10 % v/v), sodium bicarbonate (0.14 % w/v), penicillin (50 IU$(ml)^{-1}$) and streptomycin (in EMEM (10 % v/v); 0.005 % v/v). The assay medium was EMEM containing FCS (1 % v/v), cells being grown at 37 °C in a 96 well Microtest III tissue culture plate (Becton Dickinson, New Jersey), with an added aliquot (of known dilution) of toxin preparation. After 48 hours cells were viewed using a Diavert binocular microscope, at 63 x magnification, and results, in terms of proportion of cells exhibiting elongation or lysis (according to toxin examined), were gauged visually.

3.4 HAEMOLYSIS STUDIES

Haemolysis is the process by which erythrocytes break open to release haemoglobin and other intracellular contents. Broadly speaking, this process may occur either directly (for example, after addition of streptolysin O (Duncan & Schlegel, 1975)) or after a volume increase occurring as a result of osmotic influx (for example, after addition of staphylococcal α-toxin (Bhakdi & Tranum-Jensen, 1991) or suspension in hypo-osmotic conditions (Araki & Rifkind, 1981)). In isotonic media the human erythrocyte has a biconcave disc shape that imparts to it an excess surface area:volume ratio (Rand & Burton, 1963). Osmotic fragility relates the degree of haemolysis (as fraction of total) to the tonicity of the surrounding medium. Osmotic fragilities and the time courses of haemolytic reactions have been used to investigate the processes leading to haemoglobin release in either osmotically unfavorable media (Jacobs, 1932; Sidel & Solomon, 1957; Rand & Burton, 1963; Rand, 1964; Bowdler & Chan, 1969; Jay & Rowlands, 1975; Anderson & Lovrien, 1977; Maeda *et al*, 1977; Araki & Rifkind, 1981) or under conditions imposing abnormal permeability properties on the erythrocyte membrane (Bernheimer, 1947; Johnson, 1972; Inoue *et al.*, 1976; Johnson & Boese-Marrazzo, 1980; Ginsburg *et al.*, 1983, 1985; Kothary & Kreger, 1985; Weiner *et al.*, 1985; Ginsburg & Stein, 1987; Bernheimer, 1988; Deuticke *et al.*, 1991; Kreger, 1991; Kirk *et al.*, 1994).

In such reports, the rate of haemolysis has been characterised either in terms of (i) a first order semi-logarithmic plot in which the apparent rate constant of haemolysis has been defined as the steepest slope of surviving (% initial total) cells (y) against time (x) after hypotonic shock (Araki & Rifkind, 1981), or (ii) time taken for 50 % haemolysis (H^{50} (minutes)) to occur (Weiner *et al.*, 1985; Ginsburg & Stein, 1987; Deuticke *et al.*, 1991; Kirk *et al.*, 1994).

Time courses of haemolysis were assayed by measuring the haemoglobin released into supernatants by lysed cells (Ponder, 1948; Bernheimer, 1988). At each time point a sample was taken and the cells centrifuged down (CP3). The absorbance of the supernatant (after dilution) was measured, and expressed as percentage haemolysis by reference to absorbance of the solution yielded by 100 % haemolysis. There was a pronounced donor variation in the absolute H^{50} values (see Chapter 4). Accordingly, normalised H^{50} values were often used to express the rate of haemolysis relative to an appropriate control.

Haemolysis was measured either in MBS or in isosmotic solutions of the alkali metal chlorides (150 mM), which were made up in 10 mM HEPES (pH 7.4 by addition of Tris/HCl) and adjusted to 291 ± 3 mosmoles, by addition of HEPES solution, as measured by a Wescor 5100 C vapour pressure osmometer. To initiate the experiment, packed cells were added to the appropriate solution. In these iso-osmotic experiments $\{(H^{50})^{-1}\}$ is a semiquantitative indicator of the permeability (P_x) of a given ion x. Because of variation in the susceptibility of cells according to donor, the $\{(H^{50})^{-1}\}$ was also expressed as standardised data, by normalisation with respect to the appropriate control. For instance, for data describing selectivity series, the P_x (= $\{(H^{50})^{-1}\}$) of any ion was expressed relative to the P_{Cs}, since haemolysis was fastest in CsCl. The P_x/P_{Cs} index is plotted on an Eisenman plot (ie against the inverse of the crystal ion radius; Eisenman & Horn, 1983).

3.5 FLUX STUDIES

Movements of radioactive isotopes across cell membranes were followed using techniques described previously (Young & Ellory, 1982; Ellory, 1982; Ellory *et al.*, 1992). The flux of a substance S across a membrane is defined as the amount of S moved from one defined volume to another in a given period of time. Conventionally the units used

are mmoles(litre cells)$^{-1}$(hour)$^{-1}$. During the experiment the radio-isotope moves across the membrane into (i) the intracellular (for influx), or (ii) the extracellular (for efflux), compartments where it is separated out and quantified, as CPM_{sa} (sa = sample) or CPM_t (after time t), respectively.

3.5.1 Influx experiments

These experiments were performed under *initial rate* conditions with respect to the radioisotope, ie conditions under which backflux of isotope does not contribute significantly to the observed flux. The time course over which influx was measured was therefore chosen so as to ensure that backflux was negligible (see Figure 3.9). Given CPM_b (b = blank; ie those CPM attributable directly to background radiation), then:

$$\textbf{flux} \propto (\textbf{CPM}_{sa}\textbf{-CPM}_b) \quad ...(2)$$

A standard CPM (CPM_{st}) measured for a calculated amount of S is used to quantify the flux directly. For CPM_{st},

$$\textbf{amount S (mmoles)} = \textbf{vol.}_{st}\ \textbf{(l) x } [\textbf{S}]_{st}\ \textbf{(mM)} \quad ...(3)$$

For a flux experiment,

$$\textbf{amount cells (litres)} = \textbf{Hct (p) x \{flux vol. (ml)/1000\}} \quad ...(4)$$

Therefore, for a 1ml flux volume, the flux (in mmol.(l.c.)$^{-1}$(hr)$^{-1}$) of substrate (S) can be calculated:

$$\textbf{Flux (S)} = \{(\textbf{CPM}_{sa}\textbf{-CPM}_b)\ \textbf{x vol.}_{st}\ \textbf{x } [\textbf{S}]_{st}\}/\{(\textbf{CPM}_{st}\textbf{-CPM}_b)\ \textbf{x Hct x time}\} \quad ...(5)$$

wherein (and similarly for equations *6* - *8* below) volumes (vol.) are in ml, concentrations are in mM, Hct is a proportionate value, time is in hours, and the subscripts $_{sa}$, $_{st}$ and $_b$ indicate sample, standard and blank respectively.

For experiments which trace the dynamics of flux pathways, S accumulated (mmol.(l.c.)$^{-1}$) may be calculated:

$$\textbf{S.a.(l.c.)}^{-1} = \{(\textbf{CPM}_{sa}\textbf{-CPM}_b)\ \textbf{x vol.}_{st}\ \textbf{x } [\textbf{S}]_{st}\}/\{(\textbf{CPM}_{st}\textbf{-CPM}_b)\ \textbf{x Hct (p) x vol.}_{sa}\} \quad ...(6)$$

wherein units are the same as described for equation *5*.

S accumulated may also be obtained (for isolated enterocytes - see Chapter 9) in units of mmol.(10^{12} cells)$^{-1}$):

$$\textbf{S.a.}(10^{12}\ \textbf{c.})^{-1} = \{(\textbf{CPM}_{sa}\textbf{-CPM}_b)\ \textbf{x vol.}_{st}\ \textbf{x } [\textbf{S}]_{st}\ \textbf{x } 10^9\}/\{(\textbf{CPM}_{st}\textbf{-CPM}_b)\ \textbf{x vol.}_{sa}\ \textbf{x c.d.}\} \quad ...(7)$$

wherein cell density (c.d.) is measured in number of cells per ml, and other units are as described for equations *5* and *6*.

If the parameter derived from equation *6*, S.a.(l.c.)$^{-1}$, is plotted against time (mins), a

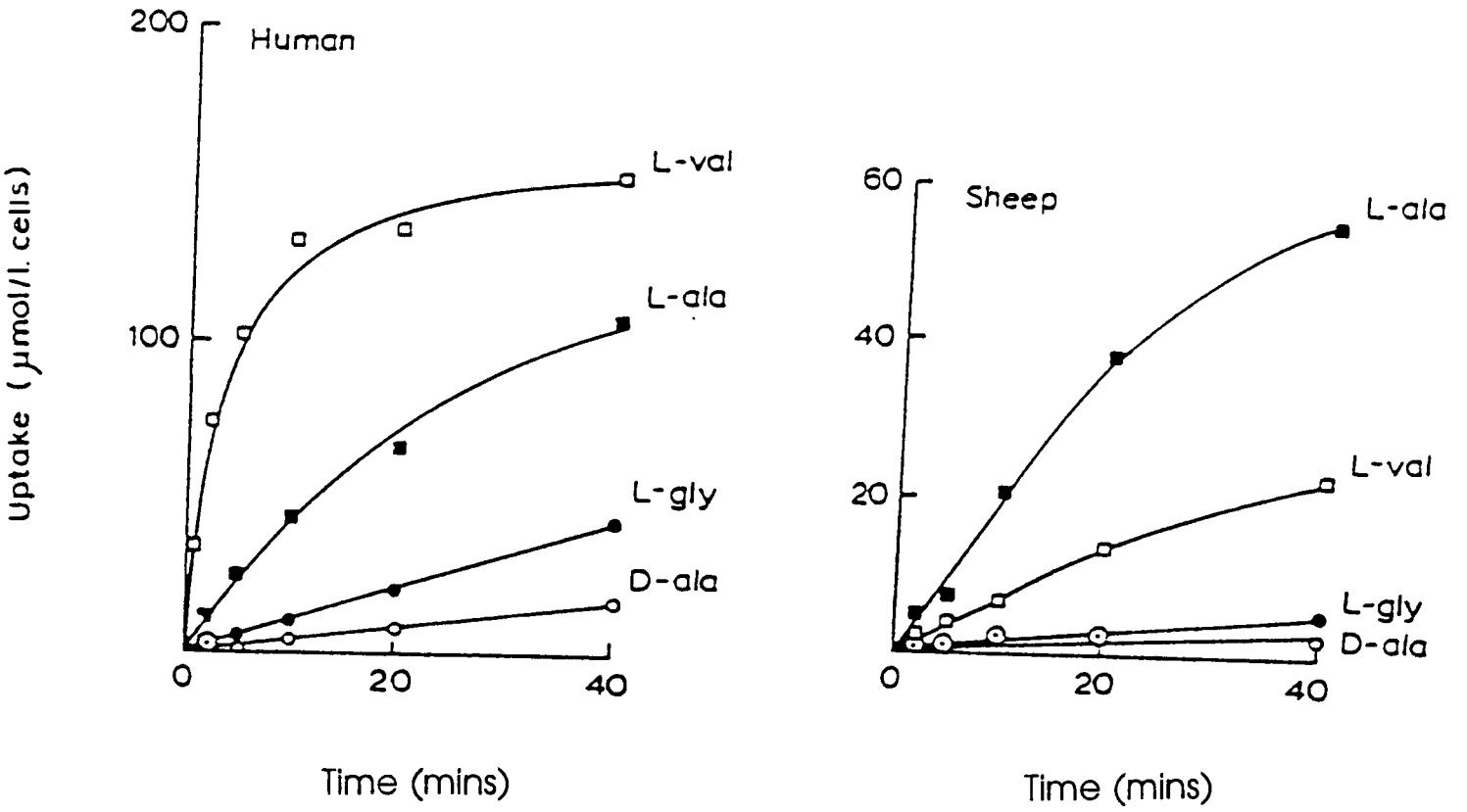


Figure 3.9 Time-courses of uptake of various amino acids into sheep and human red cells. These diagrams show how beyond the initial rate phase, backflux of isotope affects the measurement of isotopic influx. Thus a short enough flux period must be used to make backflux of isotope negligible compared with measured flux. After Young and Ellory (1982).

straight line ($y = mx + c$) may be fitted through data points so generated (x = time (mins), y = S.a.(l.c.)$^{-1}$), then:

$$\textbf{flux} = \textbf{60 x m} \quad ...(8)$$

wherein flux has its conventional units of mmol.(l.c.)$^{-1}$(hr)$^{-1}$.

For experiments investigating the multi-hit nature of cation influx, an index of substrate (S) transit is used:

$$\textbf{S transit} = \textbf{flux x Hct x 1000} \quad ...(9)$$

wherein S transit has units of mmol.(volume cell suspension (m^3))$^{-1}$(hr)$^{-1}$, flux is in mmol.(l.c.)$^{-1}$(hr)$^{-1}$ and Hct is a proportionate value.

MBS was used as the basic flux medium, containing sucrose (40 - 45 mM) to protect cells from lysis. Two techniques were used to measure influx: (i) the conventional $MgCl_2$ wash technique, as described previously (Ellory, 1982), and (ii) the DBP multiple wash technique. Two experimental procedures were used to measure inward movement of isotope, after spiking of a cell suspension with an appropriately labelled solution:

(i) $MgCl_2$ wash (MWS) technique. Cell suspensions were incubated with isotope at 37 °C for a chosen time period. Fluxes were terminated by centrifugation (CP3) and transferral of tubes to ice. The supernatant was then removed by aspiration and the erythrocytes were re-suspended in 1 ml of ice-cold MWS (stop-wash solution). The resuspension-centrifugation-aspiration cycle was repeated three times, to reduce the counts of the extracellular solution to background.

(ii) Dibutyl phthalate (DBP) multiple wash technique. This was used in the experiments delineating the dynamics of induced leaks because it allows rapid separation of cells from the medium (Ellory *et al.*, 1992). The flux was terminated by adding a 200 µl aliquot of the flux suspension to 800 µl ice-cold solution layered over 250 µl dibutyl phthalate (for HRBC) or dibutyl phthalate:dinonyl phthalate (DBP:DNP, 3:2; for enterocytes), through which the cells were then spun. The solution remaining above the oil layer was aspirated and washed off by total immersion in a large volume of water (x 3), before removal of the oil layer, and drying of the inside of the tubes by aspiration, leaving the cells below.

With the supernatant removed, 0.5 ml 0.1 % v/v Triton X-100 was added to each Eppendorf tube and mixed thoroughly to lyse all the cells, followed (after CP3) by addition of 0.5 ml 0.5 % w/v TCA to precipitate out proteins. After mixing again, the tubes were once more centrifuged (CP3). The supernatant from each tube was removed

with a Pasteur pipette, and ejected into a scintillation vial with 3 ml of distilled water. These samples were then counted for radioactivity, in a Packard Tri-carb 2000 CA scintillation counter. The standard count was made by adding a known volume of radiolabelled spiking solution to a scintillation vial (with 0.5 ml 0.1 % Triton, 0.5 ml 0.5 % w/v TCA and 3 ml distilled water). In addition, vials containing 3 ml of distilled water, 0.5 ml 0.1 % Triton and 0.5 ml 5 % TCA were used to count the background activity.

3.5.2 Efflux experiments

A small volume of packed cells (either HRBC or isolated enterocytes) was added to a pre-warmed solution (37 °C) to initiate the efflux time course. Samples of determined volumes were taken from the homogeneous suspension at specified times and centrifuged. For HRBC, fluxes were terminated by centrifugation (CP3), either directly or through DBP (250 µl). For experiments involving enterocytes, cells were centrifuged through a 250 µl layer of the oil mixture DBP:DNP (3:2; Brown & Sepulveda, 1985a,b). A volume of the supernatant was removed to 3 ml H_2O for scintillation counting.

The parameters C_t, C_o, and $C_{inf.}$ represent (i) counts present in the external medium at time t (minutes), (ii) counts of radioactivity in the external medium at time zero, and (iii) counts present inside the cells at time zero + C_o.

The rate constant k (minutes^{-1}) for efflux may hence be estimated according to the following equation:

$$k = -(1/t).\ln\{(C_{inf.}-C_t)/(C_{inf.}-C_o)\} \quad ...(10)$$

Alternatively, the rate constant k can be visualised as the negative of the slope of the graph of $\ln\{(C_{inf.}-C_t)/(C_{inf.}-C_o)\}$ (y) against time (**t**; x) (Ellory, 1982; Harvey & Ellory, 1989).

3.5.3 Particular isotopes and directions of flux

(i) K^+ influx. The $^{42}K^+$ isotope is expensive and has a half-life of c. 12 hours. $^{86}Rb^+$ is cheaper and of a longer half-life (c. 18.7 days); it was therefore used as a tracer for K^+ movement with the reservation that there could be subtle differences between Rb^+ and K^+ transport. Although this assumption has been validated for many membrane transporters (Simons, 1982; Brown & Sepulveda, 1985a; Bernhardt *et al.*, 1991), it is still questionable for most pore-forming systems. However, in this investigation, evidence is produced that K^+ and Rb^+ are handled in very similar ways by both the KH and TDH-induced lesions

(see Chapters 4 and 7, Figures 4.17 and 7.8, respectively). Carefully calculated and measured volumes of 150 mM K(+ trace $^{86}Rb^{+}$)Cl were added at time zero, at the appropriate incubation temperature and terminated after a measured time period by CP3, and transferral to ice. After processing, intracellular contents were analysed (5 minutes per vial) for Cerenkov emission in a scintillation counter (Garrahan & Glynn, 1966).

(ii) Ca^{2+} *influx.* Washed red cells were resuspended at Hct = 10 %, and BAPTA-AM (1, 2 *bis*(2-aminophenoxy)ethane N, N, N', N'-tetracetic acid acetomethoxyester) (Calbiochem, Nottingham) was added from a 100 mM stock solution in DMSO to a final concentration of 0.3 mM (0.3 % v/v DMSO final concentration). Following incubation at 37 °C for 90 minutes, the cells were washed twice by centrifugation (CP1) and resuspension in an identical medium without EGTA. A $CaCl_2$ solution (100 mM) was added to a final concentration of 0.5 mM together with c. 1 $\mu Ci(ml)^{-1}$ $^{45}Ca^{2+}$. Time courses for Ca^{2+} influx were measured using the DBP multiple wash technique. After cells were lysed in 0.1 % v/v Triton X-100 and protein precipitated with 5 % w/v TCA, the supernatant was assayed for $^{45}Ca^{2+}$ by suspension in Picofluor 40 and beta-scintillation spectroscopy.

(iii) Rb^{+} *efflux.* HRBC were loaded either overnight (8 °C) or for 4 hours (37 °C), at high density (Hct = 80 %) in MBS (+ sucrose (50 mM) as an osmotic protectant) with $^{86}Rb^{+}$ present at 10 - 12 $\mu Ci(ml)^{-1}$; prior to experimentation, erythrocytes were washed rapidly in ice-cold saline by centrifugation (CP3), aspiration and resuspension (x 6). Isolated enterocytes were loaded immediately after isolation in a concentrated cell suspension in GCS, containing $^{86}Rb^{+}$ (5 $\mu Ci(ml)^{-1}$); prior to experimentation, cells were washed twice by CP2 and resuspension in GCS.

3.6 EXPERIMENTAL MANIPULATIONS: theory and practice

3.6.1 Use of inhibitors

In some haemolysis (section 3.4) and isotope flux (section 3.5) experiments, a maximally inhibiting concentration of ouabain (100 µM; Bernhardt et al., 1991), bumetanide (100 µM; Bernhardt *et al.*, 1991) and nitrendipine (10 - 100 µM; Ellory *et al.*, 1992) was used to eliminate K^{+} movement via the endogenous membrane transporters the Na^{+}/K^{+} ATPase, the $Na^{+}/K^{+}/2Cl^{-}$ cotransporter and the Ca^{2+}-activated K^{+} (Gardos) channel, respectively (see section 2.8.1 and Figure 2.23).

3.6.2 Multi-hit analysis

The estimation of the number of interactions between entities and the membrane (commonly known as 'hits'; Inoue *et al.*, 1976; Koski *et al.*, 1983) necessary to kill or lyse cells by a factor has been attempted by assessing the effect of target concentration (eg for streptolysin O, *Clostridium perfringens* theta toxin (Inoue *et al.*. 1976), and *Pseudomonas aeruginosa* haemolysin (Johnson & Boese-Marrazzo, 1980)). For these experiments, various concentrations of target cells (erythrocytes) were treated by a constant amount of lytic agent in a constant reaction volume. The absorbance at 540 nm was measured and haemolysis (as % total) calculated (section 3.4). For a given agent, if A_{540} (an index of total - as opposed to fractional - number of cells lysed) is found to decrease as haematocrit is increased beyond a certain level, then it can be concluded that cell death is multi-hit (as opposed to single-hit) in nature.

By using isotopic tracers to measure K^+ influx (section 3.5) rather than haemolysis, and K^+ transit as the corollary of absorbance at 540 nm, the nature of the lesion itself (rather than the number of hits required for cell death) can be examined by looking for a similar relation (Koski *et al.*, 1983).

3.6.3 Effect of temperature

The effects of temperature on a wide range of physiological processes are well documented (Eckert, 1988; Prosser & Heath, 1991). In red cells there are at least three ways in which temperature can affect transport events (Ellory & Hall, 1987; Harvey & Ellory, 1989): (i) direct action on the transporter, (ii) a referred effect through the physical environment of the transporter, and (iii) chemical changes to charged groups. Generally, the temperature dependence of reactions is described by the Arrhenius equation:

$$\boldsymbol{k} = \mathbf{A}e^{-\mathbf{Ea/RT}} \quad ...(11)$$

in which $\boldsymbol{k}$ is the rate constant, **A** is a constant related to the collision frequency of the molecules, **R** is the gas constant (8.314 $J(degree)^{-1}(mol.)^{-1}$), and **T** is the absolute temperature (K). Accordingly, the activation energy ($\mathbf{E_a}$, in $kJ(mol.)^{-1}$) for a reaction can be determined experimentally, by plotting log $\boldsymbol{k}$ (y) against $\mathbf{T}^{-1}$ (x) for which the slope is designated **S**:

$$\mathbf{E_a} = \mathbf{-R.S} \quad ...(12)$$

In Chapter 4, the $\{(H^{50})^{-1}\}$ value was used as the index describing rate for this analysis. Comparisons of the values obtained can be made with the established literature (Stein,

1967; Hladky & Haydon, 1972; Cohen, 1974a,b; Hall *et al.*, 1982; Ginsburg *et al.*, 1983; Hall & Willis, 1986), to make suggestions as to the nature of underlying events.

3.6.4 Modification of membranes by neuraminidase treatment

Erythrocyte membrane structure may be modified by a variety of enzyme treatments (Carraway, 1982). In the present study, the enzyme neuraminidase was used, which reduces the negative surface charge associated with erythrocyte membranes (Stein *et al.*, 1987). Washed erythrocytes were suspended (haematocrit = 30 %) in MBS that also contained $CaCl_2$ (8 mM), BSA (0.5 % w/v; as a saturating substrate for non-specific enzymes) and 0.42 $U(ml)^{-1}$ neuraminidase type X (Sigma) from *Clostridium perfringens* (Cassidy *et al.*, 1965). Preparations were incubated at 37 °C with occasional agitation for one hour, and then washed twice (CP1) in neuraminidase-free MBS before use (Stein *et al.*, 1987).

3.7 BACTERIAL CULTURE

Standard microbiological techniques were employed (Swatek, 1967). *Vibrio* strains used, with sources, properties and references are listed in Table 3.3. Growth conditions profoundly influence both bacterial growth and gene expression (Cherwonogrodzky & Clark, 1982a,b; Nishibuchi & Seidler, 1983; Douet *et al.*, 1992). Accordingly, a variety of different media were used to culture bacteria (Table 3.4) under sterile conditions (peptone, yeast extract (YE) and Casamino acids were obtained from Difco Laboratories (Detroit, Michigan)). Media pH was measured using an Orion SA 520 pH meter (Orion Research, Boston) and corrected by addition of either NaOH (1 M) or HCl (1 M). Media (immediately after preparation) and glassware were sterilised by autoclaving at 121 °C for 20 minutes, stoppers having been slightly loosened to allow passage of steam to, and air from, closed containers.

Culture plates were made by adding agar (2 %) to LB1, mixing thoroughly and steam-cooking for 20 minutes before sterilisation by autoclave (as above). On cooling to 50 - 55 °C this media was run into petri dishes under sterile conditions. The plates were inverted after solidification of agar.

Upon receipt, each strain (Table 3.3) was saved by surface-plating onto 8 LB1 plates, and overnight culture. Bacterial cultures, either on LB1 agar plates or in liquid media (see below) were grown at 37 °C in an incubator, with a rotary shaking floor (100 r.p.m.).

genus/species/strain	source	properties	references
V. parahaemolyticus 4037	Hall, R. H.	Pathogenic strain	-
V. parahaemolyticus AQ 3815	Hall, R. H.	Pathogenic strain	Nishibuchi *et al.*, 1992
V. parahaemolyticus AQ 3815 δTDH1	Hall, R. H.	First *tdh* gene copy-deleted mutant of AQ 3815	Nishibuchi *et al.*, 1992
V. parahaemolyticus NCTC 10886	Clarke, A. G.	Fast growth; good producer of TDH	Cherwonogrodzky & Clarke, 1981
V. cholerae 8731	Hall, R. H.	Environmental strain	Hall & Drasar, 1990
V. cholerae (Ogawa) 395 N1	Hall, R. H.	derived from (395) pathogenic strain *ctx*A$^-$, *ctx*B$^+$, NANase$^+$	Kaper *et al.*, 1984 Galen *et al.*, 1992
V. cholerae CVD 101 (from Ogawa 395 N1)	Hall, R. H.	*ctx*A$^-$, *ctx*B$^+$, NANase$^+$	Kaper *et al.*, 1984
V. cholerae CVD 103 (from classical Inaba 569B)	Hall, R. H.	*ctx*A$^-$, *ctx*B$^+$	Kaper & Levine, 1990
V. cholerae CVD 103-HgR	Hall, R. H.	as above but HgR gene inserted into haemolysin locus	Levine *et al.*, 1988 Kaper & Levine, 1990
V. cholerae CVD 103-HgR2	Hall, R. H.	as for above	Kaper & Levine, 1990

Table 3.3 ***Vibrio*** **strains used.**

Medium	Constituents (g.(litre)$^{-1}$)	pH	References
Tak1	NaCl (30), peptone (10), Na_2HPO_4 (5), glucose	7.7	Takeda, 1988
Tak2	NaCl (30), peptone (10), Na_2HPO_4 (5), glucose (5)	6.35	Takeda, 1988
LB1	NaCl (5), peptone (10), yeast extract (5)	7.4	Miller, 1972 Nishibuchi et al., 1992
LB2	NaCl (5), peptone (10), yeast extract (5)	6.0	Miller, 1972 Nishibuchi et al., 1992
CC1	NaCl (30), peptone (20), KH_2PO_4 (4.08), K_2HPO_4 (5.23)	7.7	Clark & Cherwonogrodzky, 1981, 1982
CC2	NaCl (30), peptone (20), KH_2PO_4 (4.08), K_2HPO_4 (5.23)	6.2	Clark & Cherwonogrodzky, 1981, 1982
CYE	Casamino acids (30), yeast extract (4), K_2HPO_4 (0.5)	6.7	Hall & Drasar, 1990

Table 3.4 Bacterial culture media used.

Under sterile conditions, cultures were scraped clear of the agar and mixed into saving-solution (50 % (v/v) LB1, 50 % (v/v) glycerol). These aliquots were stored at - 135 °C.

Bacterial cultures were grown in the various media after 1 % innoculation from an LB1 starter culture of the designated bacterium. The increase in turbidity (A_{600}), determined in a Model 340 Spectrophotometer (Sequioa-Turner), of the nutrient broth was used to give an indication of the density of the bacterial population (Koch, 1984).

3.8 PROTEIN PURIFICATION

Separation of biological molecules from complex heterogeneous mixtures, such as bacterial culture supernatants, requires high resolution techniques such as ion exchange and gel filtration. After selective precipitation with ammonium sulphate, a combination of these techniques was used in conjunction with concentrative and dialysis steps to purify native and functional components of the culture supernatants of *V. cholerae* and *V. parahaemolyticus*.

3.8.1 Preparation of exoprotein concentrates

After culture incubation for a designated time period, the suspension was centrifuged (CP7), and the supernatant decanted off the bacterial pellet. Ammonium sulphate was dissolved in the culture filtrate to a final concentration of 45 - 60 % (w/v, dependent on the protein to be precipitated) at 4 °C. This solution was left at 4 °C for at least 2 hours. The precipitate was then collected by centrifugation (CP7), and resuspended in buffer solution (either Tris (20 mM; pH = 7.5) or *bis*-Tris (45 mM; pH = 7.1) dependent on the exoprotein desired - see Chapter 6) containing peptidase inhibitors (Leu-peptin (1.1 μM) (Aoyagi *et al.*, 1969; Wingender, 1974), pepstatin A (1 μM) (Rich *et al.*, 1985), phenylmethylsulphonylfluoride (PMSF; 0.2 mM) (Turini *et al.*, 1969; James, 1978; Moss & Fahrney, 1978) and EDTA (1 mM) (Melloni *et al.*, 1984)). The role of these inhibitors was to prevent the proteolytic degradation of the exoproteins in the biological preparation (James, 1978; Hall & Drasar, 1990). This solution was dialysed to completion against the same buffer solution at 4 °C, using the Spectra/Por 4 (molecular weight cut-off (MWCO) = 12 - 14 kd) regenerated cellulose membrane (Spectrum, Houston, Texas) and stirring the external solution with a magnetic bar. Before use, the membrane was washed in double distilled water. Exoprotein concentrate was clarified by centrifugation (CP10).

3.8.2 Filtration of exoprotein concentrate

Nalgene filter units with cellulose acetate (low protein binding) membranes (Nalge Company, Rochester, NY) of the 0.45 μm and 0.2 μm pore size specifications were used. The former was used for clarification and pre-sterilisation steps. The latter was used for sterilisation purposes since it is capable of excluding bacteria. Vacuum lines (attached to the lower chamber) were operated after addition of the sample to the (subsequently covered) upper chamber, the two chambers being separated by the filtration membrane.

3.8.3 Sample concentration

Exoprotein solutions were concentrated for either analytical purposes or application to gel filtration columns. Three protocols were used:

(i) TCA precipitation. This method was used to concentrate small volumes for analysis by SDS-PAGE. TCA (100 % w/v) was added to samples to give a 10 % final concentration. These preparations were put on ice for 30 minutes before being centrifuged (CP12). The supernatant was then drawn off using a Pasteur pipette and discarded. The precipitate was washed twice by successive resuspension (in ethanol (50 % v/v) ether (50 % v/v)), centrifugation (CP13) and aspiration. Finally the pellet was resuspended in a small volume of doubly distilled water.

(ii) Centricon-30 procedure. Centricon centrifugal microconcentrators provide fast, efficient concentration of small volume macromolecular solutions by ultrafiltration through Amicons low-adsorption, hydrophilic YM membrane. This ultrafiltration method of concentration of sample exoprotein does not cause concomittant concentration of buffer salts or sample denaturation. Sample solution is added to the reservoir, prior to centrifugation of the whole device (CP11); up to 2 ml of sample solution can be concentrated to 25 - 40 μl per centricon preparation, per run. Centrifugal force drives solvents and low molecular weight solutes through the membrane and into the solute cup. Macrosolutes with a molecular weight in excess of 30 kd are retained inside the sample reservoir, in a diminishing sample volume. In the recovery phase of the operation, the sample is transferred to the retentate cup by placing the cup over the sample reservoir and centrifuging for a second time (CP10). This method minimises film losses at the membrane and the reservoir wall.

Both haemolysins purified in this study have native M_ws > 30 kd (Honda *et al*, 1976b,c; Miyamoto *et al.*, 1980: TDH has a M_w = 42 kd; Hall & Drasar, 1990; ETH processed by

proteolysis - peptides have M_ws of 83.2, 71.6 and 60.3 kd). Accordingly Centricon-30s, in which the filtration membrane has a MWCO of 30 kd, were used to concentrate these exoproteins.

(iii) Centriprep 30 procedure. The Centriprep equipment operates on analagous principles to the centricon system outlined above. Its size and architecture are adapted so that it functions in a different range of volumes (resolving starting volumes of up to 15 ml to final volumes of 2 ml per preparation per run). Centriprep-30 (MWCO = 30 kd) was used with CP11.

3.8.4 Ion exchange

Separation is achieved on the basis of the charges carried by solute molecules. An insoluble matrix is covalently bonded to charged groups which are associated with mobile counter-ions. Recently developed ion exchangers combine good flow properties with high capacities (Fagerstam *et al.*, 1983a,b). After equilibriation of the ion exchanger, ion exchange was performed in two stages: (i) sample application and adsorption, and (ii) elution, during which substances are separated. Separation occurs because different substances have different affinities for the ion exchanger due to changes in their charge. These affinities can be controlled by altering conditions such as ionic strength (cation or anion exchange) or pH (chromatofocussing). Gradient elution leads to good resolution because, during elution, zone sharpening occurs ie the leading edge of a peak is retarded if it advances ahead of the concentration or pH required to elute it. Continuous gradients were generated by mixing two buffers (Buffer A and Buffer B) of different ionic strengths or pH together, using the Gradient Mixer GM-1 in a Fast Protein Liquid Chromatography (FPLC) system (Pharmacia, Uppsala, Sweden). The flow rate used was 1 $ml(min)^{-1}$, and the gradient time was 20 minutes.

(i) Anion exchange. An increase in ionic strength increases the competition between ions for binding-sites, causing a decreased capacity of the gel for the applied substances. The anion exchange column used was the MonoQ HR 5/5 (Pharmacia; Fagerstam *et al.*, 1983a,b; Soderberg *et al.*, 1983); this column is based on a beaded hydrophilic resin with a narrow range of particle size. The charged group on the gel is $-CH_2-N^+(CH_3)_3$ and ionic capacity of the gel is 0.28 - 0.36 $mmoles(ml)^{-1}$. Samples were eluted by an increasing ionic gradient in the direction A to B. Buffer A was Tris/HCl (20 mM); Buffer B was Buffer A + 1 M NaCl.

(ii) Chromatofocussing. Capacity is also dependent on pH, so application of a pH gradient against the column can separate biomolecules. The polybuffer exchange column used was the MonoP HR 5/20 column (Pharmacia; Fagerstam *et al.*, 1983a,b; Soderberg *et al.*, 1983; Bell *et al.*, 1984), having an ionic capacity of 0.15 - 0.2 mmoles$(ml)^{-1}$. The start buffer was 25 mM *bis*-Tris/HCl pH 7.0; eluent was polybuffer 74/HCl pH 4.0.

3.8.5 Gel filtration

In the years since its introduction (Porath & Flodin, 1959), gel filtration with Sephadex has been used extensively for purification of biological molecules, largely because of the procedure's reliability and simplicity. The stability of Sephadex and Sepharose CL and their inertness towards biopolymers under a wide range of conditions have made them the standard media for gel filtration. As solute passes down the chromatographic bed, its movement depends on: (i) bulk flow of the mobile phase, and (ii) Brownian motion of the solute particles, causing their diffusion both into and out of the stationary phase. Models proposed to account for solute behaviour in gel filtration (Flodin, 1962; Porath, 1963; Squire, 1964; Laurent & Killander, 1964; Ackers, 1967) centre on the different abilities of various molecules to enter the stationary phase. Thus, large molecules that enter the stationary phase only infrequently move through the bed faster, while smaller molecules that enter the stationary phase frequently move more slowly through the column because they spend an increased proportion of time in this stationary phase. Hence molecules are eluted in order of decreasing molecular size. For protein separation, continuous solute detection of with a UV monitor (Pharmacia UV-2) gives an elution diagram on a chart recorder (Pharmacia REC-1) in which an inferometer reading (A_{280}) (y) is plotted against elution volume (x). In the separations performed a very small sample was applied compared with the final elution volume; hence the position of peak maximum describes the elution volume (V_e) for a given solute. Sephadex and Sepharose columns generally give symmetrical peaks for a given solute (Flodin, 1962) as a result of their linear partition isotherms. The V_e itself is not a sufficient parameter to define the behaviour of a solute, because it is dependent on the total volume (V_t) of the packed bed and the way the column has been packed. By analogy with other types of partition chromatography, solute elution is best characterised by a distribution co-efficient, K_d, representing the fraction of the stationary phase that is available for diffusion of a given solute species:

$$\mathbf{K_d = \{(V_e - V_o)/V_s\}} \quad ...(13)$$

In this relation, V_o is the void volume ie the elution volume of molecules that are distributed solely in the mobile phase (since they are unable to enter the stationary phase), V_s is the stationary phase volume ie the volume of solvent within the gel that is available to very small molecules (that can move freely between mobile and stationary phases) minus V_o. In practice, V_s is rather difficult to measure and is generally substituted by (V_t - V_o); V_t is an overestimation of V_s since it contains that volume of the gel-forming substance itself that is inaccessible to all solute molecules. The parameter yielded after such a substitution is K_{av} (as opposed to K_d) representing the fraction of the stationary gel volume that is available for diffusion of a given solute species:

$$\mathbf{K_{av} = \{(V_e - V_o)/(V_t - V_o)\}} \quad ...(14)$$

Evidently K_{av} is not a true partition co-efficient but, for a given gel, it defines solute behaviour independently of bed dimensions and packing, and there is a constant ratio of K_{av}:K_d independent of the nature of the solute or its concentration.

Most models regard the partition of solute molecules between gel particles and the surrounding fluid as an entirely steric effect. Flodin (1962) divided the gel into forbidden and permitted regions in which increasing the molecular dimensions of the solute caused a greater proportion of the gel to be forbidden. In the permitted region, the solute concentration was assumed to be identical to that of the surrounding fluid. The steric approach has been extended by consideration of the geometry of pores that govern diffusion between the stationary and mobile phases. Porath (1963) derived a theoretical relationship between K_d and Stokes ionic radius assuming that Sephadex pores are conical. Laurent & Killander (1964) found a good correlation between K_{av} and molecular radius of solute, on the assumption that the gel network is composed of randomly arranged rigid rods. All such models have been successfully applied to predict solute elution behaviour. However, as noted by Ackers (1967) none may be accurate in a structural sense; results are equally in accordance with a formulation in which the fractions of the stationary phase available to solute molecules of different radii are defined by a Gaussian probability curve without assumptions being made about pore geometry. For fractionation of bacterial exoproteins of similar M_w, a Superose12 gel column (Pharmacia) was selected (flow rates as indicated).

3.9 POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

The principles underlying this methodology and the specific system used are outlined below. PAGE has been extensively reviewed previously by Hames and Rickwood (1985).

In an electric field a particle with nett charge moves towards one or other of the electrodes. A protein (at a pH other than that indicted by the *isoelectric point*) migrates towards an electrode at a rate dependent its *charge density* (ie {charge/mass}). Zone electrophoresis is designed on this basis to separate substances by progressive movement (individual entities at well-defined velocities, producing tight zones), after initial placement in the electric field as a single narrow zone at a chosen distance from the electrodes. Electrophoresis is generally performed within a supporting gel. This minimises the adverse effects of convective heat disturbances and post-electrophoresis diffusion, and furthermore may also participate in the separation process by interacting with the migrating particles.

Polyacrylamide is the paradigm supportive medium for protein electrophoresis because it is: (i) readily prepared from highly purified reagents in a reproducible manner under standardised polymerisation conditions, (ii) chemically inert over wide ranges of pH, temperature and ionic strength, (iii) transparent, and (iv) well-suited to the size fractionation of proteins. Gels with a wide range of pore sizes (the order of which is matched to the sizes of the proteins to be separated) can easily be made, the effective pore size decreasing with an increasing concentration of acrylamide. Flat slab gels were used because they allow the simultaneous electrophoresis of many samples under identical conditions so that the band patterns produced are directly comparable.

The buffer sodium dodecyl sulphate (SDS) was used to dissociate all proteins into their individual polypeptide subunits (rather than constructing a native gel system in which a non-dissociating buffer would be used in a protocol designed to preserve protein conformation, subunit interaction and biological activity). The sample was denatured by heating (100 °C for 3 minutes) in the presence of (an equal volume of) excess SDS and dithiothreitol (DTL), a thiol reagent used to cleave disulphide bonds (SDS boiling buffer is Tris-HCl (0.0625 mM; pH = 6.8) with SDS, DTL, and bromophenol blue (2, 5, and 0.002 % w/v, respectively). Under such conditions most polypeptides bind SDS in a constant weight ratio (1.4 g SDS/g peptide). The intrinsic charges of the polypeptide are insignificant compared to the negative charges provided by the bound reagent. Thus SDS-

polypeptide complexes have essentially identical charge densities and migrate in polyacrylamide gels of the correct porosity strictly according to size (Shapiro *et al.*, 1967; Weber & Osborn, 1969).

A multiphasic (or discontinuous) buffer system is present in PhastGel Gradient gels to increase the resolution of the electrophoretic process. In a multiphasic system, the sample is loaded onto a large-pore stacking gel polymerised on top of a small-pore resolving gel (in contrast, in a continuous buffer system, samples are loaded directly onto the resolving gel). Within the stacking gel, a heterogeneous buffer system acts to concentrate the proteins into extremely narrow zones prior to their separation during electrophoresis in the resolving gel. The PhastGel Gradient gels have a 13 mm stacking gel zone. The buffer system in the gels is of 0.112 M acetate (leading ion) and 0.112 M Tris, pH 6.4. The buffer system in the PhastGel SDS buffer strips (made of 2 % Agarose IEF) is of 0.2 M tricine (trailing ion), 0.2 M Tris and 0.55 % SDS (analytical grade), at pH 7.5. When electrophoresis starts, the leading and trailing ions (acetate/tricine) form a moving boundary which migrates through the stacking gel causing the proteins in the applied sample to stack between the leading and trailing ions (Ornstein, 1964; Davis, 1964; Laemmli, 1970; Hames & Rickwood, 1985). In SDS-PAGE, the SDS-polypeptide complexes are negatively charged over a wide pH range, such that the pH of the SDS-phosphate buffer in the resolving gel is not critical. In the stacking gel the pH of the SDS-discontinuous buffer system is only important in that it permits sample concentration by stacking.

As mentioned previously, acrylamide concentration is a determinant of the sizing properties of the porous nature of the gel. The gel acrylamide concentration can therefore be optimised to fractionate a heterogeneous mixture of proteins to give a high resolution display of all the components of interest. The approach used for the analysis of exoprotein mixtures was a concentration gradient acrylamide gel, the PhastGel Gradient 8 - 25, in which acrylamide concentration of the resolving gel (32 mm long) increases (and pore size decreases) from 8 to 25 % in the direction of protein migration (with 2 % cross-linking throughout). Such a system has two major advantages over uniform concentration gels: (i) the ability to fractionate proteins over a wider range of molecular weights, and (ii) the gradient in pore size causes a significant sharpening of protein bands during migration. Thus, when the stacked proteins reach the stacking/gradient gel

interface, their mobility is drastically reduced, due to the sudden decrease in pore size, and the moving boundary rapidly migrates away from the proteins. The proteins unstack and migrate (at a uniform voltage and a constant pH = 8.5) according to their mass. The process was stopped when the tracking dye reached the anode buffer strip (after approximately 60 - 65 Vh).

3.9.1 Staining techniques

Immediately after electrophoresis, the gel was subjected to one or other of the following staining protocols, each of which incorporate a fixation step preventing dispersion of zones by diffusion after electrophoresis has been terminated.

(i) Coomassie Blue staining. After electrophoresis, gels were put into Stain (Coomassie Blue R-250 (0.025 % w/v), methanol (40 % v/v), and acetic acid (7 % v/v)) and gently shaken for 2 - 4 hours. Subsequently, they were transferred to Destaining Solution I (methanol (50 % v/v) and acetic acid (10 % v/v)) for 1 hour with agitation, before being finally immersed in Destaining Solution II (acetic acid (7 % v/v), and methanol (5 % v/v)).

(ii) Silver staining. Silver staining protocols have been developed (Switzer *et al.*, 1979; Oakley *et al.*, 1980; Heukeshoven & Dernick, 1985) that are much more sensitive than Coomassie staining techniques. Although the relative density of silver stain is approximately linear with protein concentration for some proteins (eg phosphorylase), it is markedly non-linear for others (eg bovine serum albumin) over the same range (Hames & Rickwood, 1985). Thus quantification of individual protein bands after silver staining is subject to major reservations. The silver staining technique used was that described by Heukeshoven and Dernick (1985) and automated for the PhastSystem (Pharmacia, 1991). Initially, the separated protein zones were fixed and the buffer ions removed by successive washes in a fix solution (50 % v/v ethanol, and 10 % v/v acetic acid; 2 minutes at 50 °C) and (2 x) in a wash (10 % v/v ethanol, and 5 % v/v acetic acid; 2 and 4 minutes at 50 °C). The protein components were subsequently sensitised in a glutardealdehyde solution (8.3 % v/v; 6 minutes at 50 °C) which was washed out (2 x) by the wash solution (3 and 5 minutes at 50 °C) and rinsed (2 x) by deionised water (2 minutes for each rinse at 50 °C). Proteins were then stained by silver nitrate (0.25 % w/v; 13 minutes at 40 °C) and rinsed (2 x) by deionised water (0.5 minutes for each rinse at 30 °C). Gels were then exposed (2 x) to developer solution (0.04 % v/v formaldehyde, and 2.5 % w/v sodium

carbonate; 0.5 and 4 minutes at 30 °C), stop solution (5 % v/v acetic acid; 2 minutes at 30 °C) and preserving solution (10 % v/v acetic acid and 5 % w/v glycerol; 3 minutes at 50 °C).

3.9.2 Photography of stained protein bands

Wet slab gels were photographed after being laid directly onto an illuminator with an opal white screen, avoiding trapped air bubbles.

3.9.3 Molecular weight determination

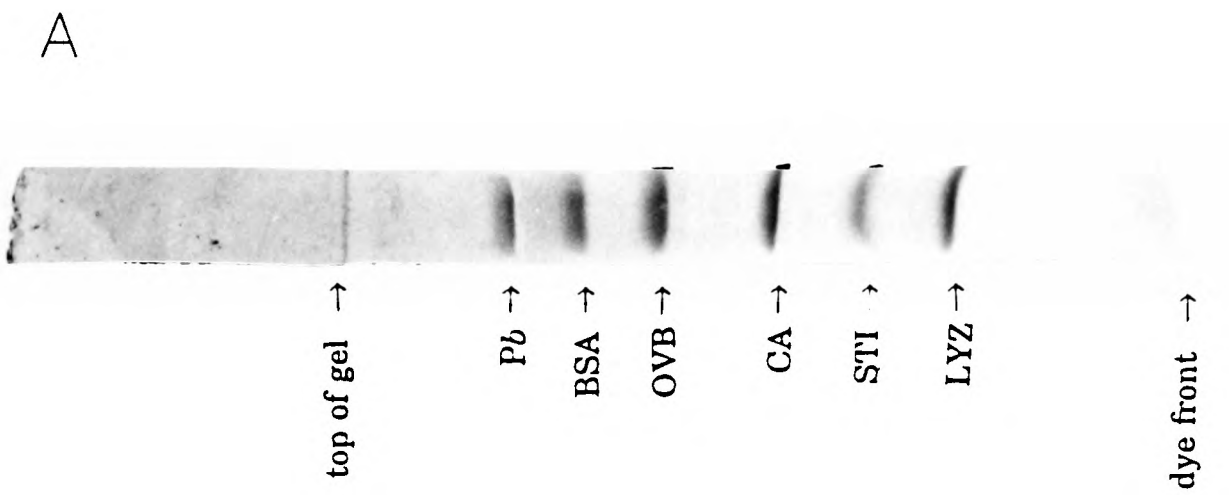
As described above, migration of bands in SDS separations is dependent not on the intrinsic electric charge of polypeptides but rather on molecular weight (Shapiro, 1967). Gels of a polypeptide mixture were run with standard proteins (low range; Bio-Rad Laboratories (Richmond, California, USA)) of known molecular weight (phosphorylase *b* (97.4 kd), bovine serum albumin (66.2 kd), ovalbumin (45 kd), carbonic anhydrase (31 kd), soybean trypsin inhibitor (21.5 kd), and lysozyme (14.4 kd)) in a neighbouring lane. Ferguson plots (Hames & Rickwood, 1985) were made in which the $\log_{10}$ polypeptide M_w was plotted against its R_f (the distance from the top of the gel to the polypeptide band, divided by the distance from the top of the gel to the dye front). A Ferguson plot derived from SDS-PAGE is shown (Figure 3.10) for the standard (M_w marking) proteins. This method was used for experiments described in Chapter 6 to estimate the M_w of purified haemolysins from R_f values.

3.10 AMINO-TERMINAL SEQUENCING

Amino-terminal sequence analysis was performed in a fully automated Model 477A Protein/Peptide sequencer system (Applied Biosystems) by an established method (Moos *et al.*, 1988). The chemistry underlying the the sequencing process is the Edman degradation reaction which occurs in the sequenator after absorption of the sample to a small disc of glass-fibre filter paper.

3.10.1 The Edman reaction

During each Edman reaction cycle, the N-terminal residue is removed from the polypeptide chain for subsequent identification. The reaction (see Figure 3.11) is divided into three steps: (i) coupling, (ii) cleavage, and (iii) conversion. The polypeptide is retained on the glass-fibre support (without covalent attachment) during the coupling and cleavage steps which occur in a temperature controlled (45 °C) glass reaction cartridge.



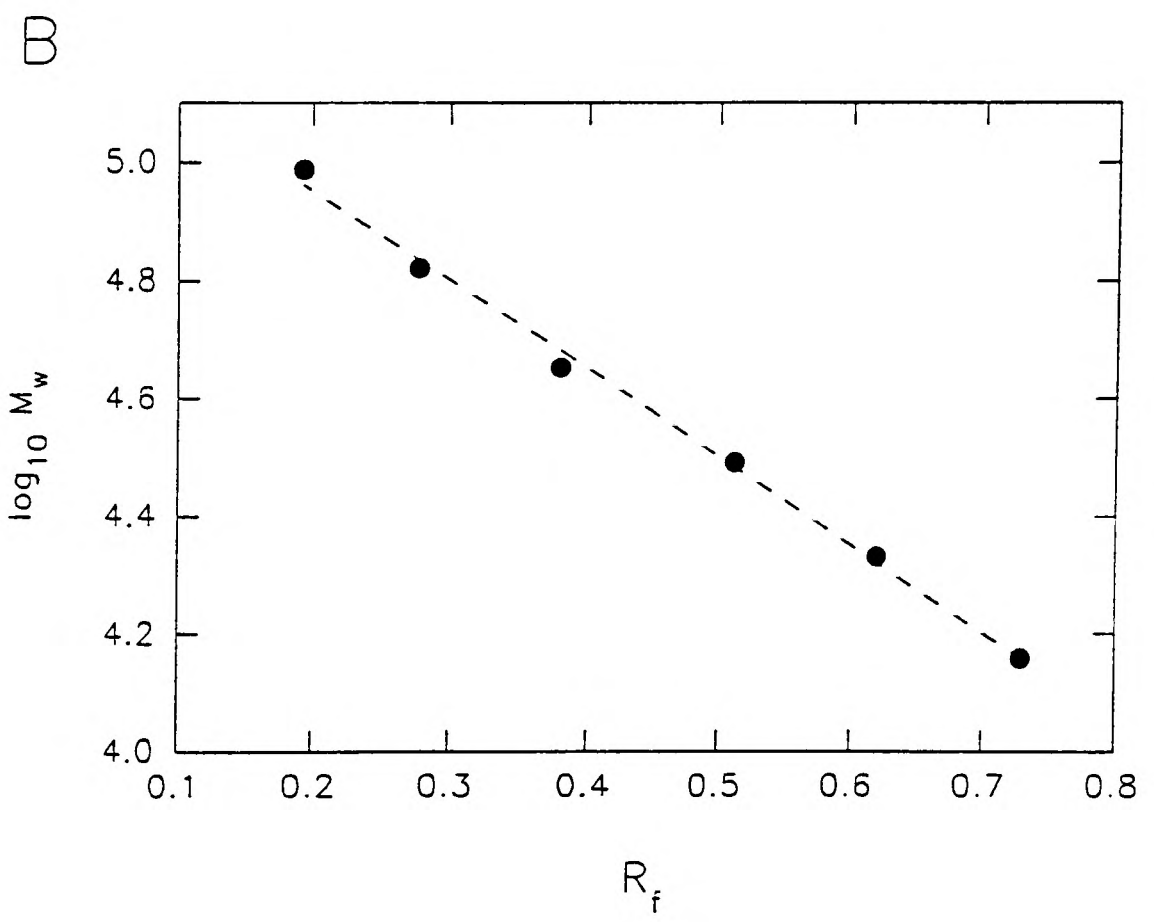


Figure 3.10 (A) Gel of molecular weight standards. Phastgel (gradient 8 - 25 %) was used to electrophorese a mixture of molecular weight markers (phosphorylase *b* (97.4 kd), BSA (66.2 kd), ovalbumin (45 kd), carbonic anhydrase (31 kd), soybean trypsin inhibitor (21.5 kd), and lysozyme (14.4 kd)). **(B) Ferguson plot for molecular weight standards.**

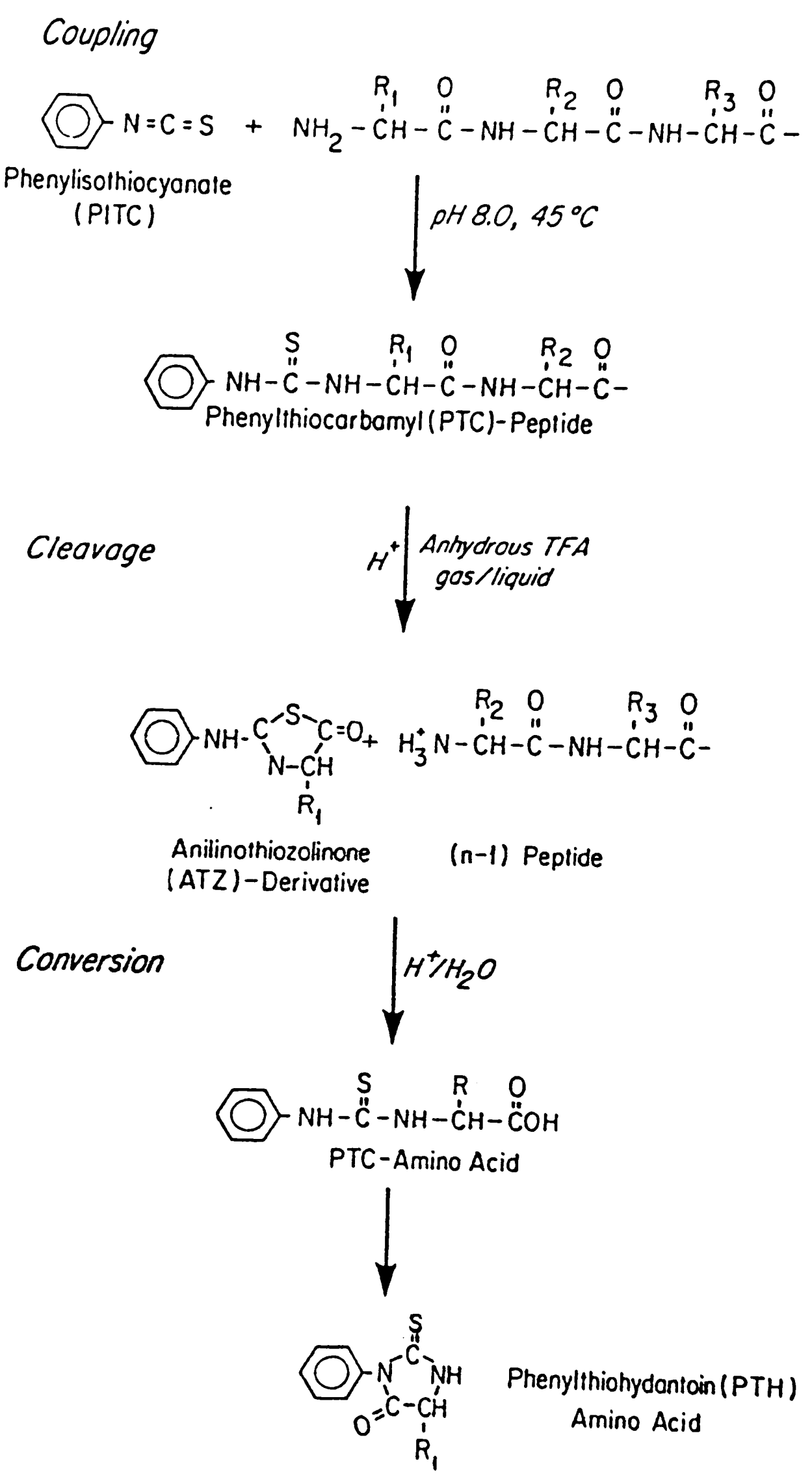


Figure 3.11 The Edman degradation reaction. The chemical reactions of the Edman degradation cycle. In the coupling step, PITC modifies the N-terminal residue of a peptide. Acid cleavage of removes the N-terminal residue as an unstable ATZ-derivative and leaves the shortened (*n* - 1) peptide with a reactive N-terminus. The ATZ-derivative is converted in the last step to a stable PTH-amino acid. After Matsudaira (1993).

The conversion reaction occurs in a separate conversion chamber in the aqueous phase. The reaction proceeds as follows:

(i) coupling: phenylisothiocyanate (PITC) modifies the α-amino group of the N-terminal residue to form a phenylthiocarbamyl (PTC) -polypeptide. Coupling at the α-amino group is favoured at pH 8.0 - 8.5, and is inhibited by N-terminal modifications such as acetylation, formylation, fatty acid acylation and cyclisization.

(ii) cleavage: the PTC-N-terminal residue is rapidly cleaved from the polypeptide chain under anhydrous and acidic conditions to liberate an unstable anilinothiozolinone (ATZ) - amino acid (aa) and the shortened (n-1) peptide with a reactive N-terminus avaiable for another reaction cycle.

(iii) conversion: the ATZ-aa is washed into the conversion chamber where it is converted into a stable phenylthiohydrazine (PTH)-derivative in a two step reaction in aqueous acidic solution. The ATZ-aa is rapidly hydrolysed into a PTC-aa before being cyclicised to a stable PTH-aa.

3.10.2 Identification of PTH-aas

PTH-aas generated during each reaction cycle were identified by high performance liquid chromatography (HPLC) and ultra-violet (UV) absorbance in an on-line system. Thus, all 20 PTH-aas were resolved from a reverse phase HPLC support, quantified by their UV absorbance, and identified by their characteristic retention time (see Figure 3.12 for a standard chromatogram). The sequence was determined by the appearance of a PTH-aa in cycle *x*, with its disappearance and the conjoint appearance of another PTH-aa (if different) in cycle *x+1*.

Some by-products of the Edman reaction protocol can also be detected in the chromatogram (eg as generated by reactions between PITC, DTL and H_2). Certain modified amino acids can be detected, in particular phosphorylated serine (as a PTH-dehydroalanine peak as opposed to a PTH-serine peak). However, glycosylated residues cannot be identified directly because normally the PTH-(glycosylated)aa is not released from the sequenator for HPLC analysis, thus generating a blank cycle.

3.10.3 Chemical limitations

Sample preparation is critical to successful sequence analysis. Contaminants that interefere with the chemistry of the Edman degradation reaction must be eliminated prior to loading. In particular, the following substances should be avoided (Matsudaira, 1993):

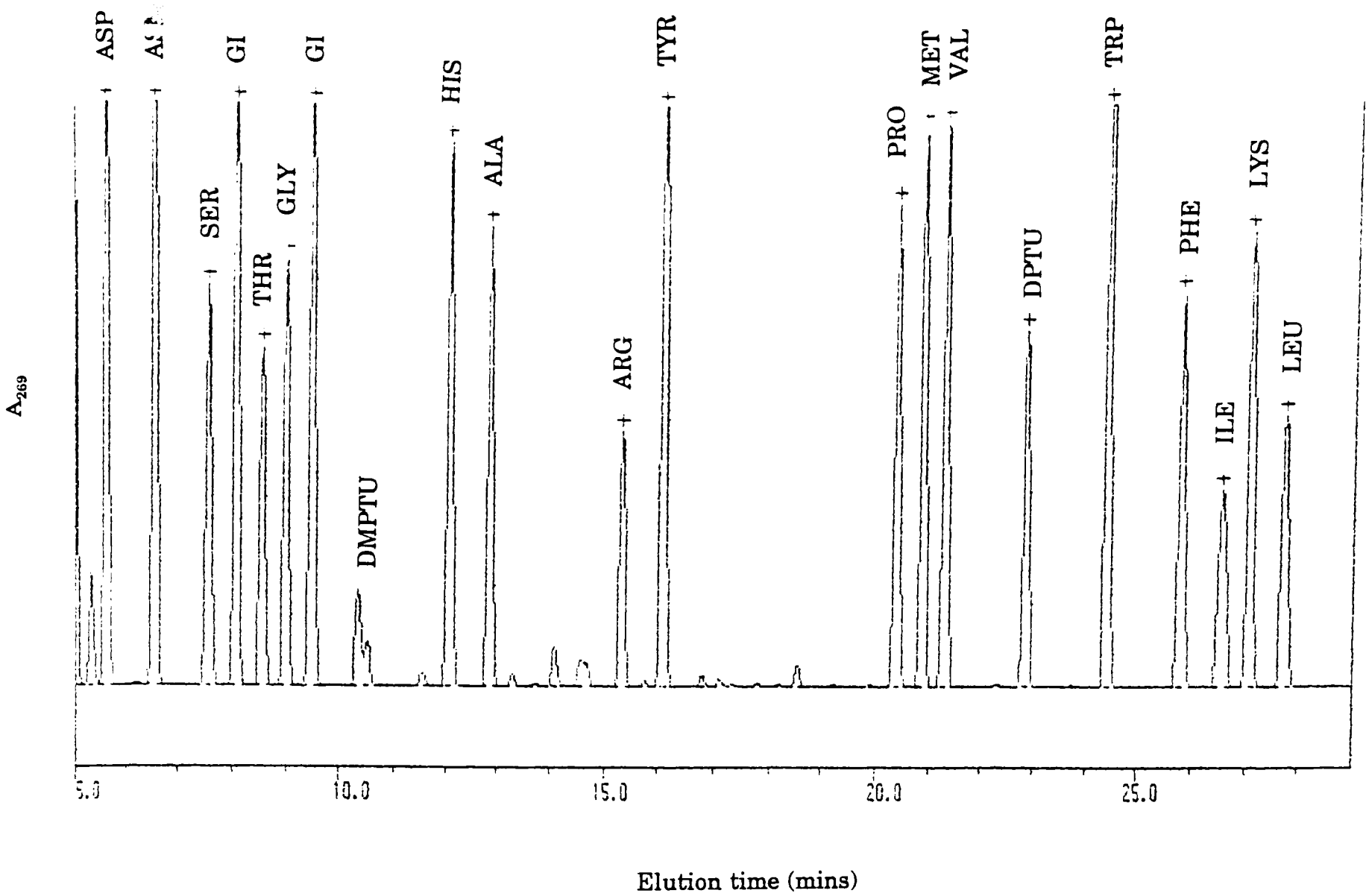


Figure 3.12 Standard chromatogram from HPLC of the amino-terminal sequencer. The elution pattern of PTH-amino acid standards (75 pmoles). DMPTU and DPTU are by-products of the sequencing reaction and serve as useful reference peaks in chromatograms.

(i) buffers - large quantities of buffer salts (with pK_a values < 8.0) can (a) induce inefficient coupling by altering the pH of the coupling step, and (b) inhibit the cleavage reaction by neutralising the cleavage acid (TFA), (ii) primary amines - these can compete with the Edman reagent, PITC, and can inhibit the coupling reaction, (iii) non-ionic detergents - these can inhibit the Edman degradation, and (iv) glycerol or sucrose - these compounds are non-volatile and leave a highly viscous residue.

3.11 DATA HANDLING

3.11.1 Graphical methods

Several types of relation were constructed, including:

(i) Haemolysis curves. These were drawn by hand for analysis of H^{50} and $\{(H^{50})^{-1}\}$ values. However, when given as figures in this dissertation, these curves were fitted to experimentally derived points using the Sigmaplot spline curve function.

(ii) Line of best fit by linear regression analysis. This was performed for a number of apparently linearly related variables.

3.11.2 Statistical significance

Results are given as mean ± standard error of the mean (S.E.M.) for *n* separate experiments, and have been tested for statistical significance (ie the threshold value for the probability of an hypothesis being correct on the basis of the data collected) by using unpaired or paired Student's *t*-tests, for normalised and non-normalised data, as appropriate. In these experiments, an hypothesis involves there being a difference between either the degree haemolysis (measured as H^{50} values), the $\{(H^{50})^{-1}\}$ values, or the mean influxes under the various conditions imposed.

CHAPTER 4

Haemolytic reaction of Kanagawa haemolysin of *Vibrio parahaemolyticus*

'They make haste to shed innocent blood' Jeremiah 59:7

4.1 INTRODUCTION

The role of the thermostable direct haemolysin (TDH) in the pathogenesis of disease associated with *Vibrio parahaemolyticus* (Takeda, 1983; Kreger, 1991; Honda & Iida, 1993), and possibly other *Vibrio* species (Honda & Iida, 1993), has already been discussed in section 2.6. Erythrocytes constitute a well-established system for studies of membrane function (section 2.8.1; Agar & Board, 1983 & references therein) and their membrane transport properties have been extensively characterised (Ellory & Tucker, 1983). The effects of toxin action on intestinal tissue may be rather complicated and difficult to interpret (section 2.4); accordingly, erythrocytes have been advocated as a model target cell in which to assess toxin action (section 2.8.1). Previously, the effects of many membrane-active toxins have been tested on red blood cells (Bernheimer, 1947; Johnson, 1972; Kanbayashi *et al.*, 1972; Inoue *et al.*, 1976; Johnson & Boese-Marrazzo, 1980; Marchlewicz & Duncan, 1981; Kothary & Kreger, 1985; Niedermeyer, 1985; Tosteson *et al.*, 1985; Weiner *et al.*, 1985; Miyake *et al.*, 1989; Krasilnikov *et al.*, 1992; Belmonte *et al.*, 1993), largely because of the factors listed in section 2.8.1, but also because the fraction of lysed cells can be derived from a simple assay of released haemoglobin (section 3.4; Bernheimer, 1988) and isotope flux methodology can be easily applied to these cells (section 3.5; Ellory, 1982; Ellory *et al.*, 1992). In addition, the mode of action of TDH itself has been partially characterised on erythrocytes (Zen-Yoji *et al.*, 1971, 1976; Sakurai *et al.*, 1973, 1975; Takeda *et al.*, 1975, 1976; Honda *et al.*, 1976c, 1992; Takeda, 1983, 1988; Toda *et al.*, 1990; Kreger, 1991; Douet *et al.*, 1992; Honda & Iida, 1993). Despite such intensive study, Bernheimer and Rudy (1986) assigned TDH to a category of individualistic agents, with no defined mechanism of action. The findings of such studies have already been summarised (section 2.6.4) but here their content is described in more detail.

Zen-Yoji *et al.* (1971) studied the effect of TDH on different kinds of erythrocyte and

found that the haemolysin showed potent haemolytic activity on erythrocytes of different species, its activity decreasing in the order: rat, dog, mouse, monkey, human, rabbit and guinea-pig. However, it was further reported that there was no activity on horse erythrocytes. Sakurai *et al.* (1975) also studied the mechanism of TDH-induced haemolysis and found that haemolysis was a temperature-dependent process, being observed at 37 °C but not at 4 °C. Although this study found that some TDH could be bound at 4 °C, this does not in itself mean that the process of toxin-binding is temperature-independent as interpreted by Freer (1986) and Kreger (1991) on the basis of these data. The binding step was followed by a lytic step which was stimulated by the divalent cations Ca^{2+}, Mn^{2+} and Mg^{2+} at 25 mM. Haemolysis occurred more rapidly in Ca^{2+} than Mg^{2+} (25 mM); however, no reference was made to appropriate osmotic control for their 'control' condition. Sakurai *et al.* also studied the kinetics of inhibition of TDH action using anti-TDH anti-serum. Free haemolysin which was not membrane-bound was completely neutralised by antiserum. The inhibitory effect on haemolysis decreased with delay in the time of addition of antiserum after addition of TDH. If it is assumed that the antiserum cannot neutralise bound TDH (which is likely to expose different epitopes after a conformational change on binding), then the kinetics of inhibition suggest a binding-phase that is not completed for 20 - 40 minutes. However, it should be noted that they did not interpret their data in these terms.

The study of Honda *et al.* (1992) is considered in more detail in section 4.3 because the experiments reported here were performed prior to publication of their report. Some of the conclusions of this latter report are at odds with data presented in the current study or by Sakurai *et al.* (1975). Comparisons of data presented (section 4.2) with the earlier reports are illuminating and discussed fully in section 4.3. The scope of the erythrocyte as a model system in which to investigate perturbations of membrane function has not been fully explored, as evinced by the simple yet revealing experiments detailed in this chapter. In particular, the application of isotope flux methodology has not been applied to analysis of the action of TDH (see Chapters 4 and 5) or many other toxins.

This chapter examines the nature of the haemolytic action of Kanagawa haemolysin (KH), the commercially available preparation of TDH (Cherwonogrodzky & Clark, 1982b). However, it is noted that the purity of this preparation has been questioned in the past, in light of the demonstration of an additional band to that given by TDH on

SDS-PAGE after silver staining (Douet *et al.*, 1992). If factors other than TDH are present (such as those described in section 2.6.3), then they may cause haemolysis themselves and/or interfere with the action of TDH. Co-operative lytic processes involving the sequential action of primary step enzymes and secondary step factors are well documented (Christie *et al.*, 1944; Munch-Petersen & Christie, 1947; Wiseman & Caird, 1967; Bernheimer *et al.*, 1979; Sterzik & Fehrenbach, 1985) and reviewed (Fehrenbach & Jurgens, 1991). Inhibitory interactions have also been shown (Fehrenbach & Jurgens, 1991), for example the haemolytic action of staphylococcal sphingomyelinase C (β-toxin) on sheep erythrocytes was inhibited by prior treatment of the target cells with small amounts of phospholipase D from either *Corynebacterium pseudotuberculosis* or the venom of the brown recluse spider (*Loxosceles reclusa*) (Bernheimer *et al.*, 1985). The possibility of such interactions between TDH and other factors in KH have been preliminarily examined in this Chapter by probing KH for certain known characteristics of highly purified TDH preparations (sections 2.6.4 and 4.2.3). No evidence for the action of other factors was found. The assay procedure used by Sigma (Sigma, 1992) to quantify aliquots of KH might be thought to suggest that haemolysis occurs by a mechanism of hot-cold haemolysis, in which haemolysis is dependent on a membrane phase transition. However, the lowering of temperature is not necessary for lytic action (Sakurai *et al.*, 1975); experiments of the current study (except those pertaining to temperature dependence) were performed at 37 °C.

Experiments involving both human and horse erythrocytes are described in this chapter. Human red cells have a well documented sensitivity to this toxin (Takeda, 1983) and are readily available, whilst horse cells have a purported insensitivity to TDH (Honda *et al.*, 1976b; Miyamoto *et al.*, 1980; Yoh *et al.*, 1986a,b; Yoshida *et al.*, 1991). The lack of effect of KH on horse erythrocytes is examined here to (i) confirm the identity of KH, and (ii) show that any apparent insensitivity is due to an insensitivity of binding rather than the ability of the horse erythrocytes to perform volume regulatory decrease by activation of an electroneutral KCl cotransporter (Gibson *et al.*, 1993) and so 'escape' haemolysis. The establishment of an insensitive cell-type is an important prerequisite to the study of the nature of receptor-toxin interactions as exemplified by a recent study (Waddell *et al.*, 1990) in which sensitivity to verotoxin of *Escherichia coli* was conferred after incorporation of the glycolipid globotriosylceramide (G_{b3}) into the otherwise insensitive

Daudi lymphoma cell-line.

The methodological approaches employed here are described in Chapter 3 (sections 3.4 and 3.5). In the section which follows, results are expressed in terms of either percentage haemolysis, time taken to reach 50 % haemolysis (H^{50}), normalised $\{(H^{50})^{-1}\}$, K^+ accumulated (mmol.(l.c.)$^{-1}$) or packed cell volume (as percentage of suspension). The implications of these data are then discussed in light of other reports (section 4.3).

4.2 RESULTS

4.2.1 Fundamental kinetic characteristics of KH-induced haemolysis

Figure 4.1B shows the time courses of haemolysis induced by KH at 37 °C in MBS (Hct = 3 %). The curves are of a non-symmetric (skewed) sigmoid nature, exhibiting a lag phase before the onset of haemolysis. After a middle phase of relatively rapid haemolysis, the rate was diminished but not stopped. The rate of haemolysis was strongly dependent on KH concentration (in the range $0 < [KH]$ (HU(ml)$^{-1}$) ≤ 1). In paired experiments to the above (ie performed on blood from the same donor at the same time), the kinetics of haemolysis were examined over a range of target cell concentrations ($0.5 \leq$ Hct (%) ≤ 6) in an unchanging initial reaction volume (5 ml) with KH at 1 HU(ml)$^{-1}$ (Figure 4.1A). Because there was a marked variation of haemolytic rate according to donor, results are expressed as standardised $\{(H^{50})^{-1}\}$ values (section 3.4). This parameter increased slightly with increasing haematocrit (Hct), indicating that a raised haematocrit (in a constant total reaction volume) slightly augmented (rather than protected against) haemolysis. This result was obtained in a KH:HRBC range in which a small decrease in KH concentration caused a pronounced decrease in haemolysis rate (Figure 4.1B). For experiments depicted in Figure 4.1B, the initial reaction volume was 5 ml, although the haematocrit was varied between 0.5 and 6 %. As the haematocrit was increased, there were corresponding small decreases and increases, in the initial extracellular volume and KH concentration respectively. The slight increase in extracellular KH concentration at raised haematocrits may explain the small increase in haemolytic rate, but essentially haemolytic rate was deemed to be independent of target cell concentration.

The effects of extracellular volume and KH concentration (Figure 4.2) on haemolysis were examined. Experiments were performed at a constant extracellular KH concentration (1 HU(ml)$^{-1}$), irrespective of extracellular volume (3.5 - 8 ml). As already noted there

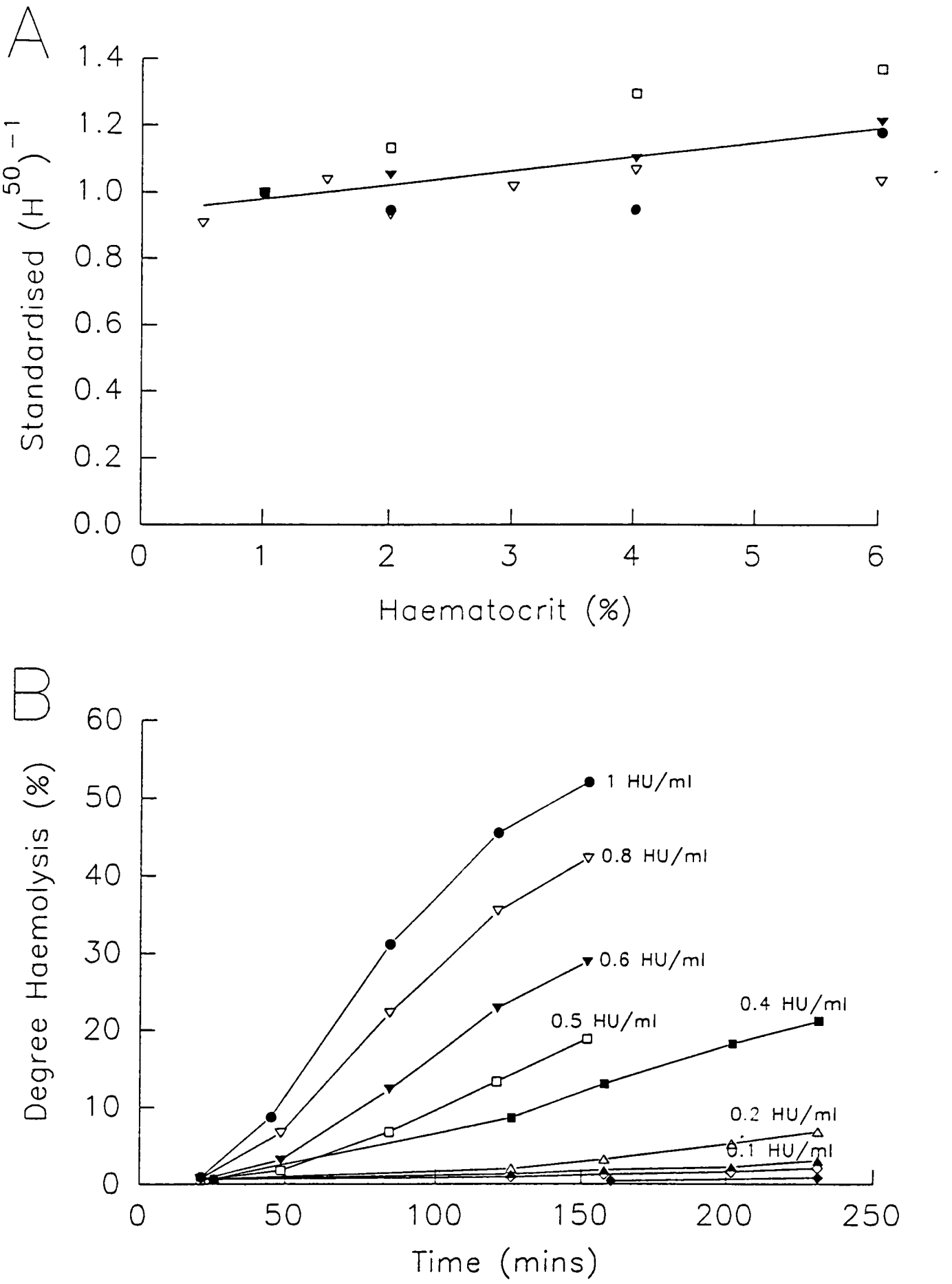


Figure 4.1 Kinetics of KH-induced haemolysis. Time-courses of KH-induced haemolysis were measured after the addition of packed cells to MBS containing endogenous transport system inhibitors and KH. (A) Pooled standardised results from four experiments (paired with (B) and each on a different donor); Hct was varied whilst using a constant total reaction volume (initially 5 ml) and (KH) = 1 HU(ml)$^{-1}$. (B) Typical results from one of four experiments showing the dose-response relation for KH. Experiments were initiated by the addition of 150 µl packed cells to MBS and the indicated amount of KH, (initial volume = 5 ml). The bottom two unlabelled lines indicate: open inverted triangles = 0.05 HU(ml)$^{-1}$, filled diamonds = no KH (ie control).

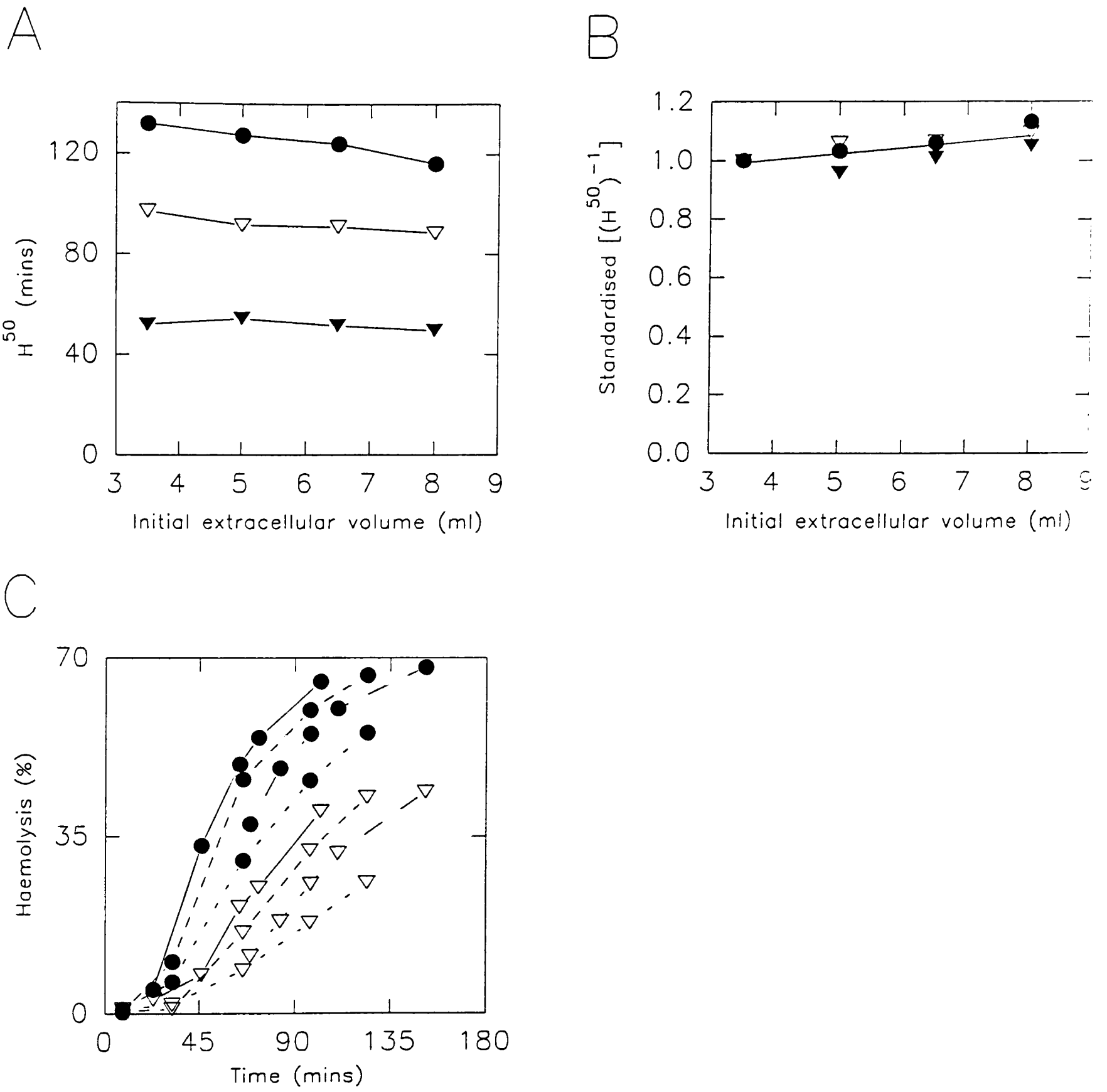


Figure 4.2 Effect of reaction volume on KH-induced haemolysis. The effect was tested at either a constant extracellular (KH) (A, B) or a constant KH:HRBC ratio (C). (A) Packed HRBC (100 µl) were added to the indicated volume of MBS containing the inhibitors and KH (1 $HU(ml)^{-1}$). Results are given as H^{50} values. Each donor is accorded a symbol-type. (B) Because of the large donor variation evinced in (A), the data have been expressed as normalised $\{(H^{50})^{-1}\}$ (an index of relative permeability). (C) Packed HRBC (100 µl) were added to MBS containing KH (1 and 0.05 $HU(ml)^{-1}$ for 5 and 10 ml systems, respectively) to initiate the time course. Results from a particular donor are accorded the same line type. Symbols indicate initial reaction volume 5 ml (filled circles) or 10 ml (open inverted triangles).

was a marked donor variation (Figure 4.2A). The H^{50} and normalised $\{(H^{50})^{-1}\}$ values (Figure 4.2A and B, respectively) for the haemolytic time courses so generated are essentially independent of extracellular volume *per se*. In fact, $\{H^{50}\}^{-1}$ increased slightly with increasing extracellular volume, indicating that no protective effect was exerted. This slight increase can be explained by the existence of a small extracellular volume in the added packed cells. This would slightly decrease the extracellular KH concentration, and would do so to a proportionately greater extent in smaller initial reaction volumes. Data in Figure 4.2C describe the effect of varying KH concentration and extracellular volume whilst maintaining a constant KH:HRBC ratio. Decreasing the KH concentration (and concommittantly increasing the extracellular volume from 4.9 to 9.9 ml) dramatically reduced haemolytic rate. Taken together, the data in Figures 4.1 and 4.2 indicate that KH-induced haemolysis is dependent only on KH concentration and donor susceptibility.

4.2.2 Underlying mechanism of lysis

Broadly speaking, haemolysins may act either by generating large lesions within the membrane through which haemoglobin can leak out, or by causing an increase in cation permeability that results in lysis after colloid osmosis. Data in Figures 4.3, 4.4, 4.5 and 4.6 confirm colloid osmosis as the mechanism underlying KH-induced haemolysis.

Osmotic protection against KH-induced haemolysis was demonstrated, either by suspension of the cells in isosmotic choline chloride, D-sorbitol or L-valine (Figure 4.3A) or by addition of extracellular sucrose (Figure 4.3B). This indicates that haemolysis is likely to occur secondary to a volume increase (after osmotic influx), rather than by direct leakage of intracellular contents.

In the absence of external colloid osmotic protectants, haemolysis due to colloid osmosis is preceded by a volume increase. Here, the volume of cells in the presence and absence of KH was followed during the haemolytic time course, by either measuring the PCV or following changes in morphology by light microscopy. Data are shown from a typical experiment, examining the PCV (Figure 4.4A) of an HRBC suspension in the presence of KH (1 $HU.(ml)^{-1}$), over the haemolytic time course (Figure 4.4B), as against untreated (control) HRBCs. The PCV increased during the lag phase prior to haemolysis supporting the contention that lysis occurs after a volume increase, secondary to colloid osmosis. The sequence of photographs in Figure 4.5 documents the changes in erythrocyte morphology as a result of KH action. Parts (a) to (f) show the field in view 0, 5, 15, 20,

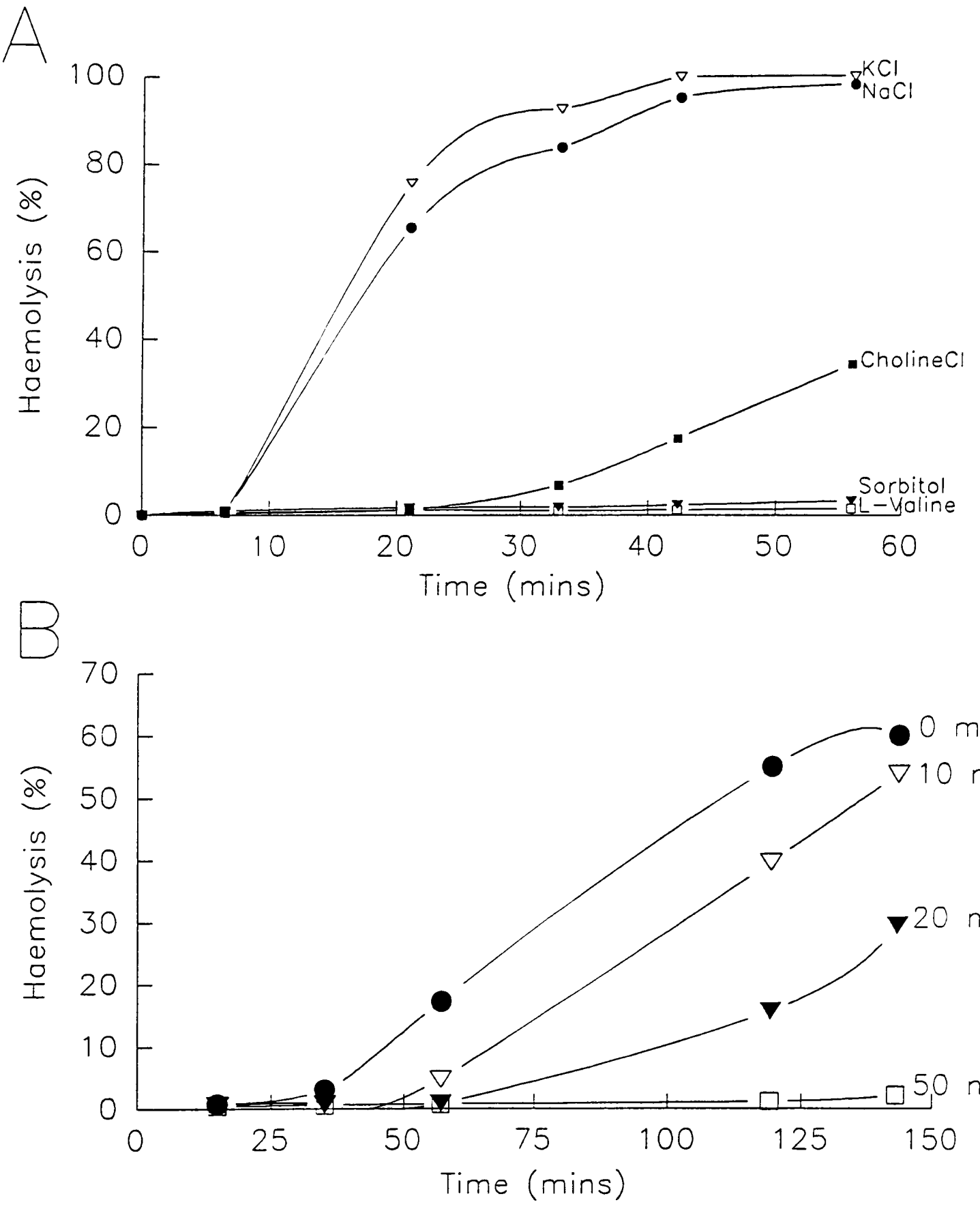


Figure 4.3 Osmotic attenuation of KH-induced haemolysis. Time-courses were initiated by the addition of packed cells to: (A) solutions indicated (295 ± 3 mosmoles(kg)$^{-1}$ at pH 7.4 (HEPES/HCl) with HEPES (10 mM) with KH (1.5 HU(ml)$^{-1}$). Hct = 2.5 %. (B) MBS containing the indicated concentration of sucrose and KH (1 HU(ml)$^{-1}$). Hct = 5%.

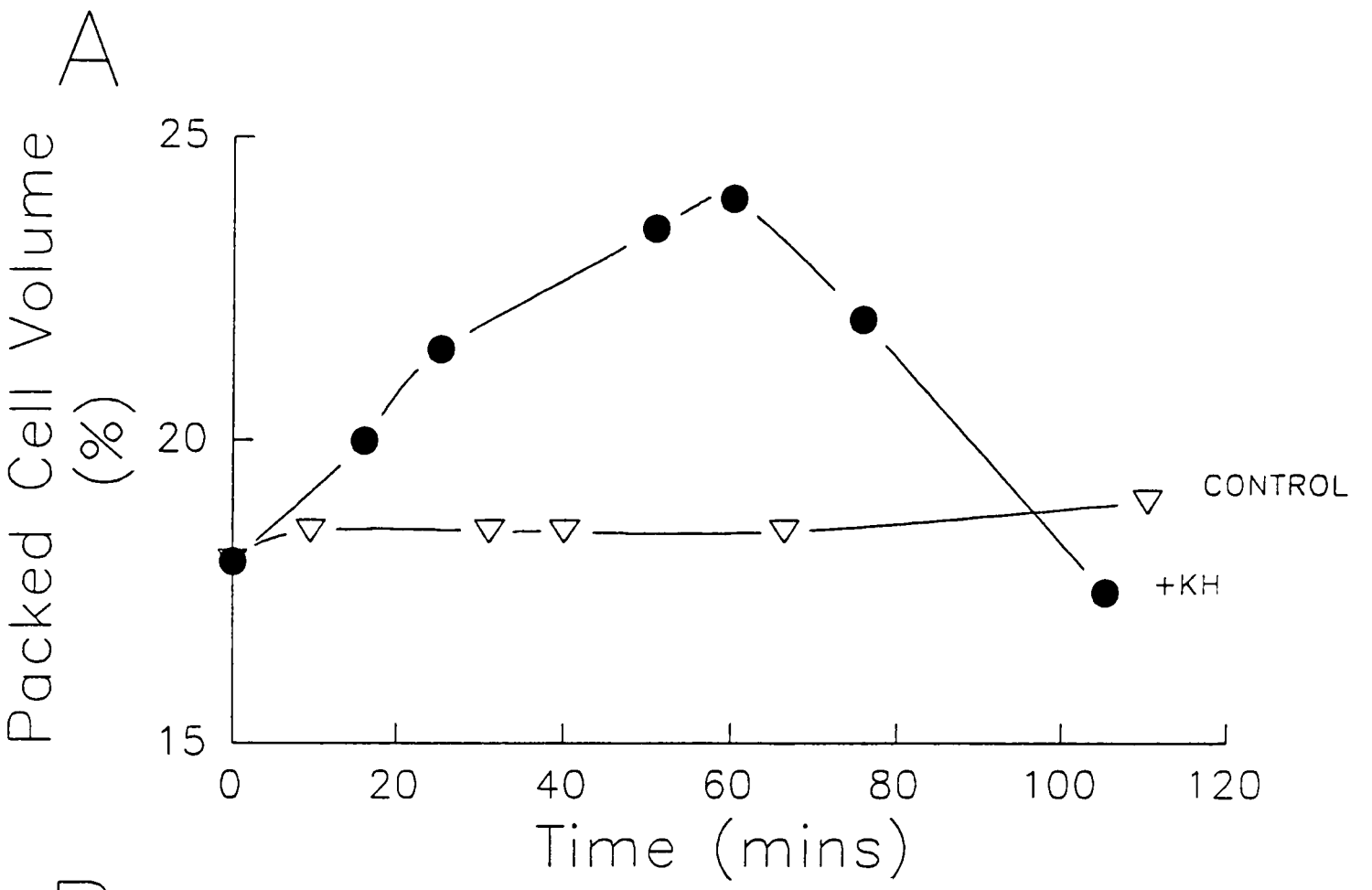


Figure 4.4 PCV changes during KH-induced haemolysis. Typical results are presented from one of three experiments. Cells were suspended at 18 % Hct in MBS in the absence (open triangles) or presence (filled circles) of KH (0.33 HU(ml)$^{-1}$). PCV changes (A) and percentage haemolysis (B) were measured.

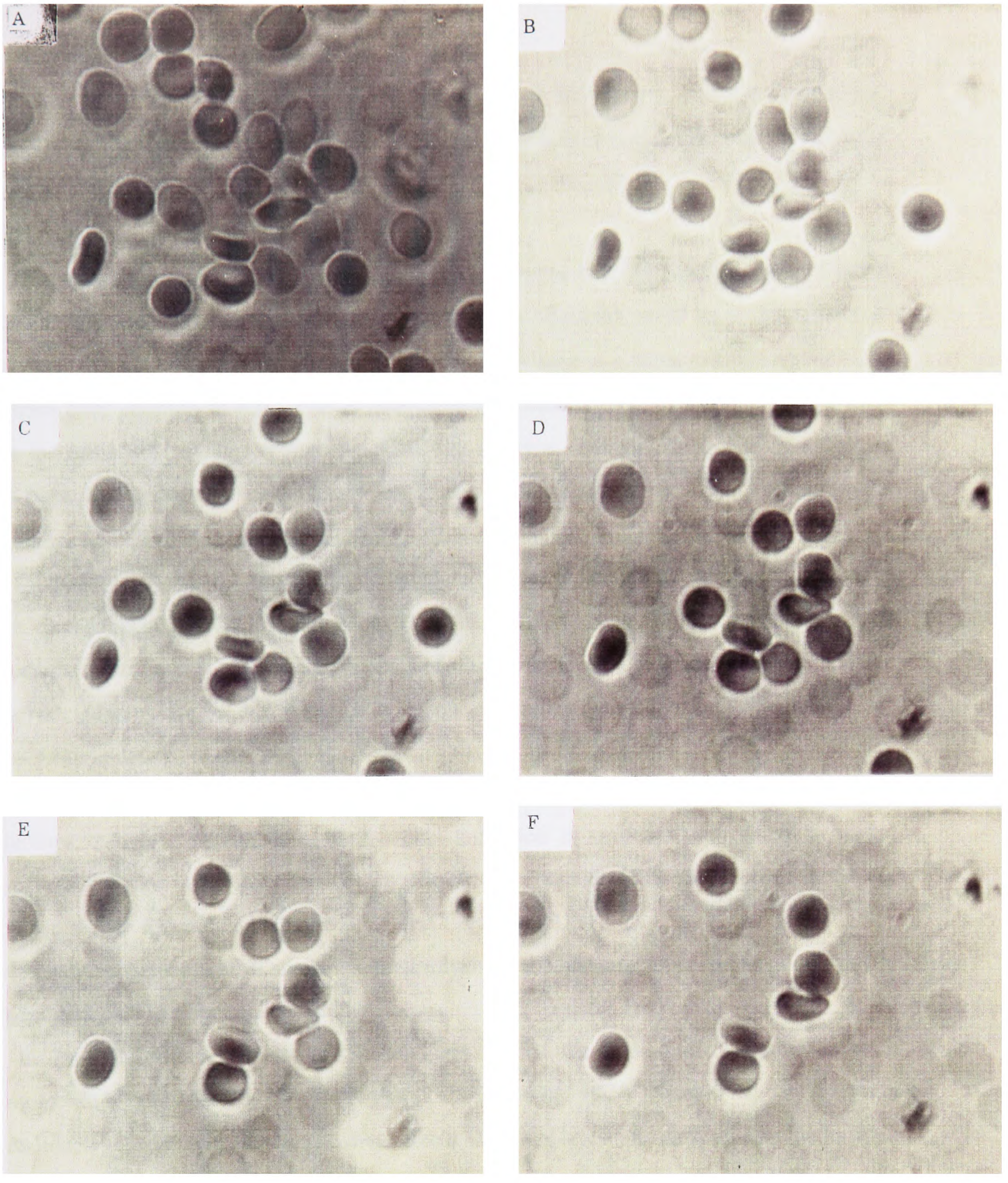


Figure 4.5 Morphological changes in erythrocytes followed by light microscopy. Parts A → F show the field of view after 0, 5, 15, 20, 25 and 35 minutes exposure (respectively) to KH (1.5 HU(ml)$^{-1}$) at room temperature. Control slides over a similar time course did not show such changes (data not shown).

25, and 35 minutes after toxin addition ([KH] = 1.5 HU.(ml)$^{-1}$), at room temperature. Cell lysis appeared to occur secondary to an increase in cell volume (see cells marked A and B). No such changes were visible in cells incubated similarly, but in the absence of KH (results not shown).

Figure 4.6 shows the dynamics of inhibitor-insensitive K^+ ($^{86}Rb^+$) influx under control conditions and in response to KH (0.15 HU(ml)$^{-1}$). Samples were taken as rapidly as possible after toxin addition. These data reveal that there is a substantially increased K^+ influx after addition of KH. This is indicative of a raised cation permeability, postulated as the basis for lysis by colloid osmosis. The increase in cation permeability was rapid in onset and appeared to be relatively constant in flux magnitude over the ten minute time course examined. Accordingly, straight lines were fitted to both control and post-KH sets of data by least squares regression. Fluxes calculated from this analysis showed that at an extracellular [K^+] of 15 mM, KH (0.15 HU(ml)$^{-1}$) significantly ($P < 0.005$, $n = 4$) increased the basal K^+ influx into HRBCs ($0.033 < Hct\ (p) < 0.07$) from (mean ± S.E.M.) 0.47 ± 0.15 to 23.12 ± 2.82 mmol.K^+(l.c.)$^{-1}$(hr)$^{-1}$. As has been noted previously for other membrane-active toxins (eg *V. cholerae* cytolysin (Krasilnikov *et al.*, 1992)), for haemolysis to occur secondary to colloid osmosis, the increase in cation influx must be instigated before haemolysis starts. This condition is well satisfied for KH as the increased cation influx occurs rapidly (Figure 4.6), whereas there is a pronounced lag phase before haemolysis (Figure 4.1A and Figure 4.4B).

4.2.3 Investigation of Kanagawa haemolysin for characteristic features of highly purified TDH preparations

A long appreciated characteristic of TDH is that the haemolytic potential of this molecule is unaffected by heat treatment (Zen-Yoji *et al.*, 1971; Douet *et al.*, 1992; Honda & Iida, 1993). Accordingly, KH was heated (100 °C for 10 minutes) and its haemolytic activity tested on HRBC. A substantial variation in haemolytic rate was noted according to donor resulting in large S.E.M.s for pooled H^{50} data prior to standardisation (methodology as given in section 3.4). No decrease in haemolytic activity was observed as a result of this treatment (Table 4.1), suggesting that thermostable factors alone, in particular TDH, account for the haemolytic activity of KH.

A second feature characteristic of purified TDH preparations is the insensitivity of horse erythrocytes. In Figure 4.7A, the lack of a response of horse erythrocytes in MBS to KH

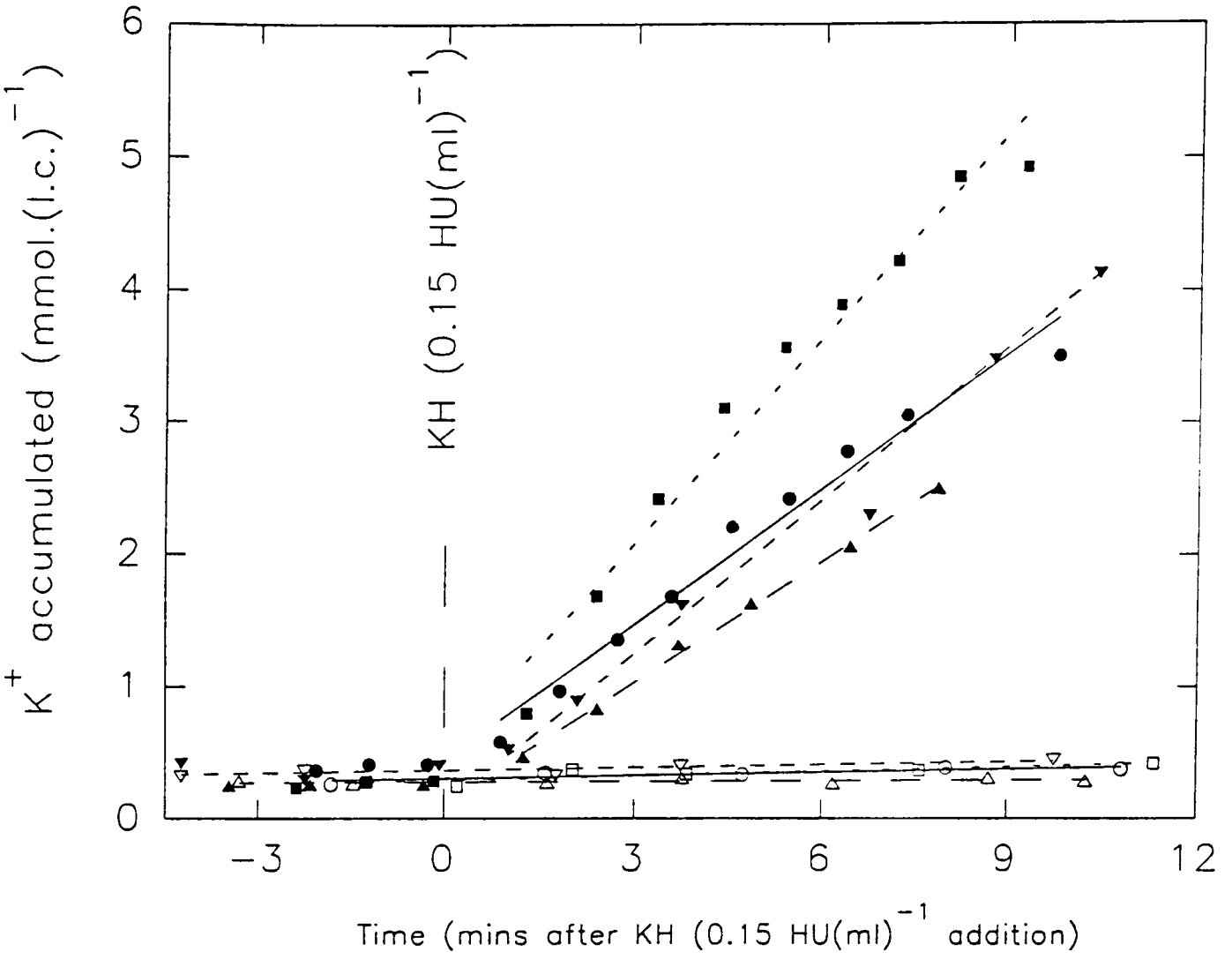


Figure 4.6 The dynamics of K^+ leak induction. K^+ ($^{86}Rb^+$) influx was measured using the multiple wash technique on cells from four donors (different symbol and line types) treated with the endogenous transport system inhibitors. Initially, 0.4 ml ($^{86}Rb^+$) KCl (150 mM) was added to 3.6 ml cell suspension (ie (K^+) =15 mM); 5 µl KH (100 HU(ml)$^{-1}$) was added to 3.4 ml cell suspension at time zero, in the experimental protocol (filled symbols); a control graph was generated for each experiment in which no KH was added (blank symbols). Lines were fitted by least squares regression.

	H^{50} (mins)		standardised H^{50} (%)	
[KH]	untreated	treated	untreated	treated
0.8 $HU(ml)^{-1}$	48.4 ± 12.38	47.0 ± 12.33	100 (st.)	96.8 ± 0.877
0.4 $HU(ml)^{-1}$	90.5 ± 28.86	86.3 ± 24.40	100 (st.)	98.3 ± 5.24

Table 4.1 Thermostability of haemolytic activity of KH. Haemolysis time courses were initiated by addition of packed cells (100 µl) to a solution comprised of CsCl (150 mM), HEPES (10 mM), ouabain (0.1 mM), bumetanide (0.1 mM), and nitrendipine (0.01 mM), at pH = 7.4, with KH at the indicated dose. For 'treated' preparations, KH was heated at 100 °C for 10 minutes, and allowed to cool to room temperature before addition to the solution.

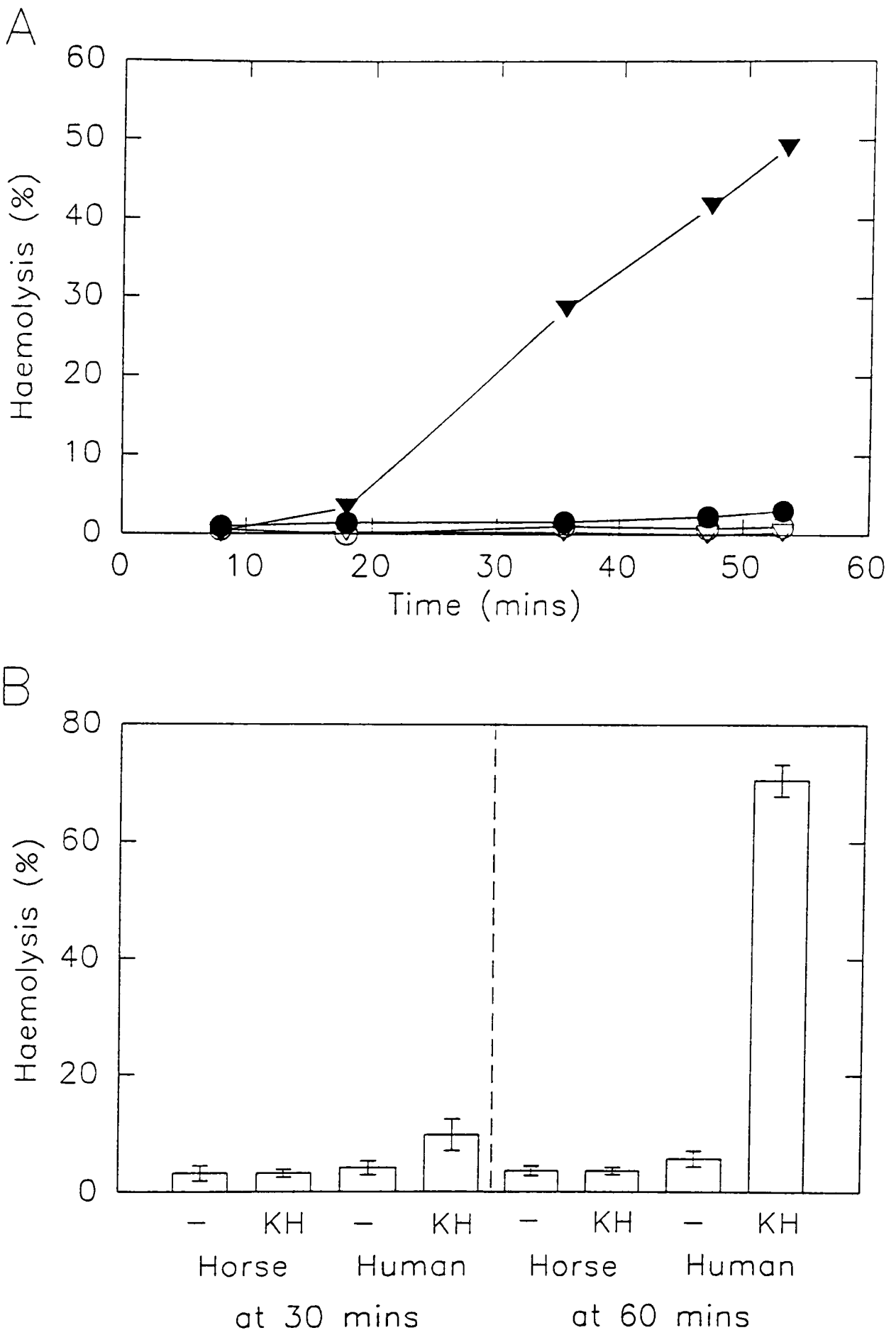


Figure 4.7 Effect of KH on horse erythrocytes. (A) Typical time course (representative of 3 experiments) of haemolysis of cells suspended (Hct = 5 %) in MBS in the presence (inverted triangles) or absence (circles) of KH (1 HU(ml)$^{-1}$). Horse cells (open symbols) are shown in relation to the positive control of human erythrocytes (filled symbols). (B) Haemolysis was assayed after 30 and 60 minutes suspension of cells (Hct = 5 %) in KCl (150 mM; pH = 7.4 with HEPES (10 mM)) containing KH (1 HU(ml)$^{-1}$).

is documented using human erythrocytes as a positive control. Because horse erythrocytes have an ability to perform volume regulatory decrease by activation of electroneutral KCl cotransport, the insensitivity to KH was further tested in 150 mM KCl solution (buffered at pH 7.4 by 10 mM HEPES), haemolysis being assayed after 30 and 60 minutes (different experiments in Figure 4.7B. There was no significant increase in horse cell haemolysis after 60 minutes incubation with KH (2 $HU(ml)^{-1}$), percentage haemolysis being (mean ± S.E.M.) 3.57 ± 0.791 and 3.57 ± 0.656 % in the absence and presence of KH, repectively. Over the same time course KH significantly ($P < 0.001$, n =3) increased haemolysis of human cells from 5.63 ± 1.358 to 70.58 ± 2.72 %.

4.2.4 Temperature-dependence of KH-induced haemolysis

Influx data (Figure 4.6) suggested that toxin binds rapidly to HRBC membranes. On the basis of this conjecture, a thermodynamic analysis (Klein, 1982; Ginsburg *et al.*, 1983) of haemolysis data was performed to gain insight into the events underlying KH action. Cells were treated with KH at room temperature, vortexed thoroughly, and transferred immediately to a waterbath at the appropriate temperature. The normalised $\{(H^{50})^{-1}\}$ was calculated as an index of permeability. The Arrhenius profile (Figure 4.8) shows an approximately linear relationship between 7 and 27 °C and an unexpected break between 27 °C and 37 °C. Indeed haemolytic rate was faster at 27 °C than at 37 °C. Standardised $\{(H^{50})^{-1}\}$ was significantly increased ($p < 0.05$, $n = 4$) by 11.9 ± 3.68 % at 27 °C from that obtained at 37 °C. The slope of the Arrhenius relation between 7 and 27 °C was calculated by least squares regression. The negative of this quantity was multiplied by the gas constant, R, to yield the apparent activation energy, E_a (Hall & Willis, 1986). This was 53.30 ± 2.79 $kJ(mol)^{-1}$, the correlation co-efficient r = -0.9687. This value is rather higher than that expected for diffusion through water-filled pores of considerably larger radii than those of the permeants (Stein, 1967; Ginsburg *et al.*, 1983). One explanation for the high E_a may be that under these conditions the binding of toxin was incomplete on transferral to the waterbath, and subsequent binding was subject to thermodynamic parameters (see section 4.3 for discussion and reservations). Therefore the nature of the interaction between KH and HRBC was investigated in more detail.

4.2.5 Nature of KH action on HRBC

(i) The binding event between KH and HRBC. In this section, experiments were performed to determine the nature of the interaction between haemolytic entities and the

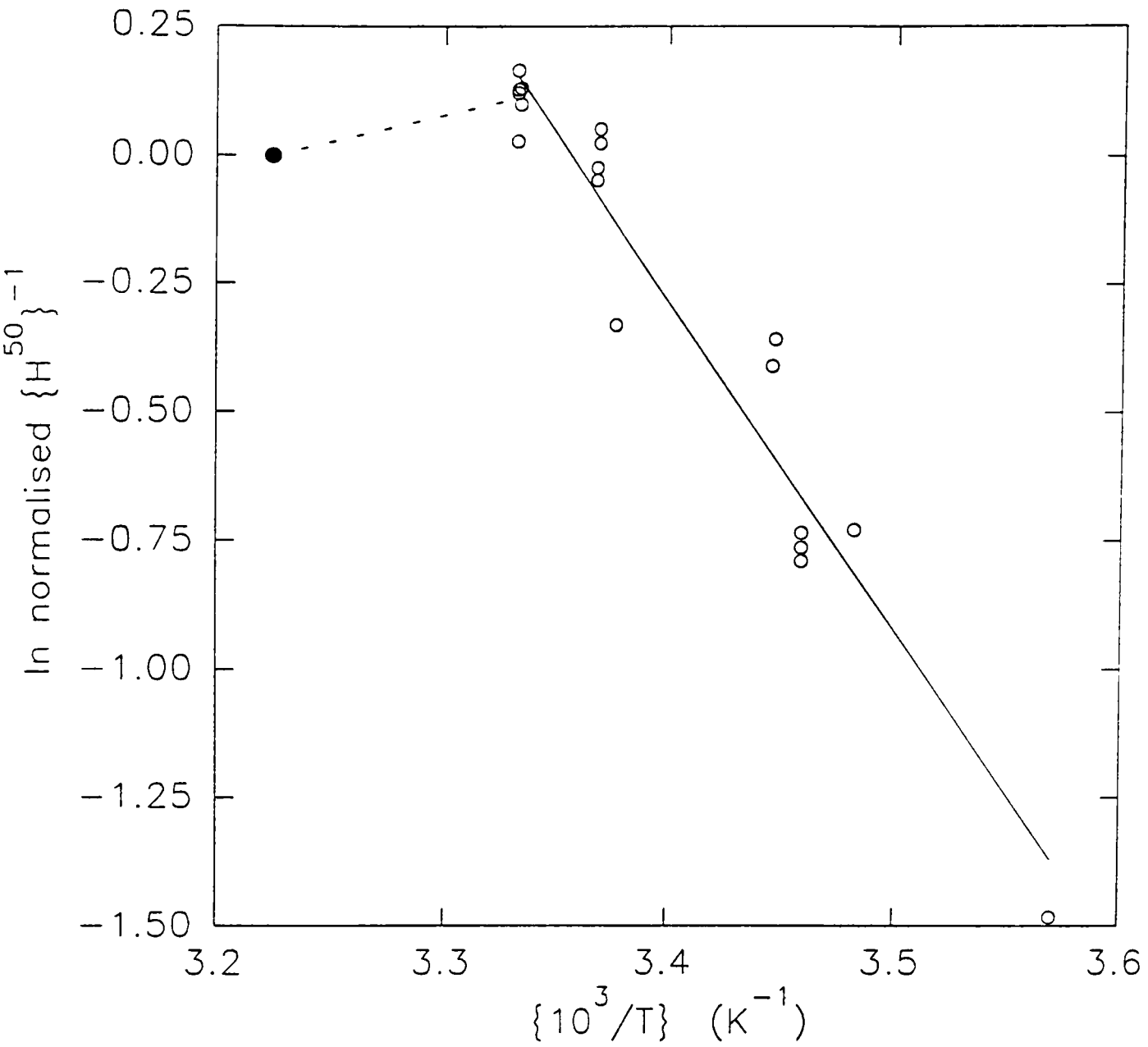


Figure 4.8 Effect of temperature on KH-induced haemolysis. HRBC (5% Hct in MBS) were treated with KH (1 $HU(ml)^{-1}$) and nitrendipine (100 μM) at room temperature, vortexed, and transferred to a waterbath at the appropriate temperature. The rate of lysis was measured for cells from three different donors. Normalised $\{H^{50}\}^{-1}$ values were calculated and results pooled as shown on this Arrhenius plot. Line from which E_a was calculated was fitted by least squares regression.

HRBC membrane. Washing out the extracellular solution (either 20 seconds or 5 minutes after KH addition at 37 °C) by centrifugation (CP3), removal of extracellular solution and resuspension, had a significant effect on subsequent haemolytic rate. Haemolysis (% total, assayed after 75 minutes at 37 °C) was significantly ($P < 0.05$; $n = 3$, for both systems) decreased, from 18.7 ± 2.5 % (unwashed system) to 3.6 ± 0.6 and 1.5 ± 0.3 % by washout after 5 minutes and 20 seconds, respectively (Figure 4.9).

There were at least two possible explanations for these results: (1) KH induced the lesion rapidly in the HRBC membrane but this was subsequently removed by the pressure exerted in the centrifugation procedure (CP3), (2) some (but by no means all) KH was bound irreversibly to the HRBC membrane (at the time of washing) - even after the phase responsible for the initial increase in cation permeability (Figure 4.6), binding of KH from the extracellular solution still contributed to the haemolytic process.

In order to distinguish between these possibilities, two strategies were employed: (1) HRBC were treated with KH in MBS containing sucrose (50 mM) as an osmotic protectant, before being spun down, haemolytic time courses being initiated by the addition of these treated cells to MBS without osmotic protectant, and (2) after KH treatment of HRBC, the cells were separated out by centrifugation (CP3) and the remaining haemolytic potential of the solution examined.

Following the first strategy, experiments were performed in which HRBC were treated by KH (0.75, 1, 1.5 $HU(ml)^{-1}$) for 5 minutes in MBS containing sucrose (50 mM) and endogenous transport system inhibitors, before being spun down (CP1) and then resuspended in either 5 or 10 ml MBS (Figure 4.10). These haemolytic time courses did not exhibit the protective effect of extracellular volume observed in Figure 4.2C. Indeed, haemolysis tended to occur at a slightly faster rate in the larger volume. This suggests that the haemolytic lesion (formed in the HRBC membrane) that remains after washout does not exist in a dynamic equilibrium with haemolytic entities in the extracellular solution. The apparent irreversibility of lesion formation was examined further. HRBC (Hct = 5 %) were treated with KH (1.07 $HU(ml)^{-1}$) in MBS with extracellular sucrose (50 mM) at 37 °C for 5 minutes before being spun down by centrifugation (CP1). These packed KH-treated HRBC (100 µl) were added to 4.9 ml MBS and vortexed (time = 0). Aliquots (1 ml) were incubated at 37 °C; after 2.5 minutes, these systems were either allowed to run as haemolytic time courses or washed by CP3 with subsequent replacement

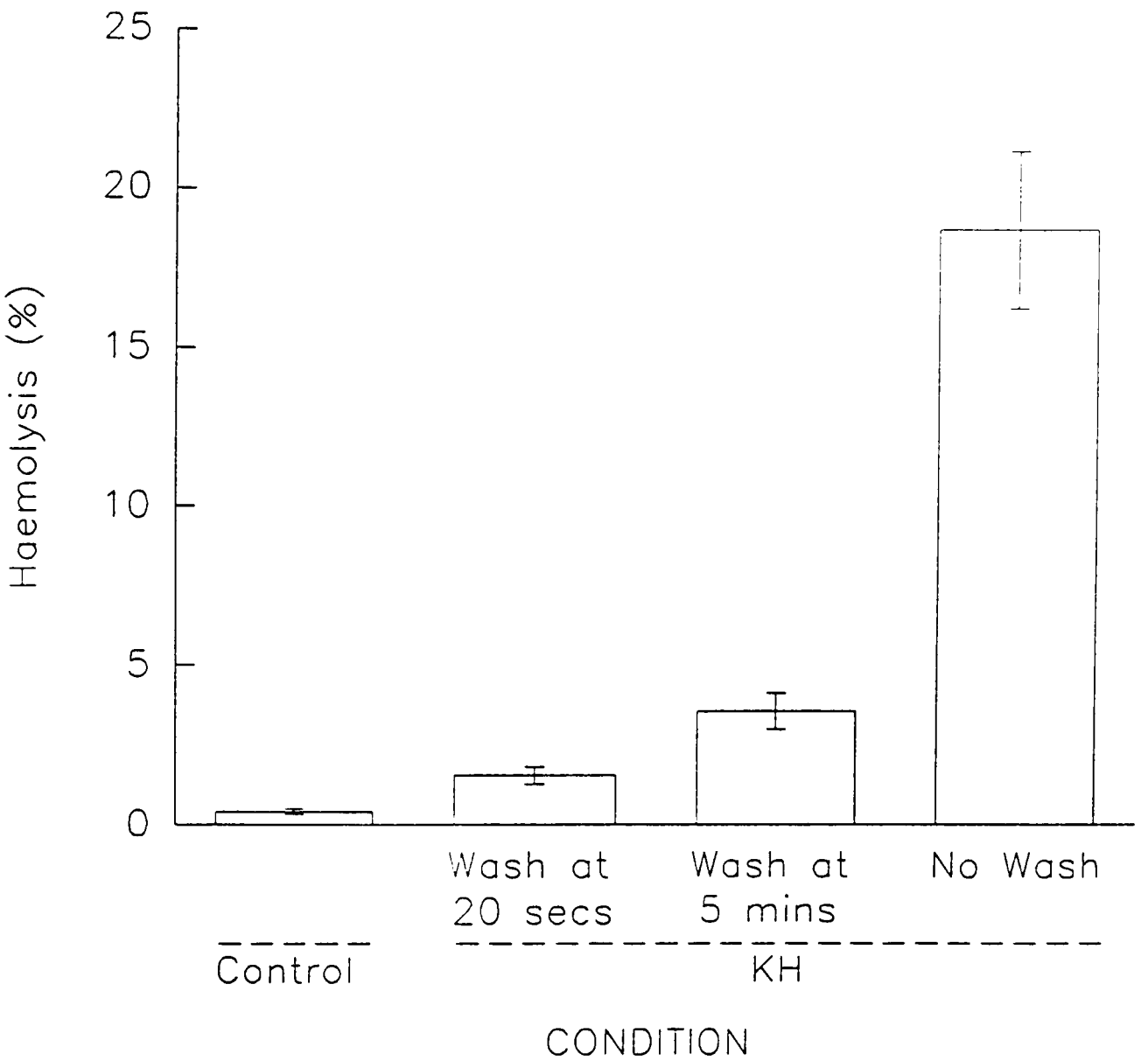


Figure 4.9 The effect of washout on KH-induced haemolysis. Results are pooled data from three separate donors. 0.5 ml MBS containing the endogenous transport system inhibitors with or without (as indicated) KH (2 $HU(ml)^{-1}$) was added to 0.5 ml of HRBC suspension (Hct = 4 %) in MBS (similarly containing inhibitors). At the time indicated, cells were spun down by CP3; 0.8 ml of the supernatant was taken off, 0.8 ml of MBS (with inhibitors) being added back to the pelleted cells which were then resuspended. Haemolysis was measured after 75 minutes from the initial addition of KH to HRBC.

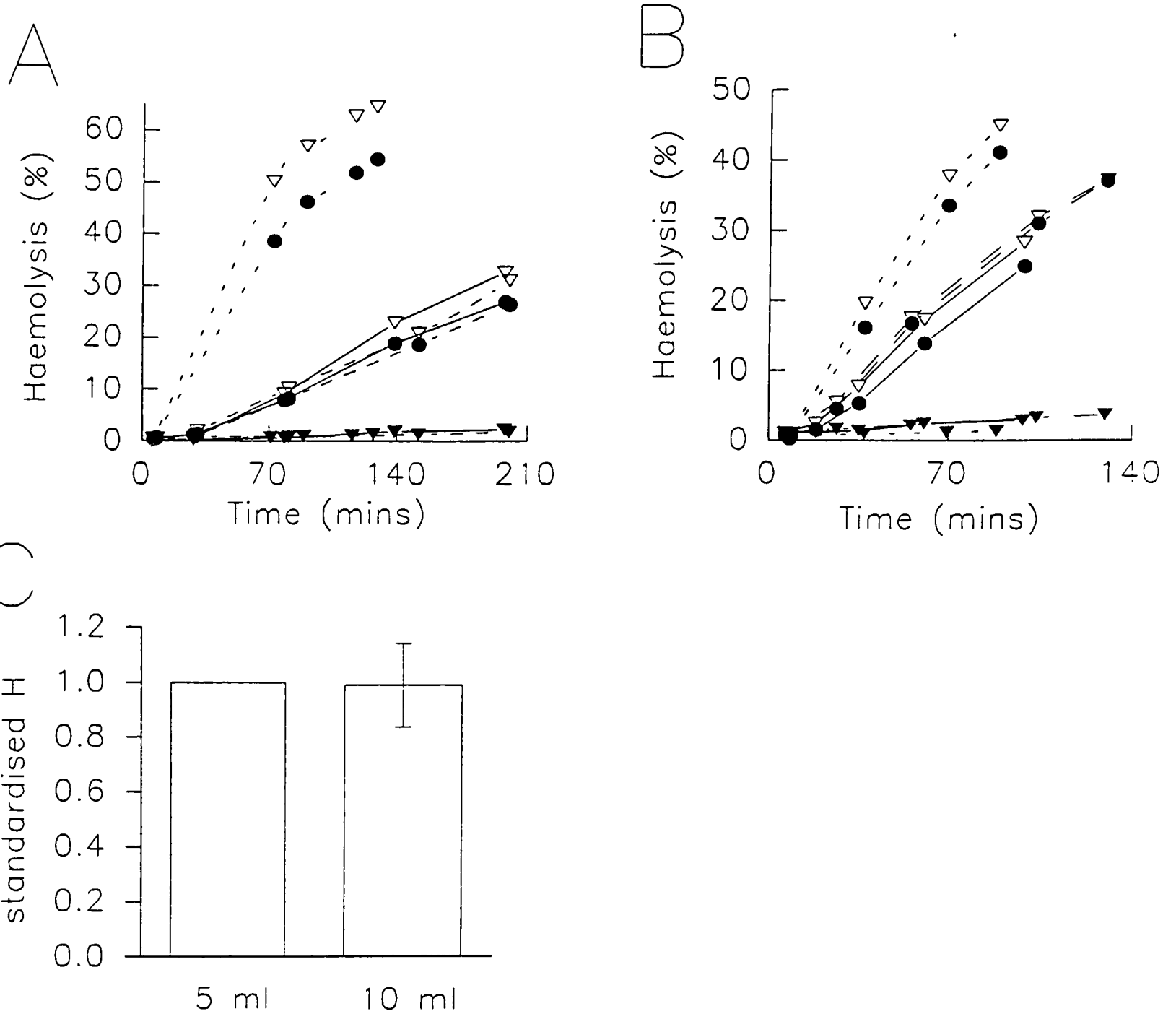


Figure 4.10 Effect of reaction volume on haemolysis of HRBC treated with KH and then washed clear of the KH-containing solution. HRBC were suspended at 37 °C in MBS (Hct = 3.5 %) containing sucrose (40 mM), endogenous transport system inhibitors and KH (doses: (A) 0.75 HU(ml)$^{-1}$, (B) 1.0 HU(ml)$^{-1}$, and (C) 1.5 HU(ml)$^{-1}$). Cells were treated for 5 minutes before being spun down (CP1 but for 3.5 minutes and at 37 °C). 100 µl of packed cells so obtained were added to either 5 (closed circles) or 10 (open triangles) ml MBS containing inhibitors. Results for A and B are given as time-courses for three donors (indicated by line type); results for C are given as pooled standardised H^{50} values. Negative controls are given by the haemolytic time courses of cells untreated by KH (closed triangles).

of 0.8 ml of the supernatant with fresh MBS before resuspension of HRBC. Haemolytic activity was assayed 75 minutes after KH exposure for both washed and unwashed systems. Results from three donors indicate that the CP3 washing procedure had no protective effect on KH-induced haemolysis (Figure 4.11).

Following the second strategy, and based on the assumption that KH binds to HRBC membranes (see above), then exposure of KH solution to HRBC (which are then separated out) should decrease the haemolytic potential of that solution. Accordingly, three haemolytic systems were set up. An HRBC (Hct = 3.5 %) suspension in MBS (0.5 ml) was added to an equal volume of (i) MBS alone (as a negative control), (ii) and (iii) MBS containing KH (such that final [KH] = 1 $HU(ml)^{-1}$). Cell suspensions were mixed thoroughly and incubated at 37 °C. After a 5 minute incubation period (during which no haemolysis was observed), the suspension from (iii) above was centrifuged (CP3) to spin down HRBC. An aliquot (0.8 ml) of the supernatant was transferred to a separate Eppendorf tube and either 50 or 16 µl packed cells (in different experiments) from the same donor were added to initiate the haemolytic time course. Haemolysis was attenuated, but not totally abolished, for KH solutions that had been previously exposed to HRBC. Data in Figure 4.12 are pooled from three separate experiments (blood from different donors) for each volume of packed cell added. The standarised H^{50} (Figure 4.12A), was used to calculate the standardised $\{(H^{50})^{-1}\}$ (Figure 4.12B), a relative index of permeability for the experimental conditions outlined. This parameter decreased significantly ($P < 0.01$ (16 µl), $P < 0.005$ (50 µl); unpaired *t* tests of normalised data, $n = 3$ for each system) to 59.6 ± 3.18 % (for 16 µl volume) and 58.1 ± 1.63 % (for 50 µl volume) of the positive control value (from system (ii)). However, this index does not differ significantly between the 50 and 16 µl systems, indicating that the effect of HRBC on the haemolytic potential of KH was not due to dilution by the addition of an extracellular volume with packed cells. Thus haemolysis was attenuated, but not totally abolished, for KH solutions that had been previously exposed to HRBC, and this attenuation resulted from decreased KH concentration due to binding to HRBC. Negative controls gave negligible haemolysis over the same time course.

(ii) The KH-membrane interaction and the effects of other agents on the rate of KH-induced haemolysis. It was possible that the attenuation (as opposed to abolishment) of haemolytic potential of KH (Figure 4.12) was due to the release by centrifugal pressure

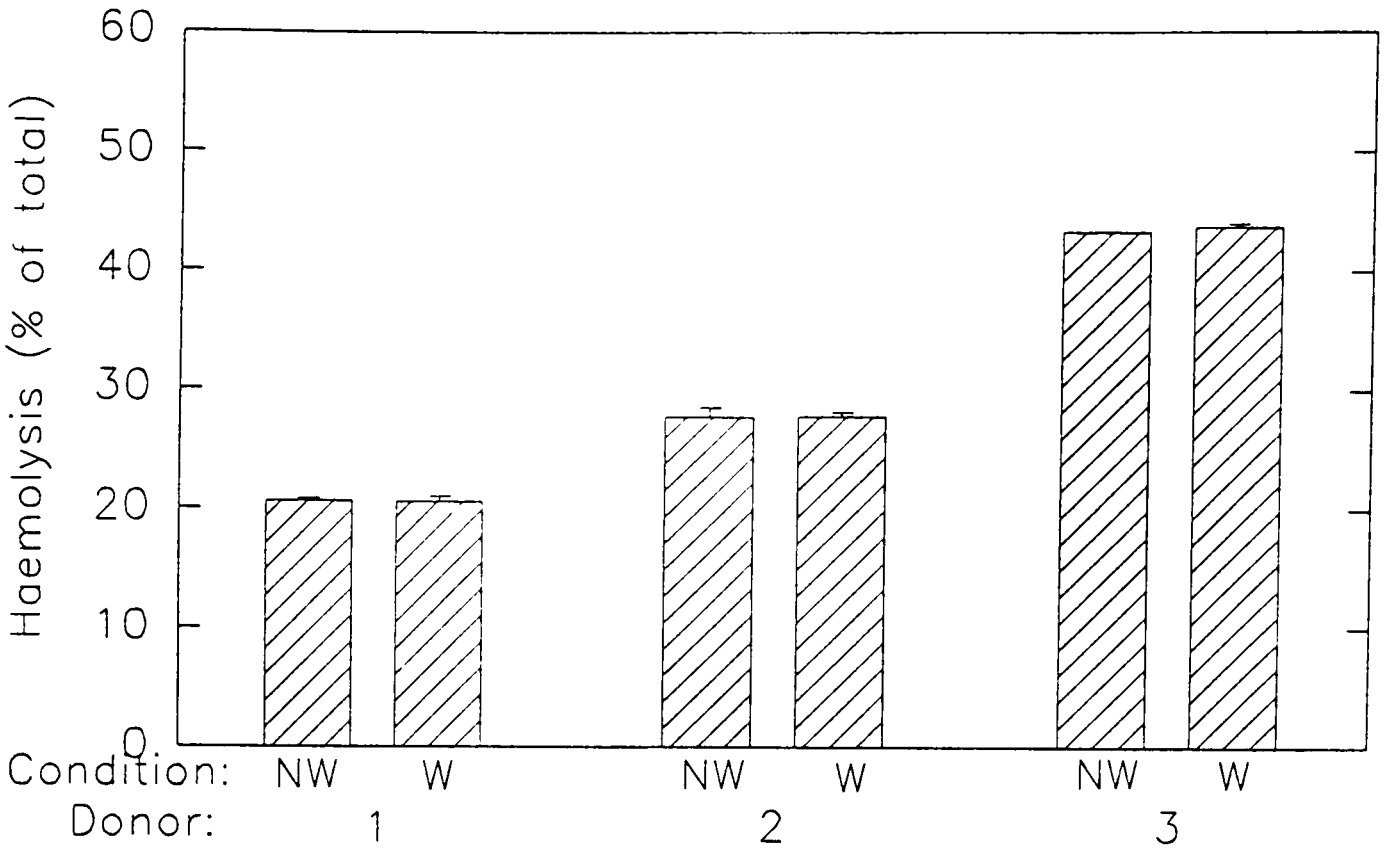


Figure 4.11 Lack of effect of CP3 on rate of haemolysis of treated and washed HRBC. HRBC were suspended (Hct = 5 %) in MBS with and without (negative control) KH (1 $HU(ml)^{-1}$) and incubated at 37 °C for 5 minutes, before being spun down (CP1). The supernatant was removed and aliquots (100 µl) packed cells were resuspended in 4.9 ml MBS. Aliquots (1 ml) of this cell suspension (in duplicate) were either not spun or spun (CP3), supernatant aspirated and resuspended to the same final volume. These systems were incubated at 37 °C for 75 minutes before % total haemolysis was calculated. Results are given for 3 donors. W = washed, NW = not washed. For HRBC untreated by KH (negative controls), haemolysis was less than 2.5 % after 75 minutes.

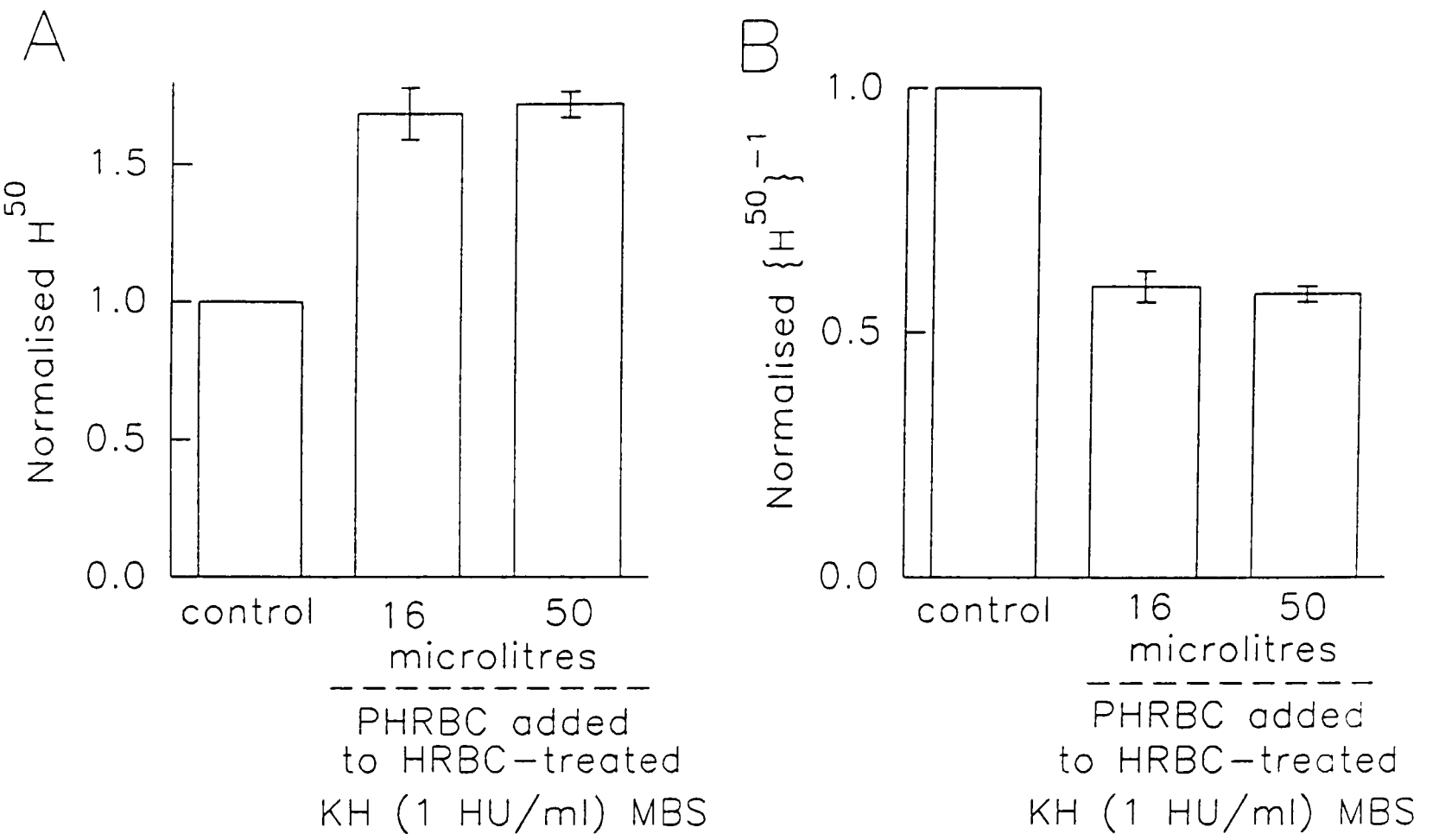


Figure 4.12 Effect of HRBC on subsequent haemolytic potential of KH. HRBCs in MBS (3.5 % Hct) were added to an equal volume of: (i) MBS alone (negative control), (ii) and (iii) MBS containing KH (final (KH) = 1 HU(ml)$^{-1}$). After 5 minutes incubation at 37 °C, HRBCs of system (iii) were pelletted and the haemolytic activity of the supernatant measured as described in text. Results are pooled data from three separate experiments for each volume PHRBC added. Experiments were performed in triplicate. Negative controls (i) gave negligible haemolysis over an equivalent time-course. PHRBC = packed HRBC.

of KH entities bound to the membrane in a reversible form. Accordingly, the DBP technique was used in an attempt to separate HRBC (with all bound KH) from the extracellular solution. An important control for such experiments was to investigate whether DBP itself had any effect on KH-induced haemolysis. HRBC (Hct = 4 %) were suspended in MBS with KH (1 $HU(ml)^{-1}$). Aliquots, with or without 250 µl DBP layered under the haemolytic mixture, were incubated at 37 °C for 60 minutes. Results (Figure 4.13A) from three different donors show that the presence of a DBP layer under the haemolytic reaction suspension decreased haemolytic rate. The inhibitory effect of DBP on KH-induced haemolysis was examined further by vigorous vortexing (1 minute in whirlimix) 0.7 ml MBS containing KH (2 $HU(ml)^{-1}$) with or without (control) 250 µl DBP. The immiscible aqueous and DBP phases were then separated by CP3. Equal volumes (0.5 ml) of HRBC suspension and aqueous KH solutions (treated as above) were combined at time zero. The haemolytic activity of the aqueous KH solution was dramatically decreased (Figure 4.13B) by DBP treatment, when compared with the appropriate control. These results suggest that there was a protein:oil interaction. There are several possibilities for the form of this phenomenon: KH may be (a) denatured by KH, (b) absorbed to the oil:water interface, or (c) partitioned into the hydrophobic phase of DBP.

Nevertheless, the DBP protocol for HRBC separation was used to separate KH-treated cells from a KH-containing solution in an experiment to test if significant haemolysis could still be induced by the extracellular solution, even after treated HRBC had been spun through DBP. These experiments were similar to those described earlier (section 4.2.5; Figure 4.9), with the exception that the DBP technique should allow separation of *all* membrane-bound KH (whether irreversibly or reversibly bound). Figure 4.14 shows data from one experiment typical of three such experiments performed on blood from different donors. Data indicate that, even using the DBP separation technique, the KH-containing solution retains haemolytic ability even after 10 minutes exposure to HRBC. Thus, it appears that KH is incompletely bound to HRBC after both 3 and 10 minutes exposure.

Haemolysis as a result of organic solute transport via a malaria-induced pathway in HRBC has been analysed previously (Ginsburg *et al.*, 1983, 1985). This pathway shows a number of similarities to the so-called Cl^- channel of other tissues (Kirk *et al.*, 1994).

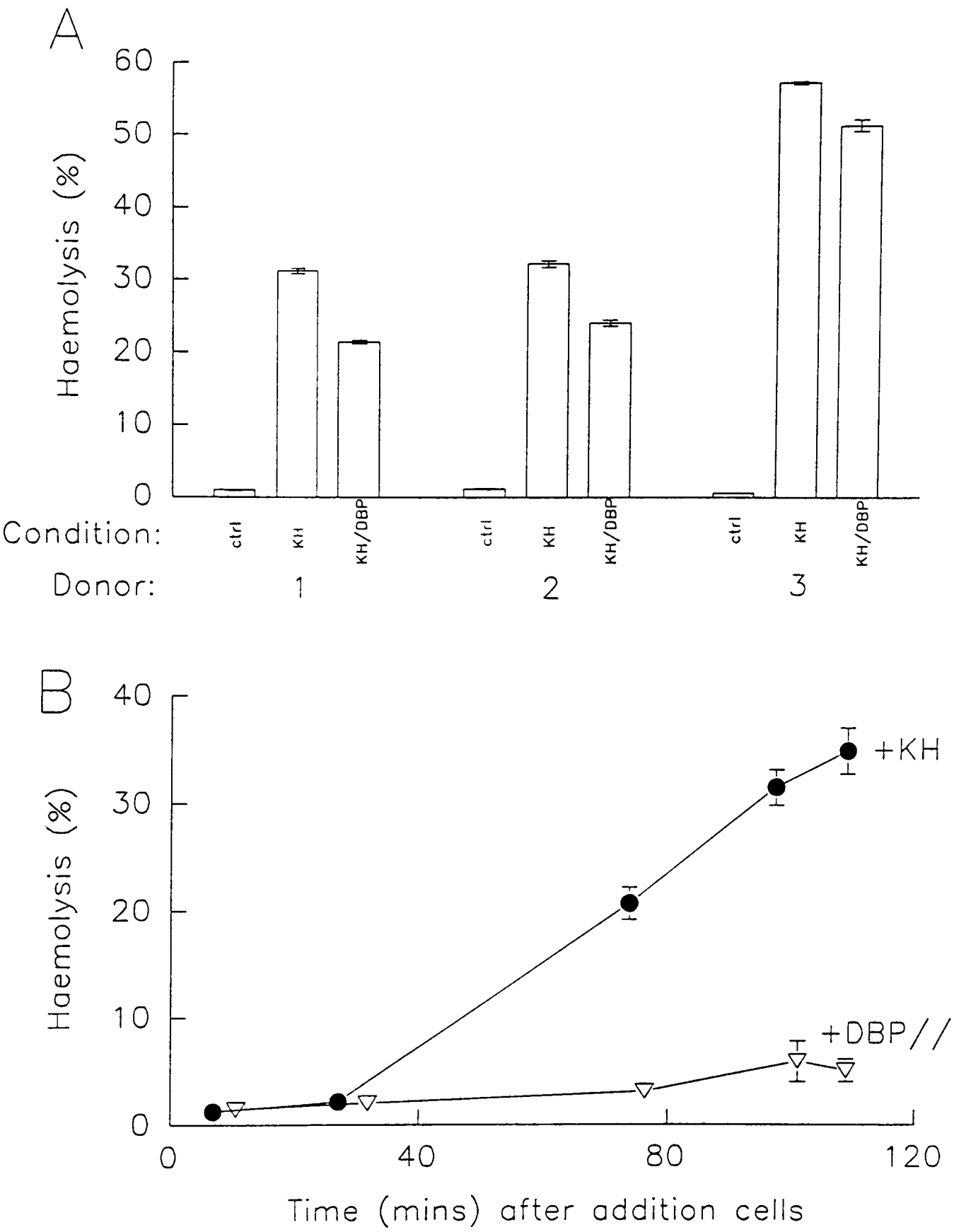


Figure 4.13 Effect of DBP on haemolytic potential of KH. (A) Packed HRBC (200 µl) were suspended (t = 0) in MBS (5 ml) with/without KH (final (KH) = 1 HU(ml)$^{-1}$). Aliquots (0.6 ml) were immediately transferred to Eppendorfs either containing/not containing 250 µl DBP as a layer underneath the cell suspension. Incubation at 37 °C was for 60 minutes. Suspensions were then centrifuged (CP3) and % total haemolysis calculated. Results are unpooled and from three donors. (B) Aliquots (0.7 ml) of KH (2 HU(ml)$^{-1}$) in MBS were vortexed (1 minute in whirlimix) either alone or with 250 µl DBP, before incubation at 37 °C for 3 minutes. Preparations were then added to equal volumes of HRBC suspension (final Hct = 3 %), initiating the haemolytic time courses (in DBP-treated KH = open triangles; in untreated KH = filled circles). Typical results are presented from one of three such experiments.

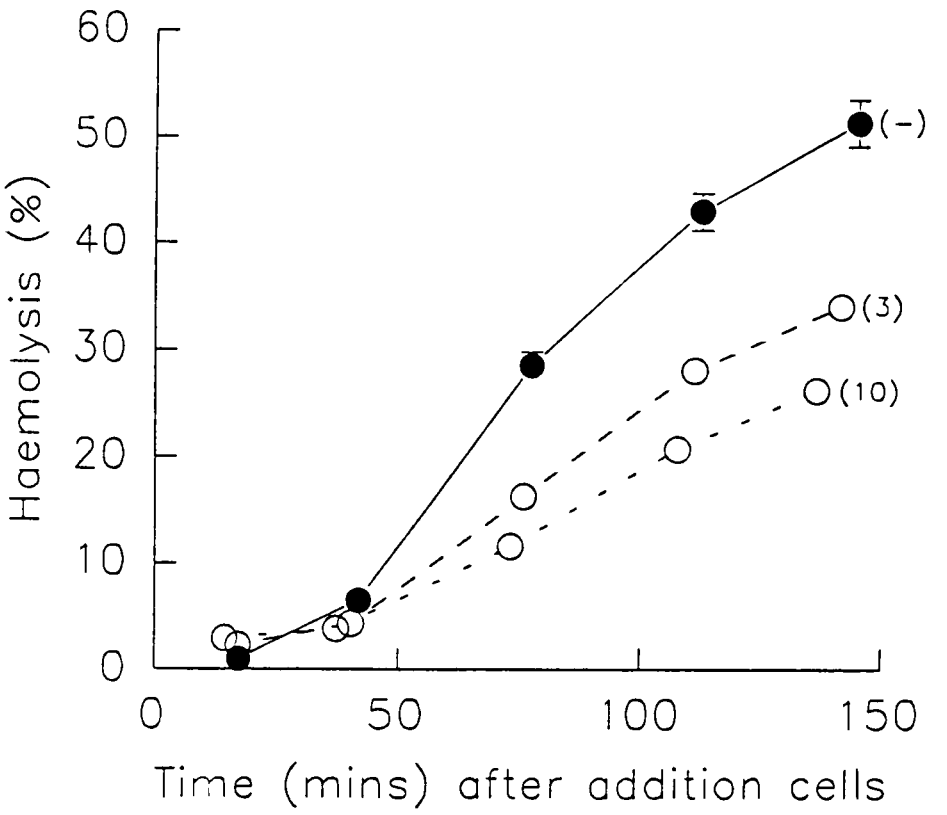


Figure 4.14 Effect of HRBC on subsequent haemolytic potential of KH when initially treated cells are spun down through DBP. HRBC were suspended (5 % Hct) in MBS with KH (1 HU(ml)$^{-1}$). After rapid vortexing, aliquots (1.2 ml) of this cell suspension were taken to Eppendorfs either containing or not containing (positive control) DBP (250 µl). Cells were either: (i) not spun (for suspension not layered over DBP; positive control = filled circles), (ii) spun (CP3) through DBP after 3 minutes, or (iii) spun (CP3) through DBP after 10 minutes. An aliquot (1 ml) of the aqueous solutions from each of (ii) and (iii) were resuspended with 50 µl packed HRBC, initiating the haemolytic time courses for these preparations (open circles, time of wash as indicated). Each condition was performed in triplicate for each donor. Typical results are presented from one of three such experiments. Negative controls for these experiments showed that % haemolysis for HRBC untreated by KH was < 1 %.

The Cl^- channel is susceptible to modulation by second messengers derived from arachidonic acid (Lambert *et al.*, 1987) and arachidonate itself has been shown to have effects on the membrane transport properties of erythrocytes (Dwight & Hendry, 1992). Therefore, the effect of arachidonate (15 μM) on KH-induced haemolysis was assessed. Normalised $\{(H^{50})^{-1}\}$ was increased from control values by 9.6 ± 3.41 %; however this increase was not significant at the 5 % level (Figure 4.15A). Moreover, under such conditions, arachidonate itself significantly ($P < 0.005$, $n = 3$) stimulated haemolysis (independent of KH), measured after 1 hour incubation at 37 °C, from 2.0 ± 0.69 to 17.5 ± 1.79 % (Figure 4.15B). In light of a previous report (Toda *et al.*, 1989) concerning the anti-haemolytic properties of tea catechins, the effects of catechin and epicatechin on haemolytic rate were assayed; normalised $\{(H^{50})^{-1}\}$ was not significantly decreased by either of these two agents at 250 μM (Figure 4.15C).

In light of the effects of DBP on the haemolytic potential of KH-containing solutions, the effect of pure corn oil (PCO) on haemolysis of both horse and human erythrocytes was measured (Figure 4.16) in 150 mM KCl . Erythrocytes were treated by a protocol analagous to that outlined for Figure 4.13B but with replacement of DBP by PCO. After the PCO had been centrifuged clear, the KH-containing solution retained an opaque appearance. The haemolytic activity of the KH solution was enhanced for human red cells, although horse cells (as previously) remained unaffected. For human erythrocytes, haemolysis after 30 minutes was significantly ($P < 0.05$, $n = 3$) increased from 9.7 ± 2.66 (presence of KH alone) to 43.6 ± 8.41 % (presence of KH treated by PCO).

4.2.6 Selectivity of the KH-induced lesion

The selectivity of the induced cation leak was examined using the technique of isosmotic haemolysis as outlined previously (section 3.4). Figure 4.17A shows the time courses of KH-induced haemolysis for a typical experiment, while Figure 4.17B shows the Eisenman plot for pooled data from four such experiments. Assuming that the identity of the extracellular ion does not affect either the action of agents that promote haemolysis other than by leak induction, or the dynamics of leak induction, then the relative haemolytic rates can be ascribed to the selectivity of the induced leak. There was a clear selectivity series for monovalent cations, $Cs^+ > Li^+ > K^+ > Rb^+ > Na^+$, a reversed Eisenman VIII sequence with a small K^+ anomaly (Eisenman & Horn, 1983).

Figure 4.18 documents (A) decreases in haemolytic rate (given as increases in

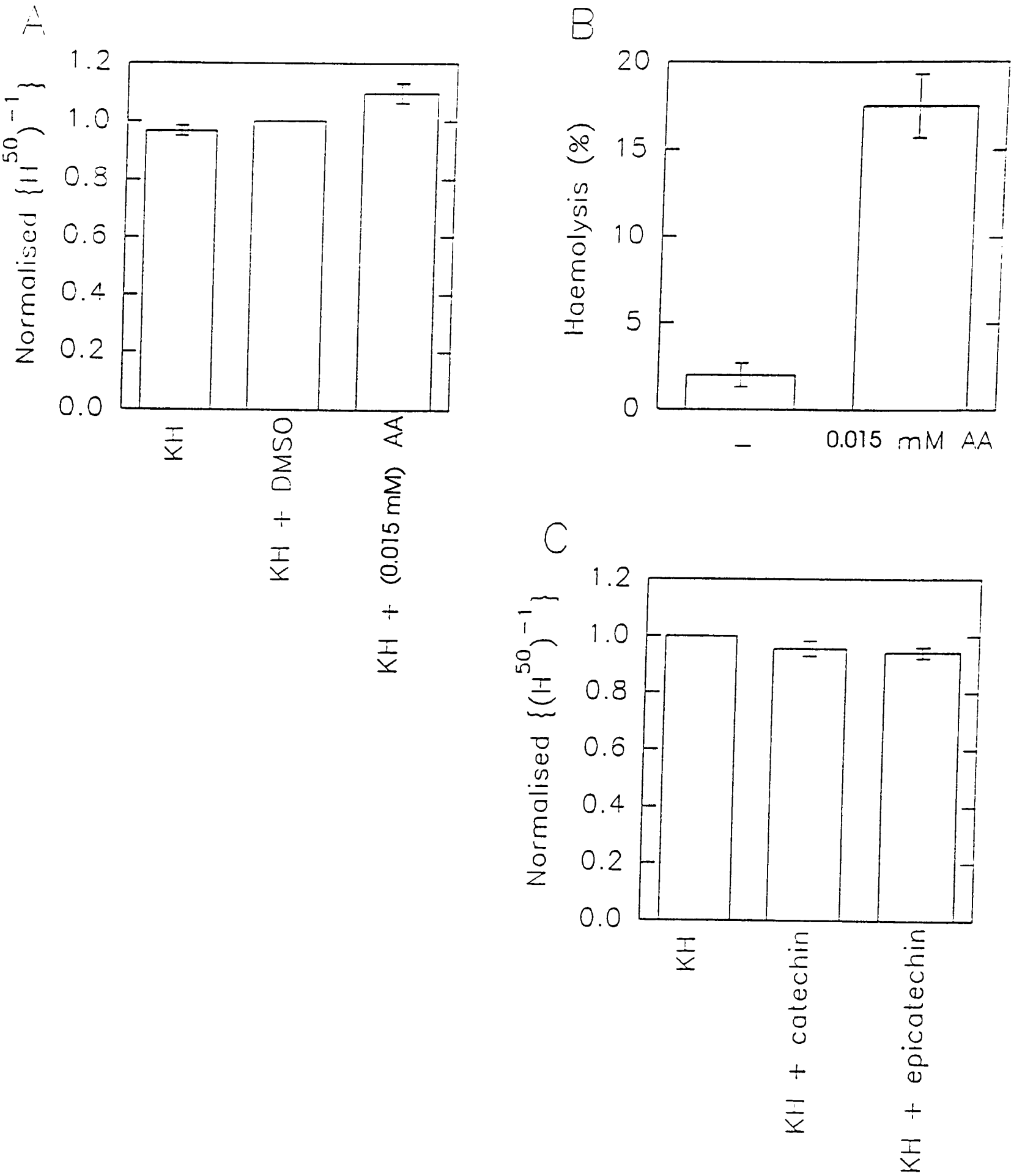


Figure 4.15 Effects of arachidonic acid, catechin and epicatechin (250 µM) on KH-induced haemolysis. HRBC were added to MBS (Hct = 5 %) containing KH (1 $HU(ml)^{-1}$) and the substance tested, arachidonic acid (15 µM), catechin (250 µM) and epicatechin (250 µM). Haemolytic time courses were followed and normalised $\{(H^{50})^{-1}\}$ calculated.

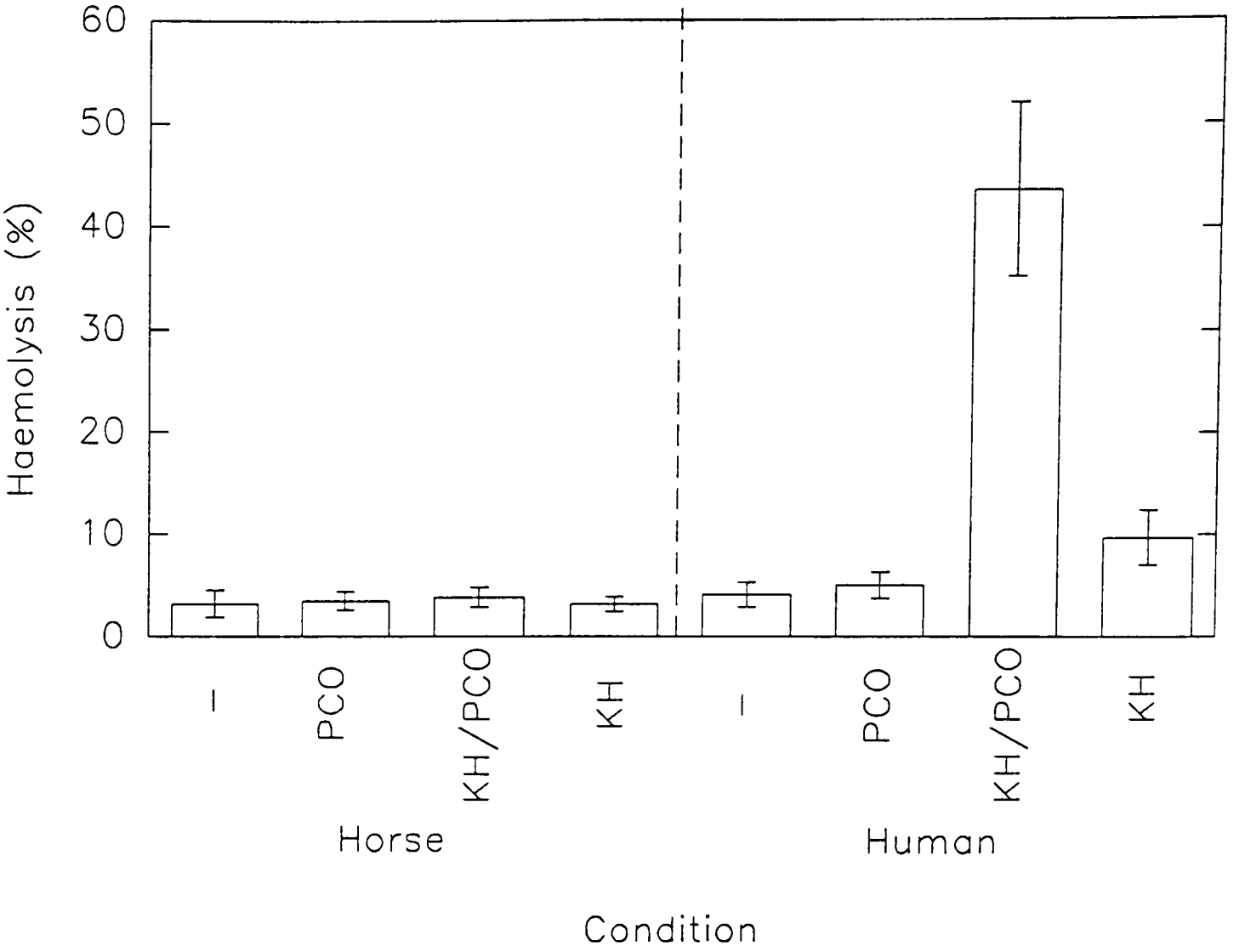
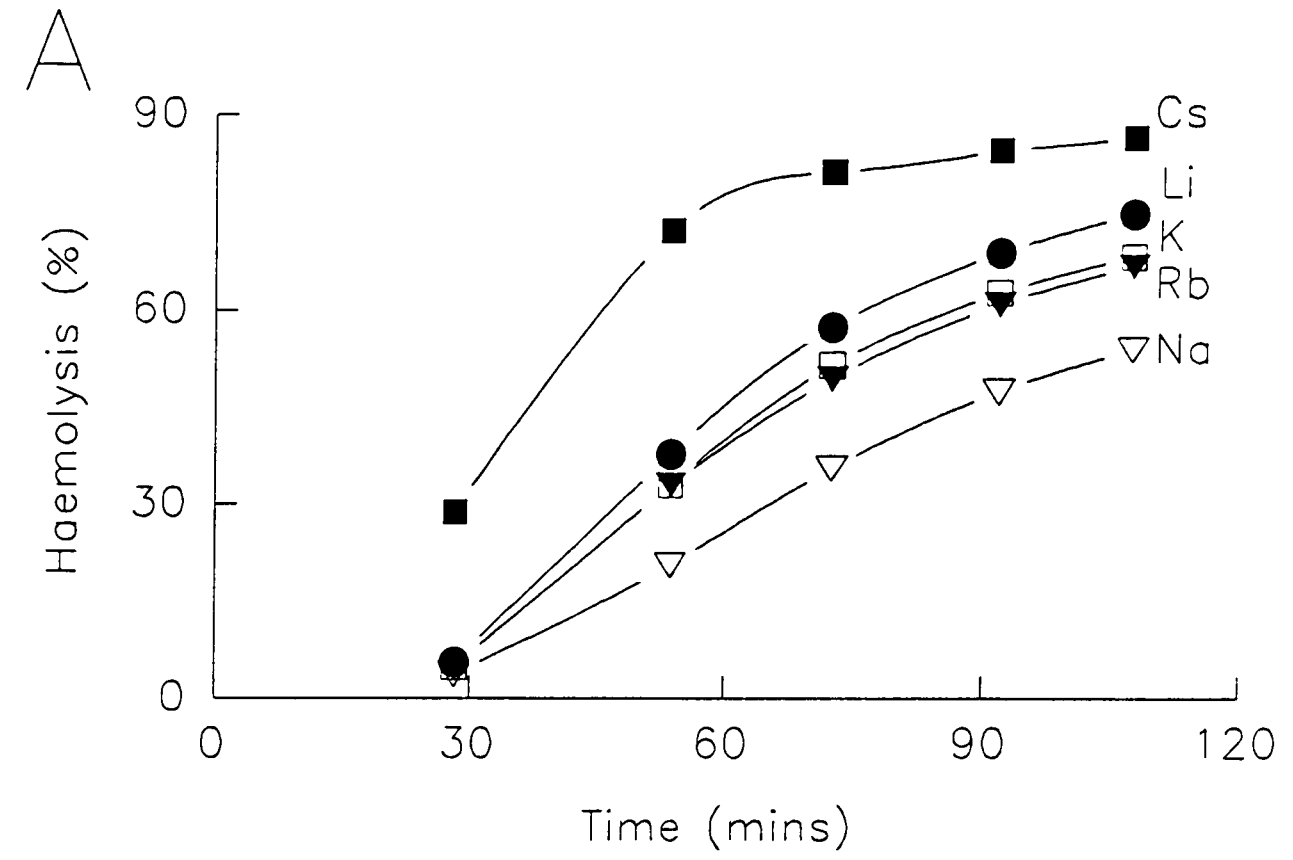


Figure 4.16 Effect of PCO on haemolytic potential of KH. MBS with/without KH was treated (or not) as described for Figure 4.13B, except that PCO was used instead of DBP. Preparations were tested on both human and horse erythrocytes by assaying haemolysis after 30 minutes.



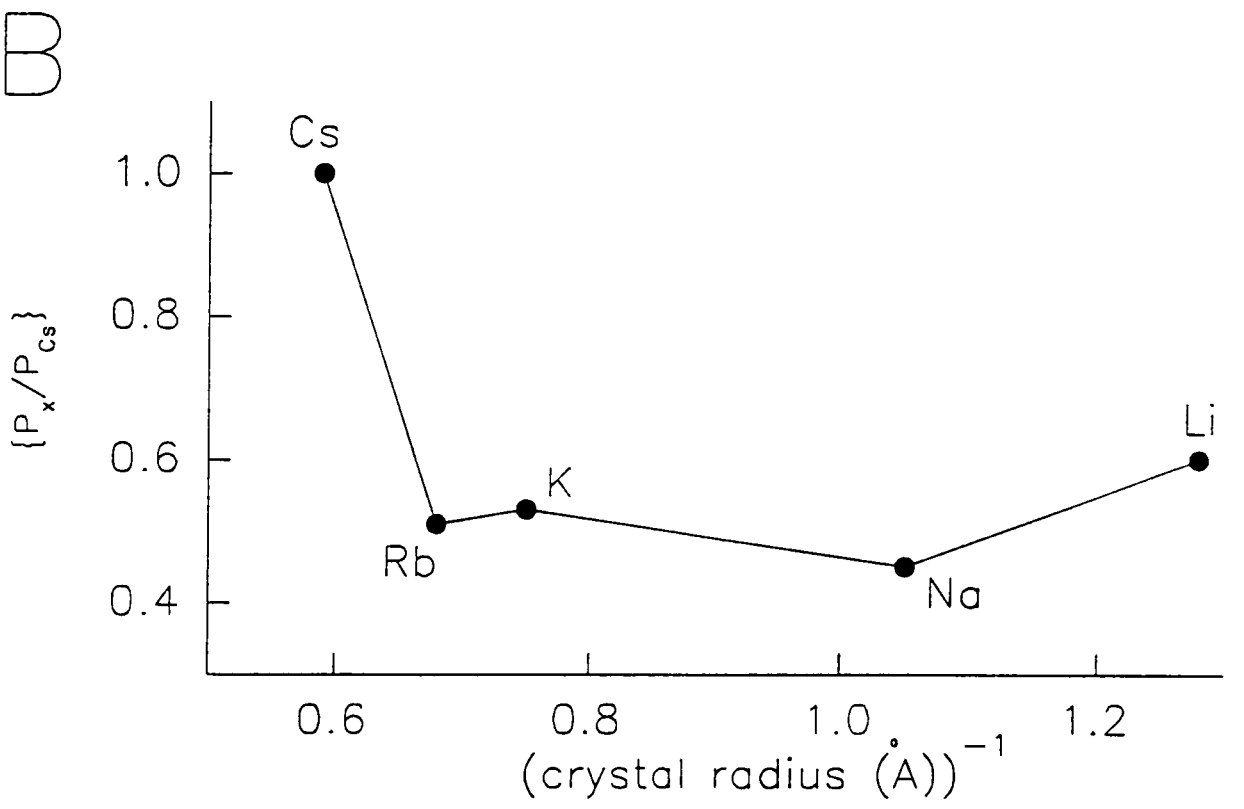


Figure 4.17 Selectivity of the lesion underlying KH-induced haemolysis. Haemolysis was measured in monovalent cation chloride (150 mM, corrected to 291 ± 2 mosmoles(kg)$^{-1}$ in HEPES (10 mM) at pH 7.4) solutions. Haemolysis time-courses were initiated by the addition of 100 µl packed cells to 4.9 ml solution containing KH (1 HU(ml)$^{-1}$). (A) Typical results. (B) Eisenman plot for pooled data (4 separate donors).

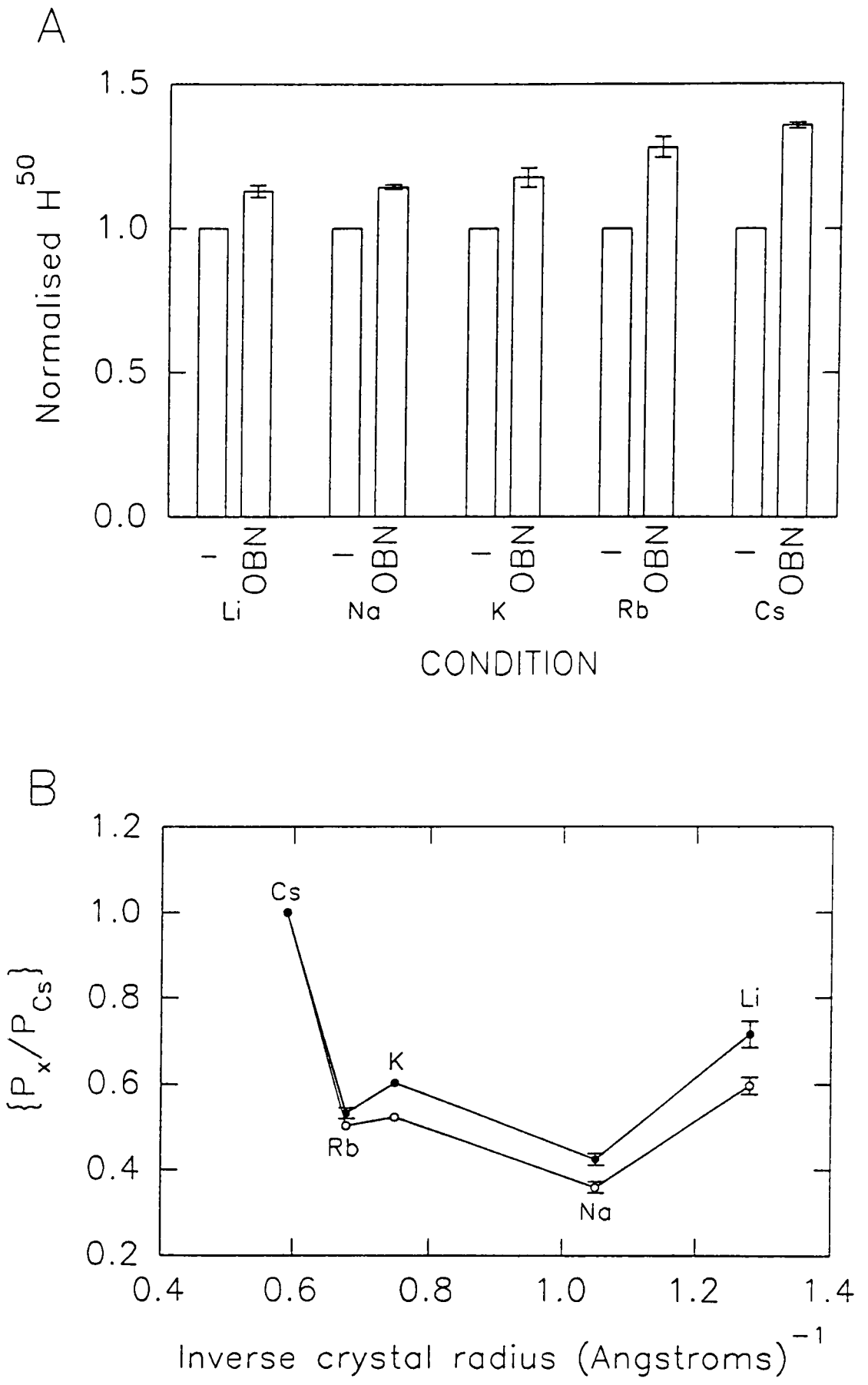


Figure 4.18 Rate of haemolysis and selectivity of the KH-induced lesion in the presence and absence of cation transport inhibitors. Haemolysis was measured as described for Figure 4.17 except that it was performed in the presence and absence of cation transport inhibitors (each at 0.1 mM) + DMSO (1 % v/v), and with (KH) = 0.6 $HU(ml)^{-1}$. (A) Example (in 150 mM NaCl) of inhibition of haemolysis by cation transport inhibitors ouabain, bumetanide and nitrendipine + DMSO (1 % v/v). (B) Selectivity series for KH-induced haemolysis of cells in either the presence (filled symbols) or absence (empty symbols) of (ouabain + bumetanide + nitrendipine (each at 0.1 mM) + DMSO (1 % v/v)).

normalised H^{50}; $P < 0.05$, $n = 3$, for each monovalent cation) afforded by the presence of a preparation containing the cation transport inhibitors (ouabain + bumetanide + nitrendipine (each at a final concentration of 100 μM)) and DMSO (in which nitrendipine was dissolved (final [DMSO] = 1 % v/v)). The surprising result that this inhibitor preparation inhibited KH-induced haemolysis was deemed most likely to be due to the effects of DMSO on membrane fluidity/osmotic fragility rather than the effects of the cation transport inhibitors themselves. Figure 4.18B shows the lack of significant effect of this inhibitor preparation on the selectivity series for KH-induced haemolysis, confirming that the apparent selectivity series of KH-induced haemolysis can be attributed solely to the direct action of the toxin as opposed to a combination of this action and the ion selective effects of endogenous transporters. Figure 4.19 shows the changes in $\{(H^{50})^{-1}\}$ for a range of concentrations of KH. Although there was a dramatic change in permeability according to KH concentration, there was no change in the monovalent cation selectivity series for the KH-induced lesion itself.

4.3 DISCUSSION

Experiments in which the erythrocyte is used as a paradigm target cell for cytolysin action are particularly appropriate for Kanagawa haemolysin of *Vibrio parahaemolyticus*, given the established correlation between the haemolytic ability of this bacterium in culture and pathogenicity (Section 2.6.4; Sakazaki *et al.*,1968; Miyamoto *et al.*, 1969, 1980; Janda *et al.*, 1988). In this study, KH-induced haemolysis was shown to be the end result of colloid osmosis. The nature of the interaction between KH and HRBC was examined in some detail, revealing that although the onset of the cation permeability increase is rapid (lag phase < 1 minute), and athough this pathway is of constant magnitude over the first ten minutes after KH addition, KH binding from the aqueous solution to HRBC membranes continues for a period in excess of five minutes and certainly greater than the 1 - 2 minutes recently proposed (Honda *et al.*, 1992; see below for discussion). Evidence was also obtained for an irreversible aqueous to membrane partition by KH. On the basis of heat stability, kinetics of action, and the insensitivity of horse cells, there is no reason to propose the existence of any biologically active factors other than TDH in the KH preparation.

The KH-induced haemolysis time course is of asymmetric sigmoid shape, reminiscent

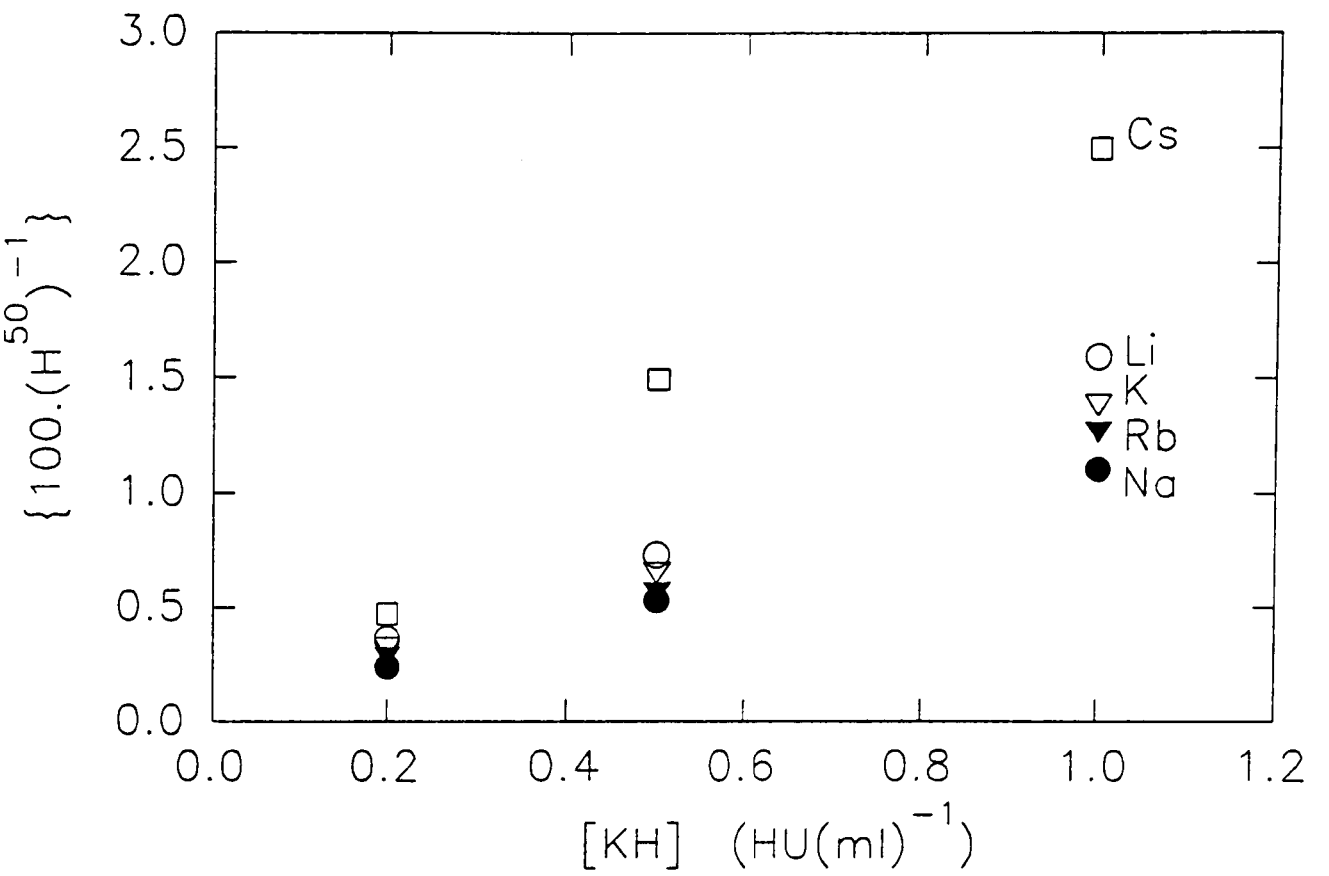


Figure 4.19 Selectivity of the lesion underlying KH-induced haemolysis. Haemolysis was measured as described for Figure 4.17 but at different toxin concentrations.

of that produced by the action of other bacterial toxins, for example *Vibrio cholerae* cytolysin (Krasilnikov *et al.*, 1992), pneumococcal haemolysin (Johnson, 1972) and streptolysin S (Bernheimer, 1946). The 'lag phase' of this sigmoid relation potentially incorporates the periods taken for (a) toxin action and (b) HRBC volume increase up to the critical volume (the largest prelytic volume to which the cell can expand). Haemolysis in the KH range examined (0 - 1 $HU(ml)^{-1}$) is strongly dependent on KH concentration (Figure 4.1A) whilst being relatively independent of target cell concentration ($0.5 \leq$ Hct (%) ≤ 6) (Figure 4.1B) and HRBC:KH ratio (Figure 4.2). These fundamental kinetic characteristics are surprising given the multi-hit nature of haemolytic reactions induced by other bacterial toxins (eg *Pseudomonas aeruginosa* haemolysin (Johnson & Boese-Marrazo, 1980), streptolysin O (Inoue *et al.*, 1976), pneumococcal haemolysin (Johnson, 1972)), for which an increasing haematocrit (at a given toxin concentration) had a protective effect not merely on % total haemolysis but also on the amount of haemoglobin released to the extracellular solution. This latter observation has been suggested to imply that in excess of one toxin entity (as it exists in the aqueous phase) is necessary for haemolysis to occur, protection being conferred by increasing target cell concentration (Inoue *et al.*, 1976). KH is fundamentally different in this respect. TDH activity on membranes is receptor-mediated and the receptors are thought to be the gangliosides G_{D1} and G_{T1a} (Takeda *et al.*, 1975, 1976). In order to explain the sole dependence of haemolysis on KH concentration (in the haemolytic range), a more complicated model may have to be developed in terms of the relationships between toxin binding, pore formation, cation flux and haemolysis.

Colloid osmosis was shown to be the mechanism underlying KH-induced haemolysis by experiments indicating that haemolysis (1) can be inhibited osmotically (Figure 4.3), (2) is preceded by an increase in HRBC volume (Figures 4.4 and 4.5), and (3) is preceded by a large increase in cation permeability, measured as $^{86}Rb^+$ influx (Figure 4.6). The raised cation flux was measured in MBS containing inhibitors to block the major endogenous routes of K^+ movement across the membrane (Bernhardt *et al.*, 1991; Ellory *et al.*, 1992). The increased inhibitor-insensitive K^+ leak is rapid in onset (lag phase < 1 minute) and appears to be constant in magnitude over the time course measured (c. 10 minutes after KH addition). The increase in cation influx (Figure 4.6) occurs prior to volume increase and lysis (Figure 4.4), in the correct temporal sequence for the

mechanism of colloid osmosis. Recently, Honda *et al.* (1992) have produced evidence suggesting that colloid osmosis underlies the action of TDH. In that report, HRBC volume increases in response to TDH were documented by following changes in the average cellular electrical resistance (comparable results to those of the present study were obtained), and TDH-induced haemolysis and volume increases were also inhibited by certain extracellular colloids. The fact that haemolysis is due to colloid osmosis suggests that the lesion (viewed as a pore) has a functional diameter rather smaller than those produced by haemolysins that act by generating large lesions through which intracellular contents leak out directly (see below). The SH-activated toxins (see Table 2.6) produce large pores (c. 15 nm diameter), consistent with a non-colloidal mechanism of action (Freer, 1986), as was indicated by early observations of concurrent release of both $^{86}Rb^{+}$ and haemoglobin by toxin-treated erythrocytes (Duncan, 1974).

No evidence presented here supports the existence of accessory haemolytic factors. In particular, the haemolytic activity of KH was unaffected by heat treatment (100 °C for 10 minutes) and horse red cells were insensitive to toxin action. There has, however, been previous indication of the impurity of KH as a TDH preparation (Douet *et al.*, 1992). Although it is possible that KH preparations vary in their precise constitution, in this study TDH appears to be the sole biologically active component. Nonetheless, the action of thermostable factors affecting haemolysis, for example, by enzymic modification of membrane structure, without affecting permeability properties *per se*, is not ruled out at this stage.

On the basis of the suggestion from flux data (Figure 4.6), and in line with the suggestion of Honda *et al.* (1992), that the TDH binding phase is rapid, a thermodynamic analysis of the permeation pathways underlying KH-induced haemolysis was attempted. The normalised $\{(H^{50})^{-1}\}$ was used to construct an Arrhenius relation (Figure 4.8). This analysis is subject to the major reservation that haemolysis is the end-result of a series of processes (the rate of each of which may depend on thermodynamic parameters) rather than being a single physical process. Therefore, an activation energy (E_a) value derived from haemolysis data may be substantially different from the value for ion transport by the pore underlying haemolysis. The E_a derived from the linear portion (between 7 and 27 °C) of this relation (Hall *et al.*, 1982; Ginsburg *et al.*, 1983; Hall & Willis, 1986) was 53.30 ± 2.79 kJ(mol)$^{-1}$. This value is higher than that: (i) expected for diffusion through

water-filled pores of considerably larger radii than the permeating substances (Stein, 1967; Ginsburg *et al.*, 1983) - the apparent E_a for free diffusion of K^+ through bulk water is c. 16.3 kJ(mol)$^{-1}$ (Bruins, 1929; Hall & Willis, 1986), (ii) given for ion movement by pore-forming ionophores (Hladky & Haydon, 1972; Cohen, 1974a,b) - gramicidin-mediated K^+ transport across an artificial membrane has an E_a of c. 22.6 kJ(mol)$^{-1}$ (Hladky & Haydon, 1972), and (iii) given for sorbitol-induced lysis of human erythrocytes infected by *Plasmodium falciparum* ($E_a = 41.8 \pm 4.18$ kJ(mol)$^{-1}$; Ginsburg *et al.*, 1983). However, the E_a obtained is similar to that obtained for ouabain and bumetanide insensitive K^+ influx (in the presence of EGTA) into human erythrocytes, between 18.5 and 37 °C ($E_a = 63.0 \pm 5.5$ kJ(mol)$^{-1}$; Hall & Willis, 1986). In addition to the reservation given above for Arrhenius analysis of haemolysis data, possible explanations for the high E_a include: (1) the binding phase is incomplete by the time of transferral to the appropriate temperature, and the ongoing process of binding/pore formation is itself dependent on thermodynamic parameters, (2) binding and pore formation is complete but ion movement through the KH-induced lesion is restricted (compared to ion movement by diffusion through a large water-filled pore), and (3) temperature-induced changes in membrane structure secondarily affect either binding of KH or perturb pore function. Explanations (1) and (3) could also potentially explain the observed break in the Arrhenius relation.

As discussed in section 4.1, a previous study (Sakurai *et al.*, 1975) has delineated two steps in TDH action: (i) toxin binding, and (ii) a lytic process. It was found that some TDH-binding did occur at 4 °C, interpreted by both Freer (1986) and Kreger (1991) as meaning that the binding step is temperature-*independent* although no such claim was in fact made by Sakurai and his colleagues. By contrast Honda *et al.* (1992) suggest that the TDH binding (or absorption) phase is temperature-dependent whereas the lytic step is temperature-*independent.* Their data are, however, quite inadequate to make such a claim. Haemolysis experiments were performed under certain conditions (ie in Tris-buffered saline at pH 7.2) at a TDH concentration (0.5 μg(ml)$^{-1}$). Haemolysis started after about 10 minutes incubation at 37 °C and was almost completed within 35 minutes. The rate of haemolysis was thus rather faster than that observed in our studies. Unfortunately, time taken to cause a certain degree of haemolysis (section 4.2) was not used as an index of haemolytic rate in their study. This would have been difficult because under their conditions, haemolysis occurred rather rapidly. Rather, the measure employed was %

haemolysis after a given period of time. If haemolysis so assayed was in the mid-range (and haemolysis was still occurring) then comparisons between different conditions would be practicable. However, this is not the case when 100 % haemolysis has occurred since no indication of *rate* is then given. The data of Honda *et al.* (1992) documenting temperature-dependence is therefore flawed in this respect. Their initial experiments showed that no haemolysis occurred within 40 minutes for cells incubated with toxin at 4 °C; however, on warming to 37 °C, haemolysis occurred as for cells plus toxin incubated at 37 °C. Their subsequent results show that, after 1 hour incubation in saline free from extracellular colloid impermeants, 100 % haemolysis had occurred for cells preincubated (with extracellular colloid impermeants) at 37 °C with TDH for a range of times. Because 100 % haemolysis occurred for all preincubation times except those under two minutes, a binding phase of 1 - 2 minutes was proposed. However, their results make no distinction between the haemolytic rates for haemolytic systems preincubated for longer than 2 minutes. Either haemolysis after a shorter time period or time taken to reach a degree of haemolysis should have been measured. In addition, neither Honda *et al.* (1992) nor Honda and Iida (1993) make reference to the report of Sakurai *et al.* (1975) to which they produce conflicting conclusions. Their rather simplistic interpretation of results has been largely corrected by studies presented here which show that the binding phase continues for a much longer period than 1 - 2 minutes (see below), even though the pathway responsible for increased cation flux appears rapidly and is constant in magnitude for the first ten minutes. It is possible that the hypothesis (outlined in (1) and/or (3) above) underlies the irregularity in the Arrhenius relation whereby KH-induced haemolysis is significantly faster at 27 than 37 °C.

The nature of the binding event was studied in more detail by assessing the effect of washout on subsequent haemolytic rate (Figure 4.9). Although some KH was bound after 1 - 2 minutes (Figure 4.6), the binding of KH by HRBC membranes appeared to be incomplete even after 5 minutes of toxin exposure. To check that bound KH was not released, the effect of extracellular volume was assessed on HRBC which had been treated with KH for 5 minutes in MBS + sucrose (50 mM) before being washed and resuspended in MBS (Figure 4.10). The protective effect of extracellular volume (noted in Figure 4.2C) is not observed for either of these conditions. This adds an important feature to the working model of KH action. After pore formation, there is a stabilisation phase in the

membrane and KH in the membrane-bound form is not in equilibrium with aqueous KH.

The irreversibility of lesion formation is further documented by data shown in Figures 4.11 and 4.14, which demonstrate that the entities responsible for the haemolytic lesion are not washed out under centrifugal pressure. Data in Figure 4.12 show that not all KH is bound by HRBC after 5 minutes toxin exposure. The simplistic explanation, that KH is bound after this time but that some of it is only weakly associated (and hence reversible by centrifugation) was discounted by experiments (Figure 4.14) showing that if after 3 or 10 minutes exposure to KH, the treated HRBC were spun through DBP, the aqueous solution still retained a considerable portion of its haemolytic ability. This occurred even though the presence of DBP itself is inhibitory to KH activity (Figure 4.13).

The organic solvent DBP is immiscible with water. Experiments presented in Figure 4.13 showed that vigorous vortexing of KH with DBP dramatically decreased the haemolytic activity of the aqueous solution, suggesting a protein-hydrophobic environment interaction; KH could either partition into DBP, be absorbed to the oil-water interface, or be denatured by DBP. These data mirror previous experiments (Takeda *et al.*, 1975, 1976) in which incubation of TDH with ganglioside preparations decreased its subsequent haemolytic activity, providing good evidence for ganglioside mediation of TDH action. In preliminary experiments, other membrane-active agents, arachidonic acid (15 μM), catechin (250 μM) and epicatechin (250 μM) had no significant effect on the rate of KH-induced haemolysis (Figure 4.15). For these latter two agents, the concentration used was in excess of twice that for which a significant effect had been claimed previously (Toda *et al.*, 1989), using a different technique to assess haemolytic rate. Results herein did not rule out a small inhibitory effect of these compounds; further investigation of the effects of these compounds was not merited.

Vortexing of a KH solution with pure corn oil dramatically enhanced the haemolytic activity of the aqueous solution. However, horse cells remained insensitive to this treated KH preparation, suggesting that subsequent membrane-insertion of KH-containing lipid in the emulsion was unlikely or that if it occurred it was still dependent on the receptor interaction present in human but absent in horse erythrocytes. PCO had only a small effect on haemolysis in the absence of KH. Therefore it is suggested that PCO increases the osmotic fragility of cells, thus potentiating the lytic action of KH. The possibility that PCO enhances the rate of KH binding or the magnitude of the leak induced was not ruled

out.

The monovalent cation selectivity series was derived for the KH-induced lesion: $Cs^+ > Li^+ > K^+ > Rb^+ > Na^+$, conforming to a reversed Eisenman VIII series (Eisenman & Horn, 1983) with a small K^+ anomaly (Figure 4.17). The presence of a preparation of cation transport inhibitors (ouabain, bumetanide and nitrendipine; section 3.6.1) and DMSO caused a slight decrease in the rate of haemolysis but not affect the selectivity series (Figure 4.18), indicating that the contributions made by endogenous cation transport systems during the haemolytic process are unlikely to affect the apparaent selectivity. DMSO is the most likely agent in the preparation to affect haemolytic rate (Figure 4.18A) in this way, presumably by an effect on membrane fluidity/osmotic fragility. Because the selectivity sequence is unchanged over a range of haemolytic KH doses (Figure 4.19), it is suggested that even with increasing KH concentration, there is no fundamental change in the nature of the lesion itself - rather, it is the *number* of lesions that increases. Electron microscopy of erythrocyte membranes using the freeze-fracture method shows that TDH-treatment causes the appearance of homogeneous circular arrays in the membrane. These rings had external and internal diameters of 40 and 30 nm respectively (Honda *et al.*, 1992). On the basis of these data, it was suggested that TDH is a pore-forming protein. By measuring the inhibitory effects of extracellular osmotic impermeants (of known molecular radius) on haemolysis (similar to experiments performed in Figure 4.3), these workers obtained a measurement of functional pore size; raffinose (1.14 nm diameter) and inulin (2.8 nm) were as protective as PEG, indicating a pore-diameter of approximately 1 - 2 nm. A similar discrepancy between structural and functional pore-sizes has been reported for staphylococcal α-toxin (Fussle *et al.*, 1981). Interestingly, TDH does not appear to act by a progressive barrel-stave mechanism in which its pore-size would be increased above this diameter by recruitment of more toxin molecules to line the pore. This assertion is made on the basis of evidence provided by Honda *et al.* (1992), that even at higher doses of toxin small colloids such as inulin were capable of protecting against haemolysis, together with evidence in this chapter (Figure 4.19) that the selectivity of the lesion is unaffected by the KH concentration. As already discussed, the pore size is small (c. 1 - 2 nm diameter). It is therefore conceivable (by analogy with staphylococcal α-toxin; Bhakdi *et al.*, 1986b) that the important effect(s) of toxin action *in vivo* may not be due to a lytic effect but rather to a more subtle effect on cellular

physiology (see sections 5.2.4 and 5.3). This is especially the case given the ability of the pathophysiological target cells (enterocytes) to swell (Knutton *et al.*, 1976) and repair membrane damage (Bhakdi *et al.*, 1986a,b). Therefore, it was deemed important to examine the effects of smaller doses of KH on cation fluxes across the erythrocyte membrane, not only by the induced pore but also by the endogenous membrane transporters.

By application of simple and well established techniques relating to use of the erythrocyte as a model membrane target, fundamental features of KH action have been delineated and a rather more complex picture of membrane attack has emerged than that outlined by Honda *et al.* (1992). Isotope flux methodology can yield information on the more subtle effects of (lower doses of) TDH on membrane function and will be described in the next chapter. Such perturbations of cation transport may indicate more appropriate pathophysiological parameters from which to extrapolate to the alimentary dysfunction associated with *V. parahaemolyticus*.

CHAPTER 5

Cation flux studies on erythrocytes treated by Kanagawa haemolysin of *Vibrio parahaemolyticus*

'...I stood hearing a foreign language in my blood... '
Jon Stallworthy, from So Much in Common *in* Hand in Hand

5.1 INTRODUCTION

The previous chapter has described work delineating the haemolytic action of Kanagawa haemolysin (KH) of *Vibrio parahaemolyticus*. The release of haemoglobin in an all-or-none fashion by lysed erythrocytes (Ponder, 1948; Bernheimer, 1988) makes for a simple assay of toxin action used not only in Chapter 4, but throughout this dissertation. However, haemolysis is a complex process, especially if instigated by bacterial toxins, when lysis is the end-result of several sequential processes. Evidence has been presented in Chapter 4 and elsewhere (Honda *et al.*, 1992), implicating colloid osmosis secondary to an increase in cation permeability as underlying toxin-induced haemolysis. Thus the steps occurring in series and envisaged as underlying TDH-induced haemolysis are: (i) toxin binding, (ii) pore formation causing an increase in cation permeability, (iii) increased net cation influx, (iv) water following the cation flow by osmosis, causing cell-swelling, and (v) the cell membrane rupturing at the critical volume, releasing haemoglobin to the solution.

Isotope flux methodology (section 3.5) is well established for studies on erythrocytes (Garrahan & Glynn, 1966; Ellory, 1982; Harvey & Ellory, 1989; Ellory *et al.*, 1992) particularly in terms of cation transport (Ellory & Tucker, 1983; Kirk *et al.*, 1994). This technique has been applied to the study of bacterial toxin effects on erythrocytes (eg streptolysin O (Duncan & Schlegel, 1975), *Escherichia coli* haemolysin (Bhakdi *et al.*, 1986), and *V. cholerae* haemolysin (Hall *et al.*, 1986; Krasilnikov *et al.*, 1992)) and other cells (eg staphylococcal α-toxin (Bashford *et al.*, 1989), and diphtheria toxin (Alder *et al.*, 1990)), but not in the case of TDH.

Many advantages are afforded by radioisotope techniques, in particular: (i) examination of only one of the above processes (namely the step instigating a raised cation permeability) that contribute to haemolysis, and (ii) experiments should be possible at lower toxin doses. The latter is especially important since (a) experiments may measure

a more pathologically relevant response - TDH does not have to cause cell lysis in order to instigate a diarrhetic effect (Nishibuchi *et al.*, 1992), and (b) experiments may be more economical in terms of using valuable supplies of purified toxin.

As has been described in sections 4.1 and 4.3, there is some confusion in the literature about the steps involved in the process of pore formation by TDH (Sakurai *et al.*, 1975; Freer, 1986; Kreger, 1991; Honda *et al.*, 1992; Honda & Iida, 1993) and their individual characteristics. Data presented in Chapter 4 suggest that the binding/membrane-stabilisation phases of TDH action may be rather longer than the 2 minutes recently suggested (Honda *et al.*, 1992). However, in section 4.2.2, K^+ ($^{86}Rb^+$) influx was measured over a ten minute time course after KH addition and the increase in cation influx was rapid in onset and appeared to be constant in flux magnitude. Isotope flux methodology is well suited to examining the dynamics, and other features, of toxin action more thoroughly.

In this chapter, the use of the erythrocyte as a model membrane system with which to examine toxin action has been extended with these techniques. Results are collated in four sections, considered below.

(i) Nature of the cation leak. The route of raised K^+ influx was considered in relation to the major components of endogenous K^+ influx, the Na^+/K^+ ATPase, the $Na^+/K^+/2Cl^-$ cotransporter and the Ca^{2+}-activated K^+ channel (Figure 2.23). Subsequent attention was focussed on the induced leak insensitive to ouabain, bumetenide and nitrendipine. The dynamics, dose-response, and stability and other features of toxin-induced influx were examined.

(ii) Effects of non-enzymic agents on the induced leak. Clarification of the nature of the induced lesion was attempted by examining the effects of several non-enzymic agents on inhibitor-insensitive KH-induced K^+ influx. The membrane-active agents used were divalent cations (Sakurai *et al.*, 1975; Menestrina, 1986; Pasternak, 1987, 1991; Bashford *et al.*, 1988a,b, 1989; Miyake *et al.*, 1989), phloretin (which both decreases the endogenous dipole potential and inhibits a variety of membrane transport processes (Owen *et al.*, 1974; Andersen *et al.*, 1976; Jennings & Solomon, 1976; Melnik *et al.*, 1977; Deuticke *et al.*, 1991)) and DIDS (20 μM; as an inhibitor of Band 3 aggregation (Benz *et al.*, 1984)). The effect of vortexing KH with DBP on subsequent leak inducing potency of the KH preparation was examined in light of earlier results (Figure 4.13) indicating that

such treatment causes a reduction in haemolytic activity.

(iii) Effect of neuraminidase treatment of HRBC on subsequent KH action. Previous reports have suggested that the receptor entities for TDH are the neuraminidase-sensitive gangliosides, G_{D1a} and G_{T1} (Takeda *et al.*, 1975, 1976). As has been previously discussed (sections 2.3.3 and 3.6.4), neuraminidase successively cleaves sialic acid from higher order glycoproteins and gangliosides, decreasing the order of such structures. This effect has been assessed previously in erythrocytes by measuring decreased surface charge by microelectrophoresis (Stein *et al.*, 1987). The effect of neuraminidase treatment of erythrocytes on subsequent KH action was examined using both haemolysis and flux measurements, it was originally deemed likely that such treatment would be protective against KH.

(iv) Effects of KH on endogenous membrane transport function.

It has been noted previously (section 2.8.1) that the erythrocyte has membrane transporters similar to those characterised in other tissues, and that effects on membrane transport (rather than lysis) may be important in the pathogenic effects instigated by TDH (section 2.6.4 and Figure 2.22). Therefore, the effects of KH on HRBC endogenous K^+ influx pathways were examined under different external conditions.

The methodological approaches employed here have been described in Chapter 3. The implications of data presented in this chapter are discussed in light of previous reports (section 5.3) and a working scheme for the plausible pathogenic effects of TDH is outlined.

5.2 RESULTS

5.2.1 Nature of the cation leak induced by KH

Maximally inhibiting concentrations of cation transport inhibitors were used as described previously (section 3.6.1), ouabain for Na^+ pump (Bernhardt *et al.*, 1991) bumetanide for the $Na^+/K^+/2Cl^-$ cotransporter (Bernhardt *et al.*, 1991) and nitrendipine for the Gardos channel (Ellory *et al.*, 1992)), in order to observe the action of KH on the endogenous components of K^+ influx through the cell membrane. Measurement of fluxes was performed over the ten minute period following KH addition. Figure 5.1 shows that KH addition (0.1 $HU(ml^{-1})$) to cells suspended in nominally Ca^{2+}-free (NOM.) MBS ($[K^+]_o$ =

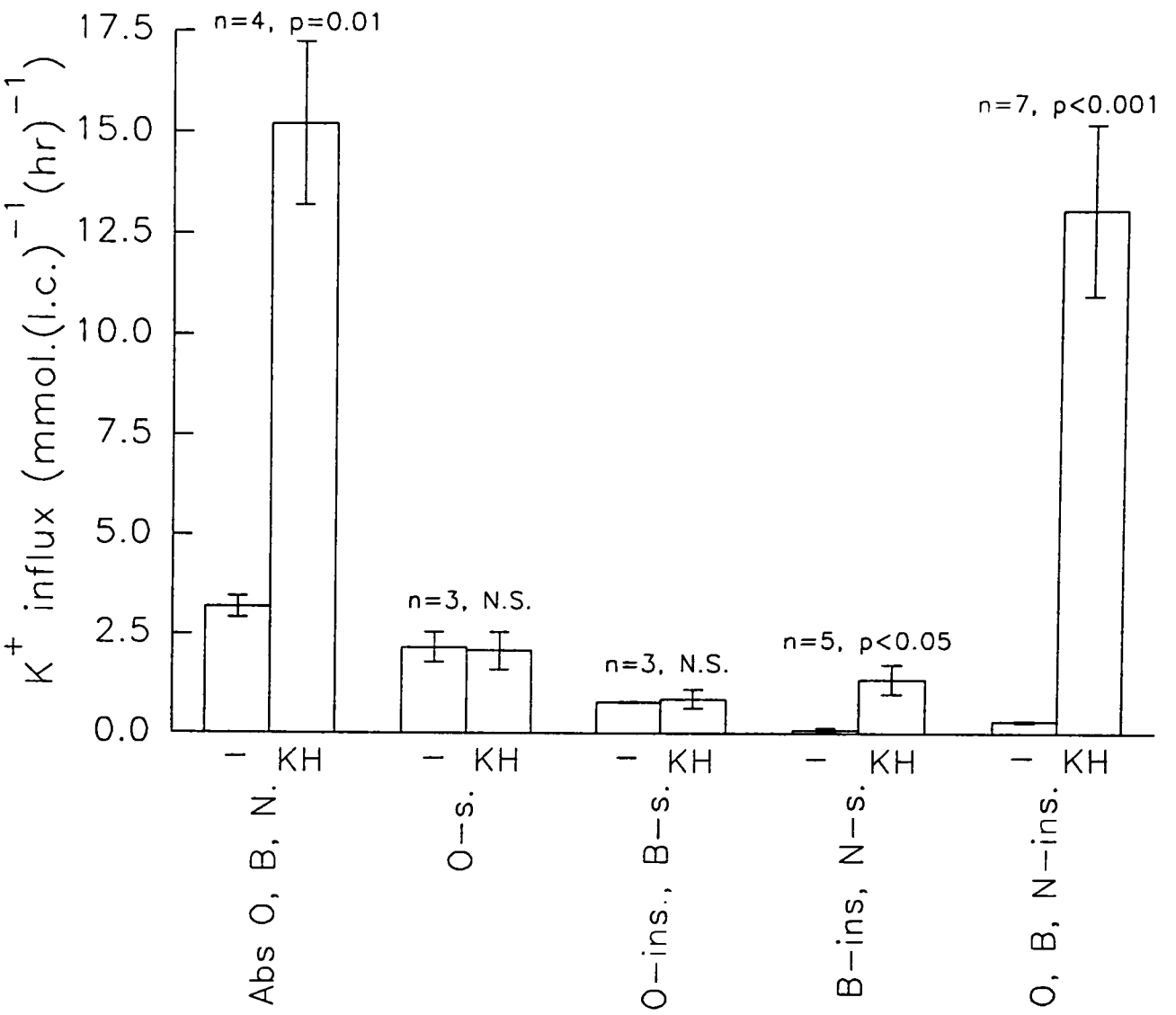
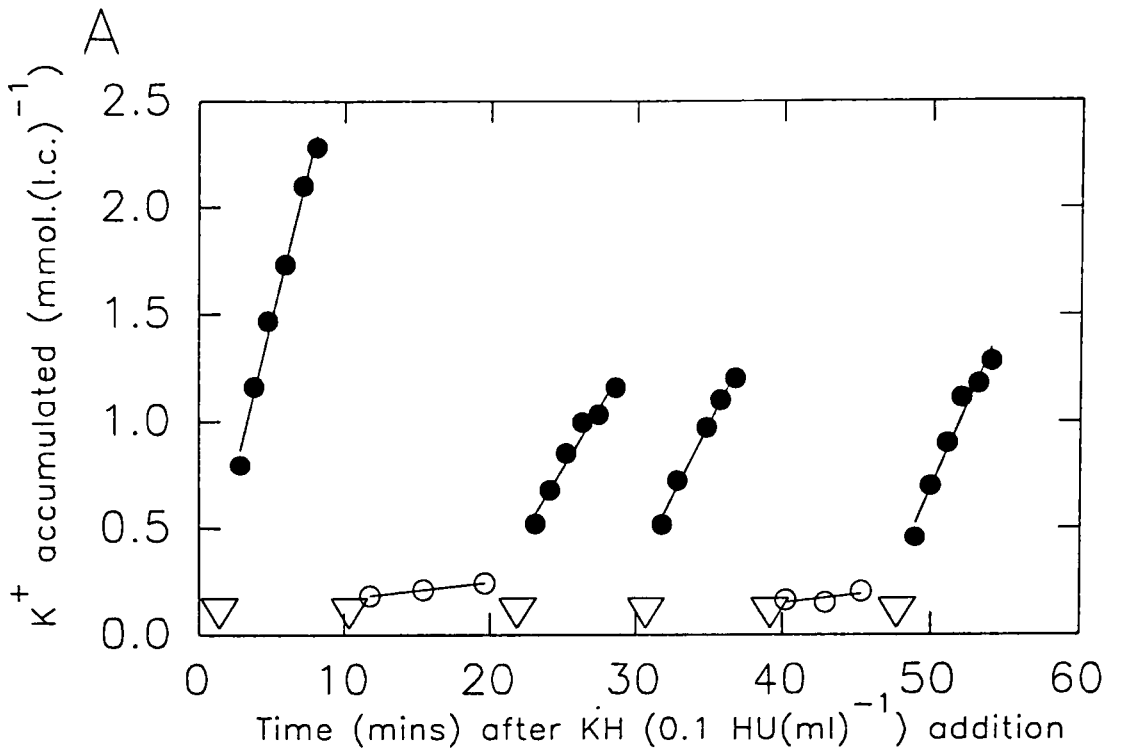


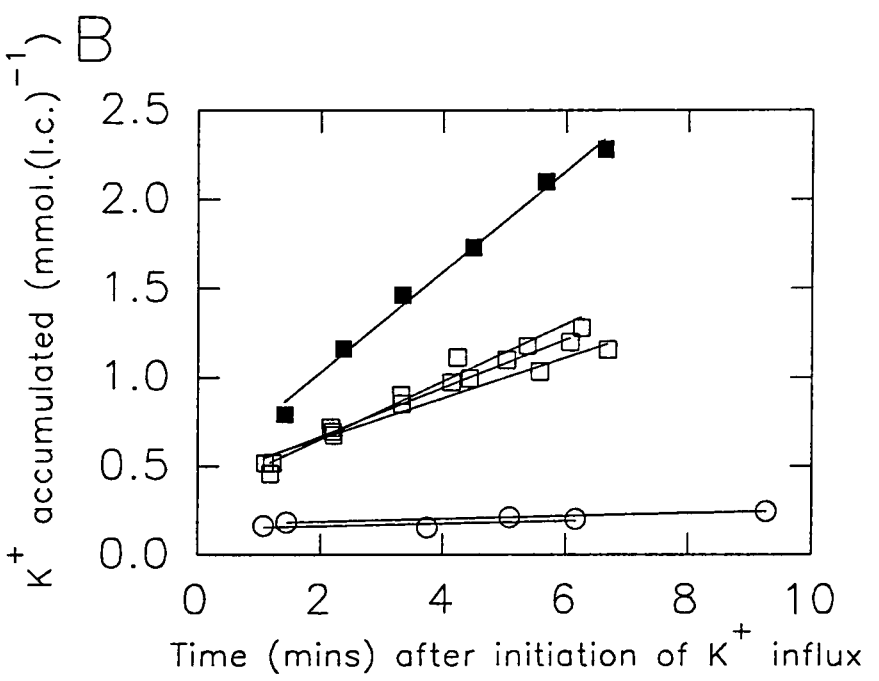
Figure 5.1 Effects of KH on endogenous components of K^+ influx. HRBC were incubated in the presence or absence of KH (0.1 HU(ml)$^{-1}$) for 1 minute at 37 °C before addition of ouabain (100 μM), bumetanide (100 μM) and/or nitrendipine (10 μM) as indicated. K^+ influxes (10 minute periods) were initiated by the addition of 100 μl K(^{86}Rb)Cl (150 mM) to aliquots (0.9 ml) of cell suspension. $(K^+)_o$ = 15 mM, $(Na^+)_o$ = 135 mM, Hct = 5 %; (in)s = (in)sensitive. Results are means ± S.E.M. for at least 3 separate experiments. Significances of differences between control and KH columns were tested by unpaired *t*-test.

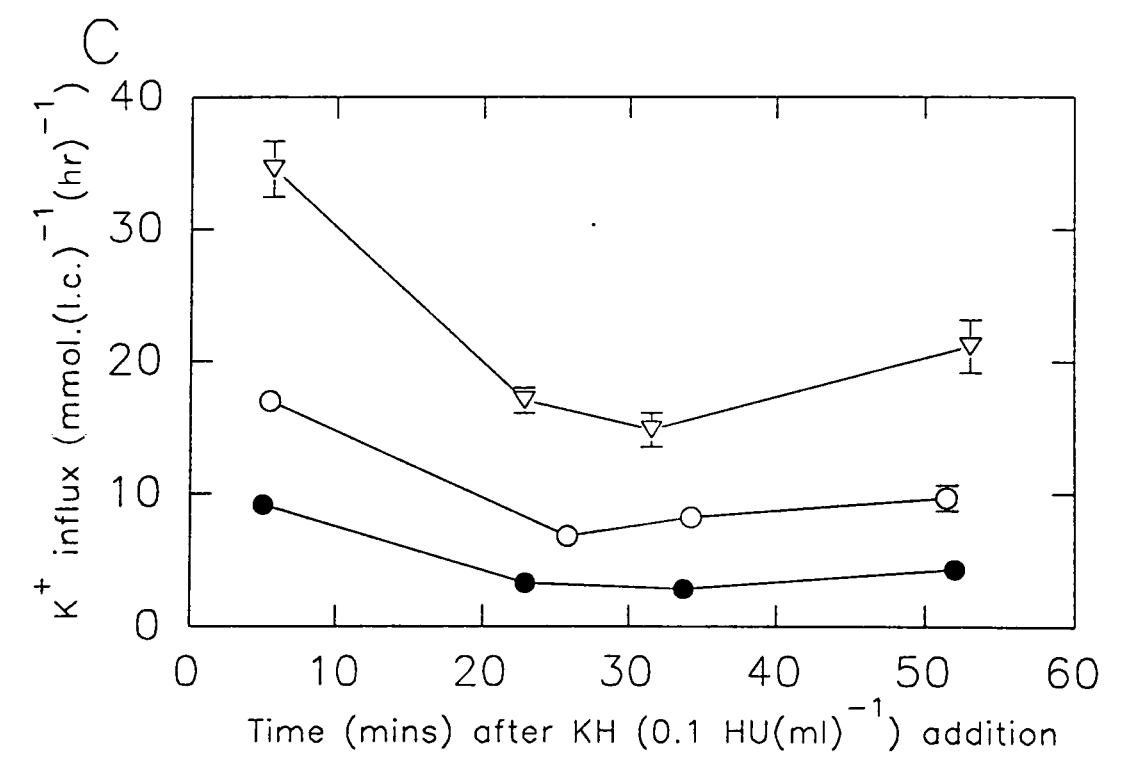
15 mM) caused significant (differences between control and KH-treated results were compared using unpaired *t*-tests) increases in the residual (ie insensitive to all the inhibitors) leak ($P < 0.001$, $n = 7$) from (mean ± S.E.M.) 0.30 ± 0.026 to 13.09 ± 2.124 mmol. $K^+(l.c.)^{-1}(hr)^{-1}$ and in the ouabain and bumetanide-insensitive, nitrendipine-sensitive influx ($P < 0.05$, $n = 5$) from 0.08 ± 0.050 to 1.37 ± 0.371 mmoles K^+ $(l.cells.)^{-1}$ h^{-1}. The effects on the ouabain-sensitive component (Na^+ pump) and the ouabain-insensitive, bumetanide-sensitive component ($Na^+/K^+/2Cl^-$ cotransport) were not significant. The significant effect on the nitrendipine-inhibited K^+ influx is considered in more detail in section 5.2.4. Evidently, the major component of increased K^+ influx was that which was insensitive to all the inhibitors used.

The dynamics of the KH-induced inhibitor-insensitive K^+ influx pathway were examined over a ten minute time course in Chapter 4 (Figure 4.6). Figure 5.2 details the magnitude of K^+ influx by this pathway over an extended time period, circumventing the problem of isotope equilibration/backflux by incubating with KH for different periods prior to measuring flux. Within the ten minute time period subsequent to KH addition (and similarly to data depicted in Figure 4.6), a high K^+ influx was noted that appeared to be constant in magnitude with respect to time. However, fluxes followed at later times were relatively unchanged with respect to each other, and although considerably raised with respect to control cells, were lower than those observed within the first ten minutes after toxin addition (Figure 5.2B). K^+ influx was estimated for each of the groups of K^+ accumulated data, and for each donor, by linear regression (Figure 5.2C). Although there was a profound donor dependence of induced K^+ influx (as noted previously for KH-induced haemolysis), the pattern of dynamics of KH-induced inhibitor-insensitive K^+ influx was the same for all three donors.

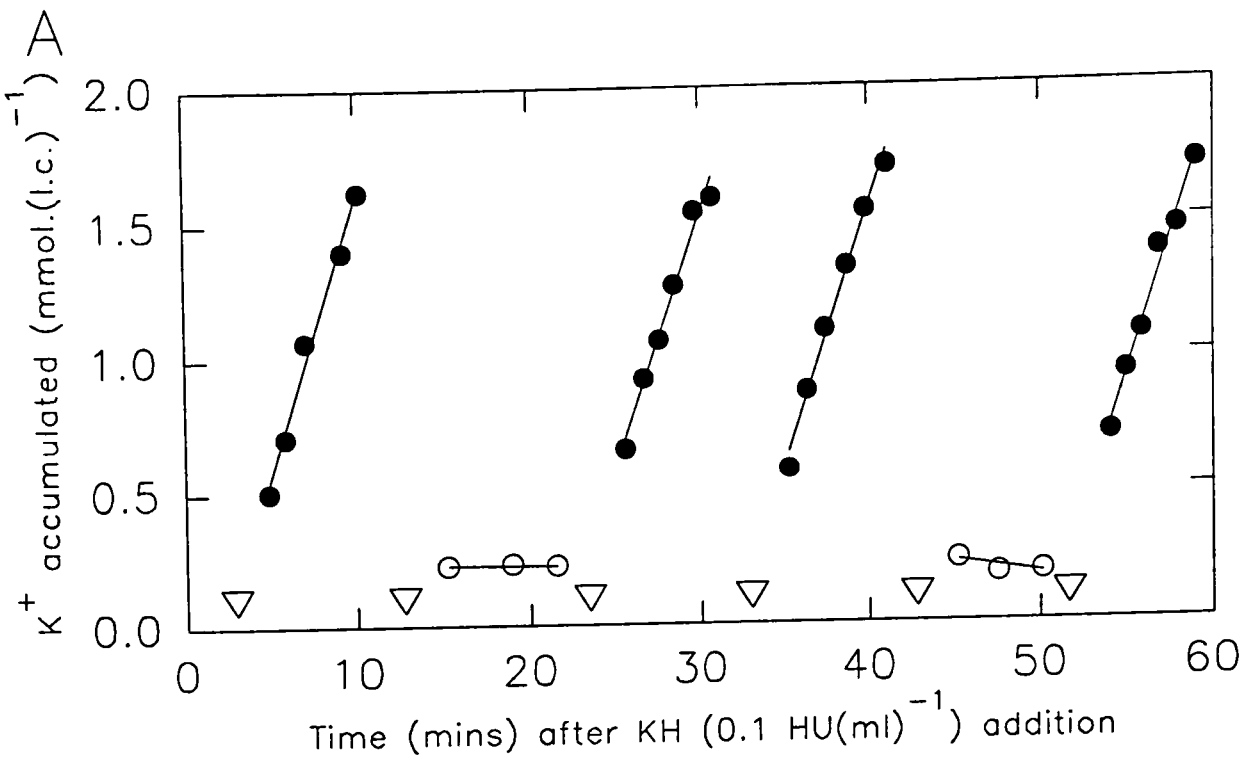
Similar experiments were performed (Figure 5.3) but KH was added to a cell suspension that was kept on ice rather than being incubated at 37 °C. Immediately after addition of (^{86}Rb)KCl, the aliquot of cell suspension was incubated at 37 °C for 2 minutes (the time taken for temperature equilibriation (Ellory, 1982)), prior to measuring K^+ accumulated (still at 37 °C) as performed previously for results given in Figure 5.2. However, under these conditions (in comparison with results shown in Figure 5.2), K^+ influx did not decrease with time after KH addition. Thus, although toxin may bind at low temperatures (Honda *et al.*, 1992) it does not appear to induce a flux pathway which progresses through

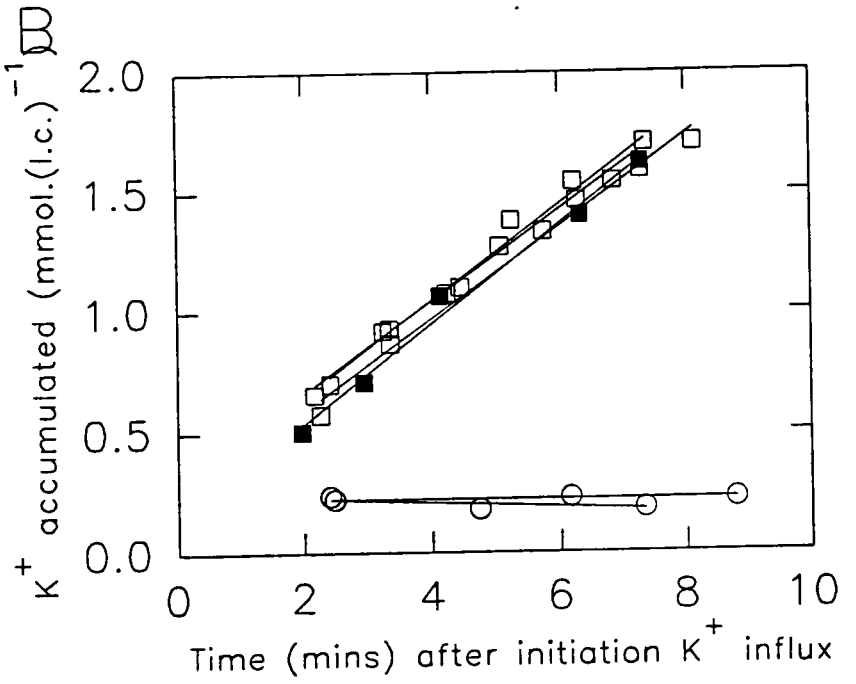

A
K^+ accumulated (mmol.(l.c.)$^{-1}$)
2.5
2.0
1.5
1.0
0.5
0.0
0
10
20
30
40
50
60
Time (mins) after KH (0.1 HU(ml)$^{-1}$) addition

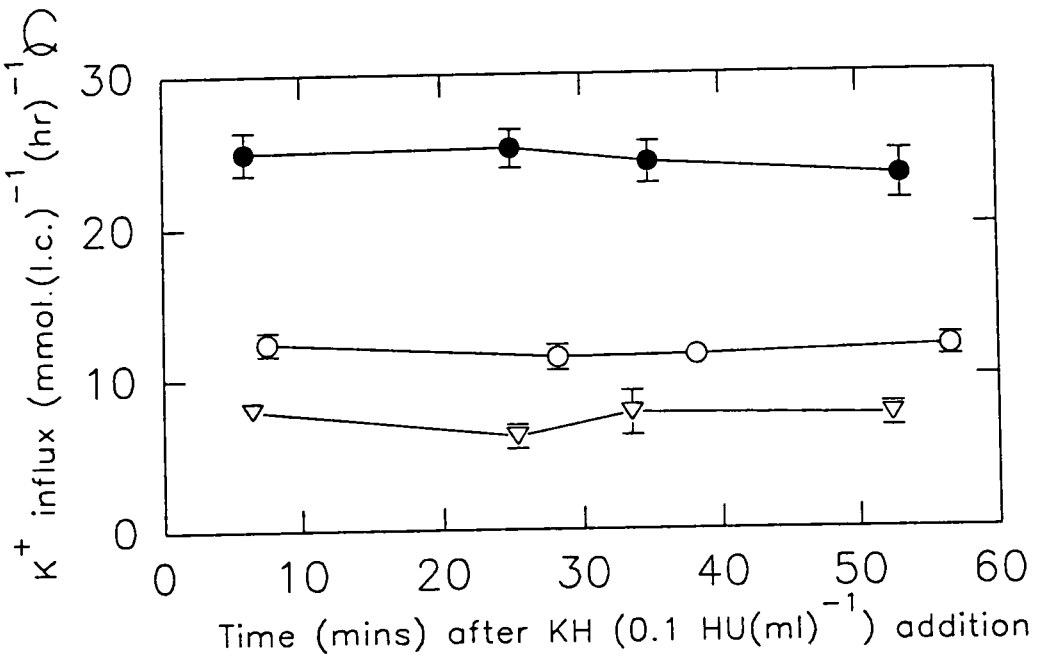

B
K^+ accumulated (mmol.(l.c.)$^{-1}$)
2.5
2.0
1.5
1.0
0.5
0.0
0
2
4
6
8
10
Time (mins) after initiation of K^+ influx


C
K^+ influx (mmol.(l.c.)$^{-1}$(hr)$^{-1}$)
40
30
20
10
0
0
10
20
30
40
50
60
Time (mins) after KH (0.1 HU(ml)$^{-1}$) addition

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Figure 5.2 The extended dynamics of KH-induced inhibitor-insensitive K^+ influx. HRBC were suspended (Hct = 6 %) in MBS with sucrose (50 mM) and ouabain, bumetanide and nitrendipine (each inhibitor at 100 μM). KH (0.1 $HU(ml)^{-1}$) was added/not added to the cell suspension, and immediately transferred (time = zero) to a water bath at 37 °C. Subsequently cell suspension aliquots (1.17 ml) were spiked with 130 μl K(^{86}Rb)Cl (150 mM) and K^+ uptake ($([K^+])_o$ = 15 mM) was measured, using the DBP technique (section 3.6), for a time period of 1 - 7 minutes after spiking. (A) Results from a typical experiment representative of three such experiments. K^+ accumulated is plotted against time after KH addition for HRBC either treated (closed circles) or untreated (open circles) by KH after addition of KCl at times indicated (open inverted triangles). (B) Analysis of data shown in (A); for ease of comparison, results are similarly given as K^+ accumulated, but against time after spiking. Results obtained in the first ten minutes after KH addition are given as closed squares; those from subsequent periods are given as open squares. Results for untreated cells are given as open circles. (C) Results from all three donors are given as K^+ influx (calculated by linear regression from data for which typical results are given in (A)), against the median time of the influx period.


A
K^+ accumulated (mmol.(l.c.)$^{-1}$)
2.0
1.5
1.0
0.5
0.0
0
10
20
30
40
50
60
Time (mins) after KH (0.1 HU(ml)$^{-1}$) addition


B
K^+ accumulated (mmol.(l.c.)$^{-1}$)
2.0
1.5
1.0
0.5
0.0
0
2
4
6
8
10
Time (mins) after initiation K^+ influx


C
K^+ influx (mmol.(l.c.)$^{-1}$(hr)$^{-1}$)
30
20
10
0
0
10
20
30
40
50
60
Time (mins) after KH (0.1 HU(ml)$^{-1}$) addition

previous page:

Figure 5.3 The dynamics of KH-induced inhibitor-insensitive K^+ influx, when the cells are kept on ice. HRBC were suspended (Hct = 6 %) in MBS with sucrose (50 mM) and ouabain, bumetanide and nitrendipine (each inhibitor at 100 μM), and kept on ice. KH (0.1 $HU(ml)^{-1}$) was added/not added to the cell suspension, and the cell suspension maintained cool on ice. Subsequently cell suspension aliquots (1.17 ml) were added to 130 μl $K(^{86}Rb)Cl$ (150 mM) and incubated at 37 °C; K^+ uptake ($(K^+)_o$ = 15 mM) was measured, using the DBP technique (section 3.6), for a time period of 2 - 8 minutes after spiking, as 2 minutes is required for temperature equilibriation with the external environment. (A) Results from a typical experiment representative of three such experiments. K^+ accumulated is plotted against time after KH addition for HRBC either treated (closed circles) or untreated (open circles) by KH after spiking (at times indicated by open inverted triangles). (B) Analysis of data shown in (A); for ease of comparison, results are similarly given as K^+ accumulated, but against time after spiking. Results obtained in the first ten minutes after KH addition are given as closed squares; those from subsequent periods are given as open squares. Results for untreated cells are given as open circles. (C) Results from all three donors are given as K^+ influx (calculated by linear regression from data for which typical results are given in (A)), against the median time of the influx period.

the same characteristic dynamics as those outlined above. Similar effects of donor dependence were noted as for Figure 5.2.

A possible (although unlikely) explanation for the observed dynamics of KH action at 37 °C was that the raised cation permeability caused a depolarisation of the membrane potential and hence a decrease in the electrical driving force for K^+ entry. Accordingly the dynamics of $^{86}Rb^+$ efflux in response to KH were also followed (Figure 5.4). HRBC were loaded with $^{86}Rb^+$, by overnight suspension (Hct = 80 %) in MBS (+ $^{86}Rb^+Cl$ (10 $\mu Ci(ml)^{-1}$)) at 8 °C, and then washed 6 times in ice-cold MBS using CP3, before transferral of packed cells to prewarmed (37 °C) MBS with sucrose (50 mM) and ouabain, bumetanide and EGTA (each at 0.1 mM). Typical results are given in Figure 5.4, in which the $\ln\{(C_{inf.}-C_t)/(C_{inf.}-C_o)\}$ index (y) is plotted against time (x) such that the rate constant of the reaction (k) is given by the negative of the gradient. Similar dynamics were observed by the efflux protocol, contraindicating an effect on membrane potential as underlying the dynamics of KH-induced K^+ influx.

Results in Figure 5.5 indicate that KH-induced K^+ influx in the presence of ouabain, bumetanide and nitrendipine, was linearly dependent on external K^+ concentration in the range 0 - 30 mM, the magnitude of the leak being dependent on the donor. However, the relation between K^+ influx and KH concentration was more complex (Figure 5.6). This dose-response relation was followed at a fixed extracellular K^+ concentration (15 mM), but at different haematocrits and under a variety of extracellular conditions (see Figure 5.6), which may partly account for the quantitative differences observed. These experiments were also performed using KH from different batches which, in addition to the donor dependence (Chapter 4 and Figures 5.2, 5.3 and 5.5) may also contribute to the large variance observed. Subsequent experiments, for which pooled data are presented, were performed using the same KH batch to achieve internally consistent results. Increasing KH concentration in the range 0 - 0.45 $HU(ml)^{-1}$ caused a progressive increase in inhibitor-insensitive K^+ influx (actual inhibitor preparations varied as given in the legend to this figure). However the dose-response relation was not monotonic; at lower toxin doses the gradient of this relation was less than that observed for higher doses.

Figure 5.7A depicts the magnitude of inhibitor-insensitive K^+ influx in response to variation of haematocrit (Hct as proportionate value). The same patterns were seen in all three experiments, although the results were not directly quantitatively reproducible.

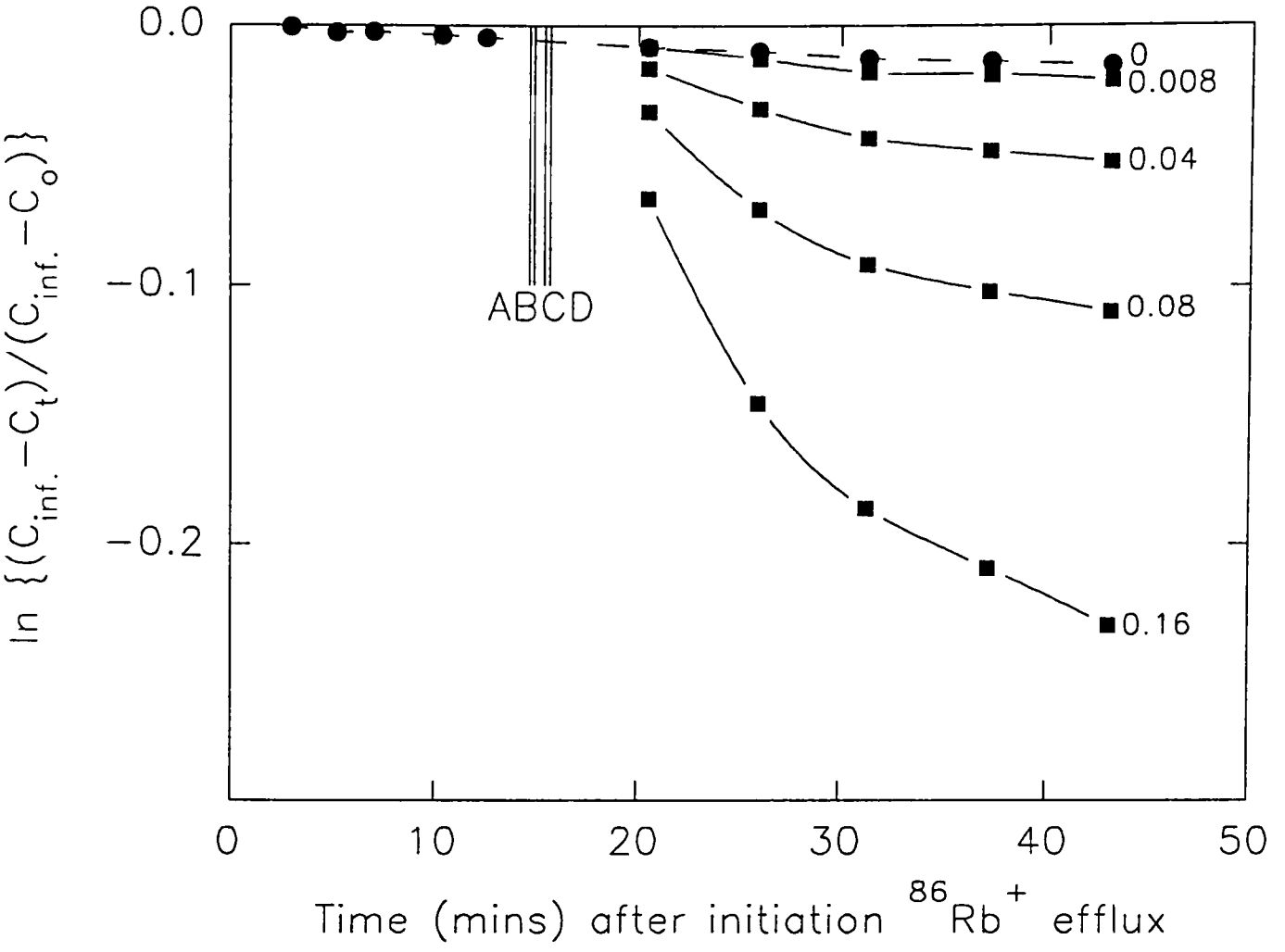


Figure 5.4 The dynamics of KH-induced $^{86}Rb^{+}$ efflux. HRBC were loaded with $^{86}Rb^{+}$ by overnight suspension (Hct = 80 %) in MBS with $^{86}Rb^{+}$ (10 μCi(ml)$^{-1}$) at 8 °C, before being washed (CP3) 6 times in ice-cold MBS and resuspended (Hct = 2 %) in pre-warmed MBS with sucrose (50 mM). Sample aliquots (1 ml) were centrifuged (CP3) and the supernatant (0.75 ml) removed for counting. After the first 5 time points the cell suspension was split five ways and added to KH at the indicated dose (HU(ml)$^{-1}$).

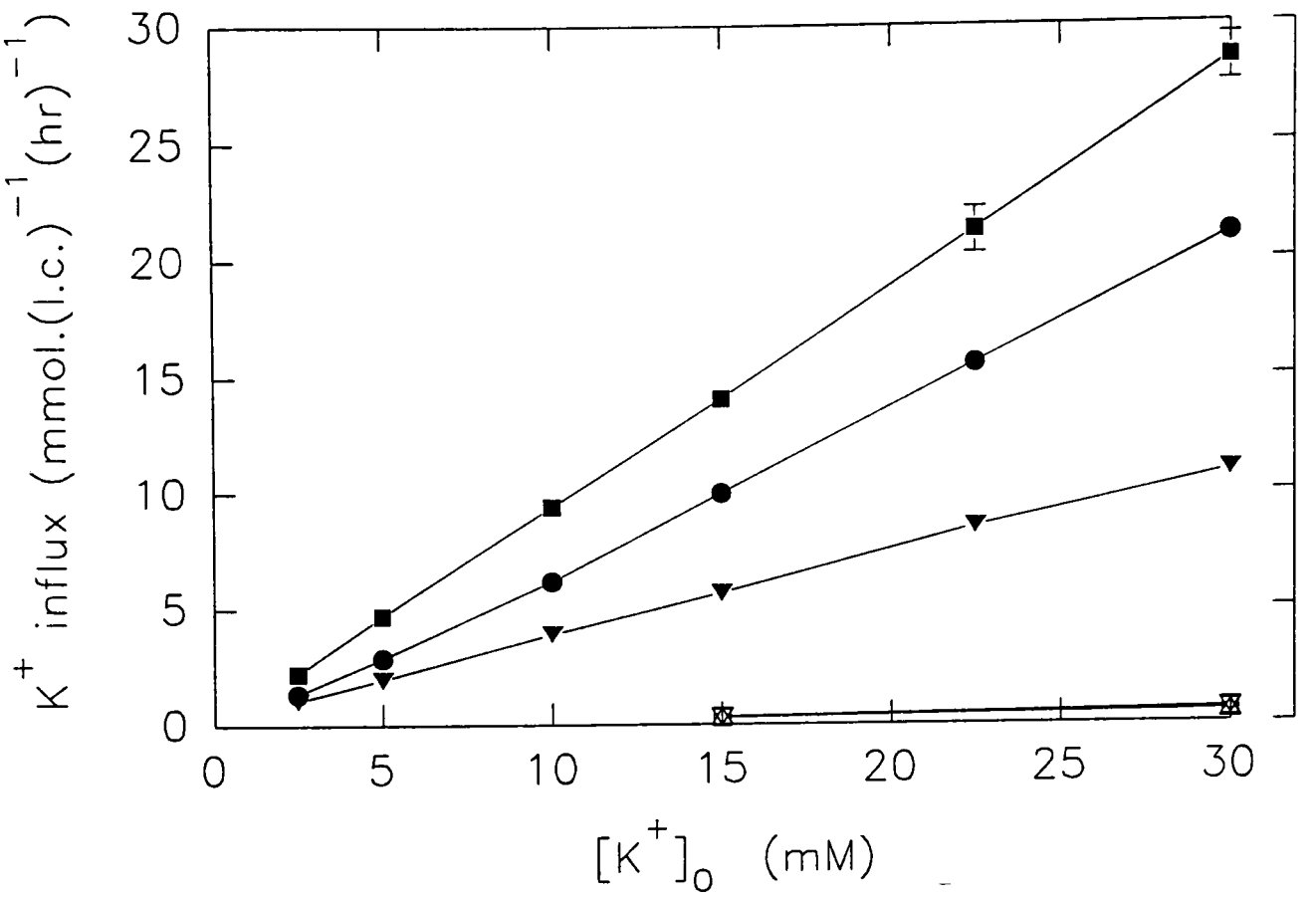


Figure 5.5 Dependence of magnitude of K^+ influx on extracellular K^+ concentration. HRBC suspended (4 % Hct) in MBS with sucrose (50 mM) and ouabain, bumetanide and nitrendipine (each at 0.1 mM) were vortexed either with or without KH (0.1 HU(ml)$^{-1}$). Aliquots (0.8 ml) of these cell suspensions were spiked with 200 µl solution comprised of (K(^{86}Rb)Cl (150 mM)/NaCl (150 mM)), giving a range of extracellular K^+ concentrations, as indicated. Filled and empty symbols represent data from KH-treated and untreated HRBC respectively. Each symbol type represents blood from a particular donor.

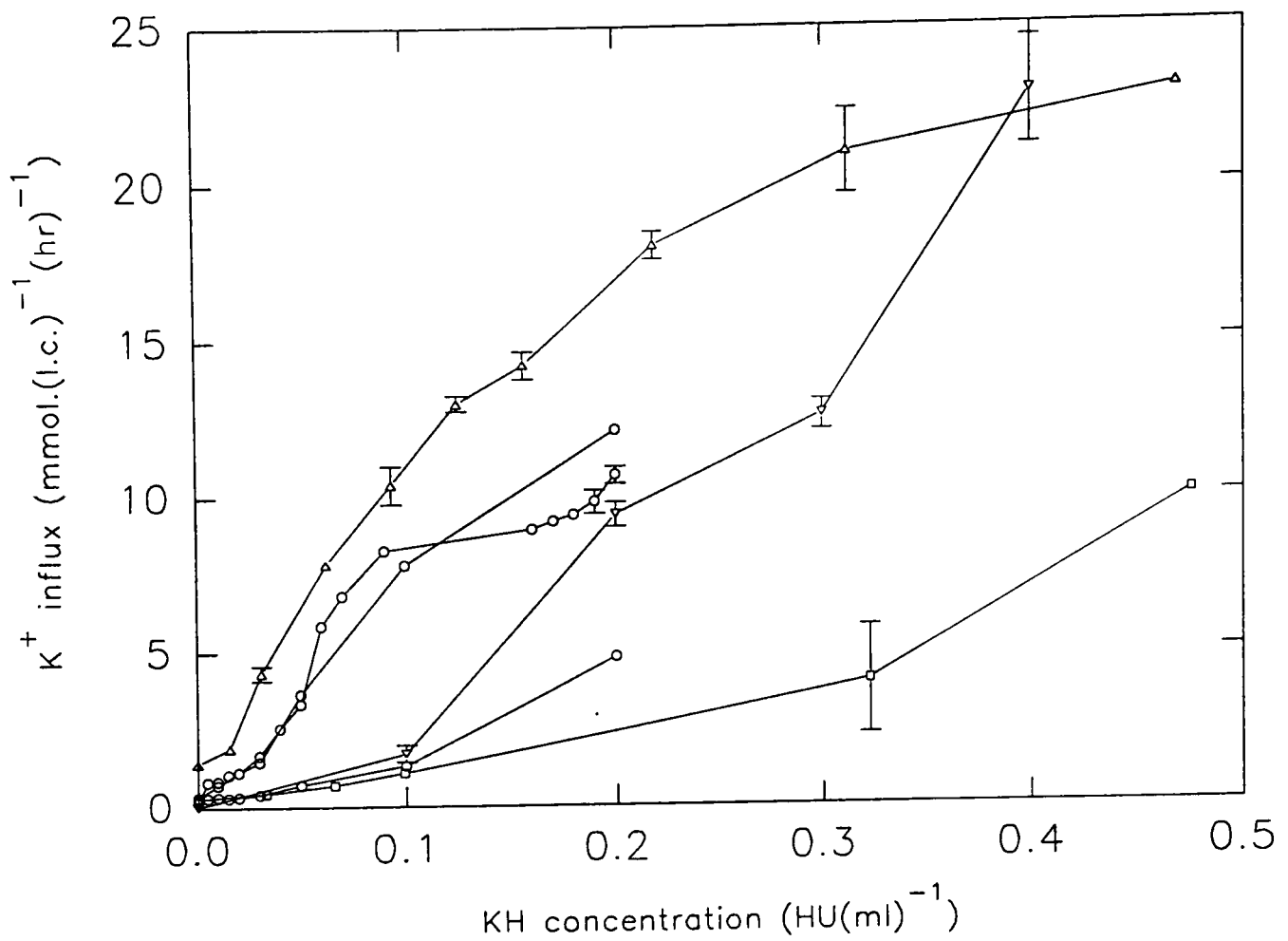


Figure 5.6 Dependence of magnitude of K^+ influx on KH concentration. Fluxes were initiated by the addition of 100 µl $K(^{86}Rb^+)Cl$ (150 mM) to 0.9 ml HRBC suspended ($3 \leq$ Hct (%) ≤ 7) in MBS with sucrose (50 mM), KH (as indicated), ouabain (100 µM) and bumetanide (100 µM) (i) alone (squares), (ii) + nitrendipine (10 µM) (inverted triangles), (iii) + quinine (300 µM) (triangles), and (iv) + nitrendipine (10 µM) and EGTA (100 µM) (circles). These results were obtained from different KH preparations, at various Hcts, and from different donors; any (or all) of these factors may play a role in the variable response of K^+ influx to KH.

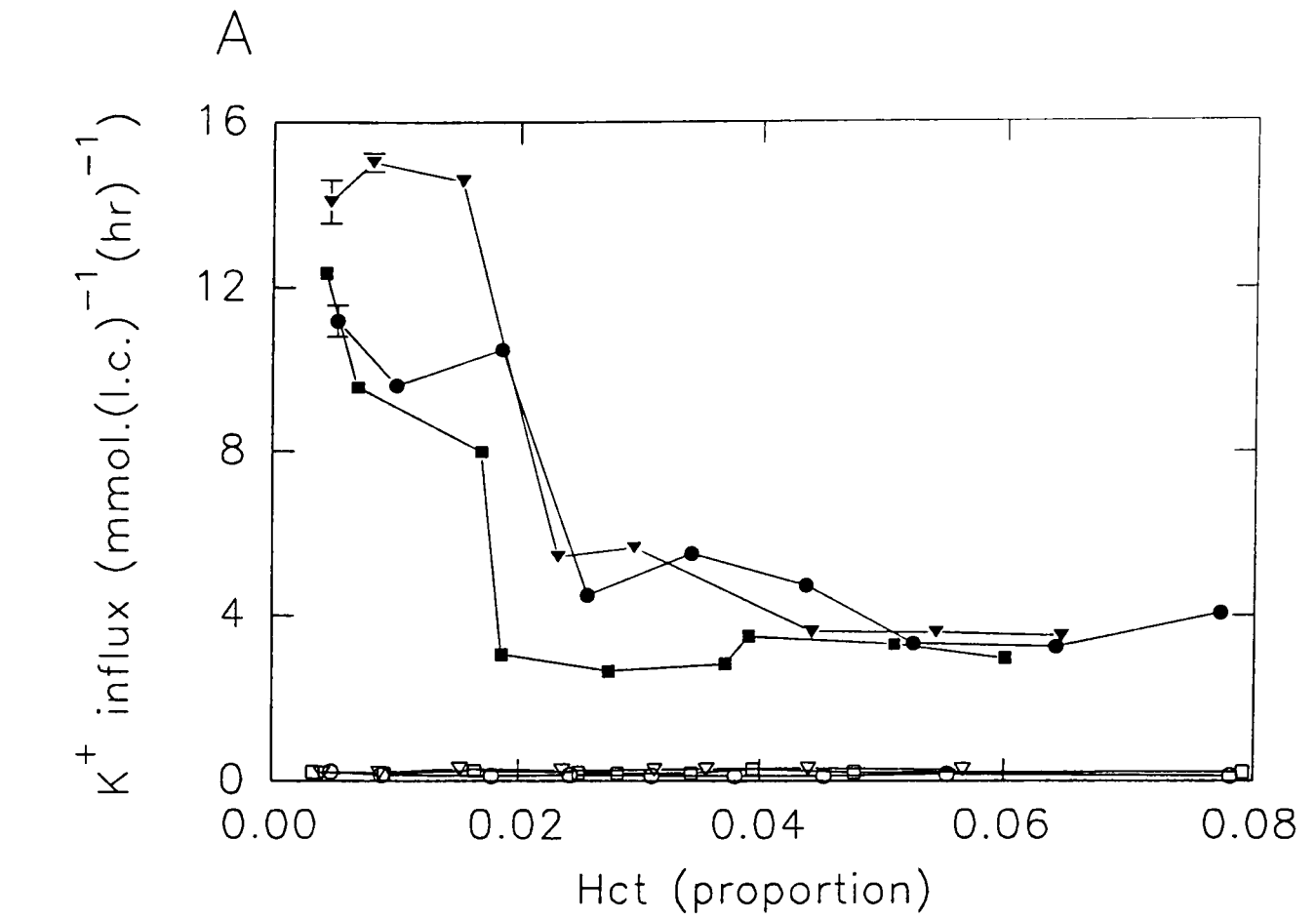
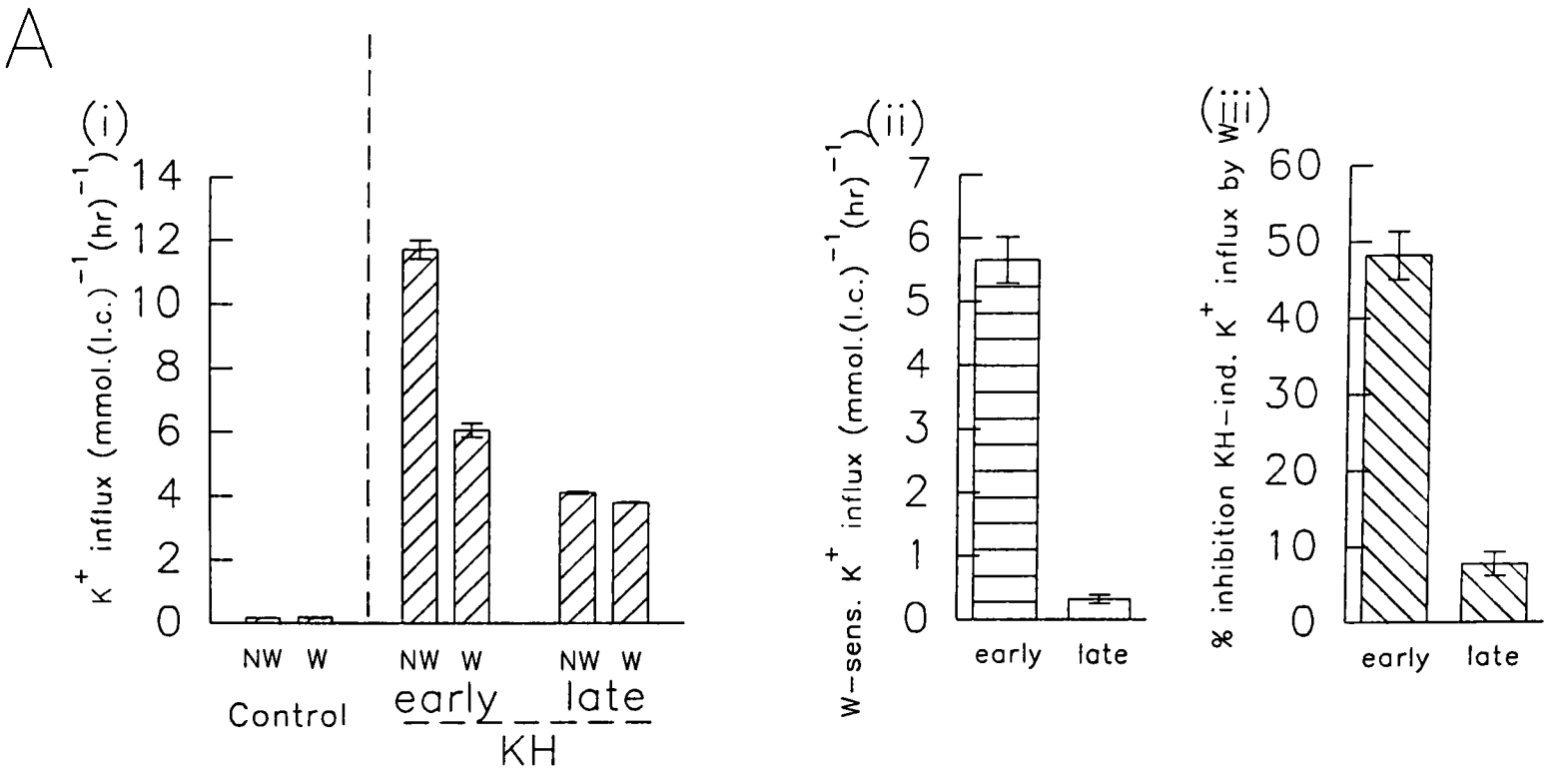


Figure 5.7 Multi-hit analysis of KH-induced K^+ influx. Fluxes were initiated by the addition of 100 µl K(^{86}Rb)Cl (150 mM) to 0.9 ml of HRBC suspension, in MBS with ouabain, bumetanide and nitrendipine (each at 0.1 mM) ± KH (0.06 HU(ml)$^{-1}$)) of varying Hct as indicated, such that in the flux tube $(K^+)_o$ = 15 mM, $(Na^+)_o$ = 135 mM. Each symbol type represents a different donor. KH was present (filled symbols) or absent (open symbols).

Increasing the haematocrit (ie the target cell concentration) beyond a certain level (c. 0.02, depending on donor) caused the induced flux to fall dramatically, before reaching a plateau phase (at a haematocrit greater than 0.035). It should be noted that the basal leak, in KH-treated cells, obtained at haematocrits > 0.035 did not change with increasing haematocrit but was greater than the basal leak (by approximately 20 x) in untreated control cells (the control data show that in untreated cells K^+ influx does not change with increasing haematocrit). Furthermore, as shown in Figure 5.7B, an index of total counts accumulated (K^+ transit) by the cells was observed to increase with increasing haematocrit over the lowest range (up to c. 0.02). In the middle haematocrit range (c. 0.02 to 0.03), K^+ transit actually decreased rather than flattening off at a higher value. This suggests that there was a protective effect of higher target cell concentration, K^+ transit values being lower than those which would have been predicted if KH acted only by a single-hit mechanism. In the higher haematocrit range, K^+ transit increased again; the slope of this relation was less marked than that observed in the initial phase but greater than that for the control cells untreated by KH (reflecting the increased basal leak caused by TDH-treatment).

In light of data (Figures 5.2 and 5.4) indicating a transition from higher to lower magnitude of KH-induced cation permeability, experiments were performed to investigate the relative susceptibilities of these pathways to washout by centrifugation (CP3), aspiration (of 0.8 ml supernatant) and resuspension (Figure 5.8). KH (0.1 $HU(ml)^{-1}$) was found to significantly (unpaired *t*-test; $P < 0.05$, $n = 3$, for both early and late phases) increase K^+ influx into ouabain, bumetanide and nitrendipine inhibited HRBC (Figure 5.8B(i)) from 0.22 ± 0.036 to 12.21 ± 2.456 (early period; ten minute flux, median time = 11.1 minutes after KH addition) and 4.22 ± 0.882 (late period; ten minute flux, median time = 39.25 minutes after KH addition) mmol. $K^+(l.c.)^{-1}(hr)^{-1}$. As noted previously, donor variability resulted in quite large S.E.M.s for absolute K^+ influxes (Figure 5.8B(i)), but the pattern of high and low magnitude phases for KH-induced K^+ influx was found (similarly to Figures 5.2 and 5.4). When results were normalised (Figures 5.8B(ii),(iii)), a remarkable degree of consistency was achieved. The late phase of KH-induced K^+ influx was significantly (unpaired *t*-test of normalised data; $P < 0.001$, $n = 3$) less than the early phase, being reduced to 34.4 ± 0.49 % of the early phase value (Figure 5.8B(ii)). Furthermore, the % inhibition of K^+ influx by washout was also significantly (unpaired



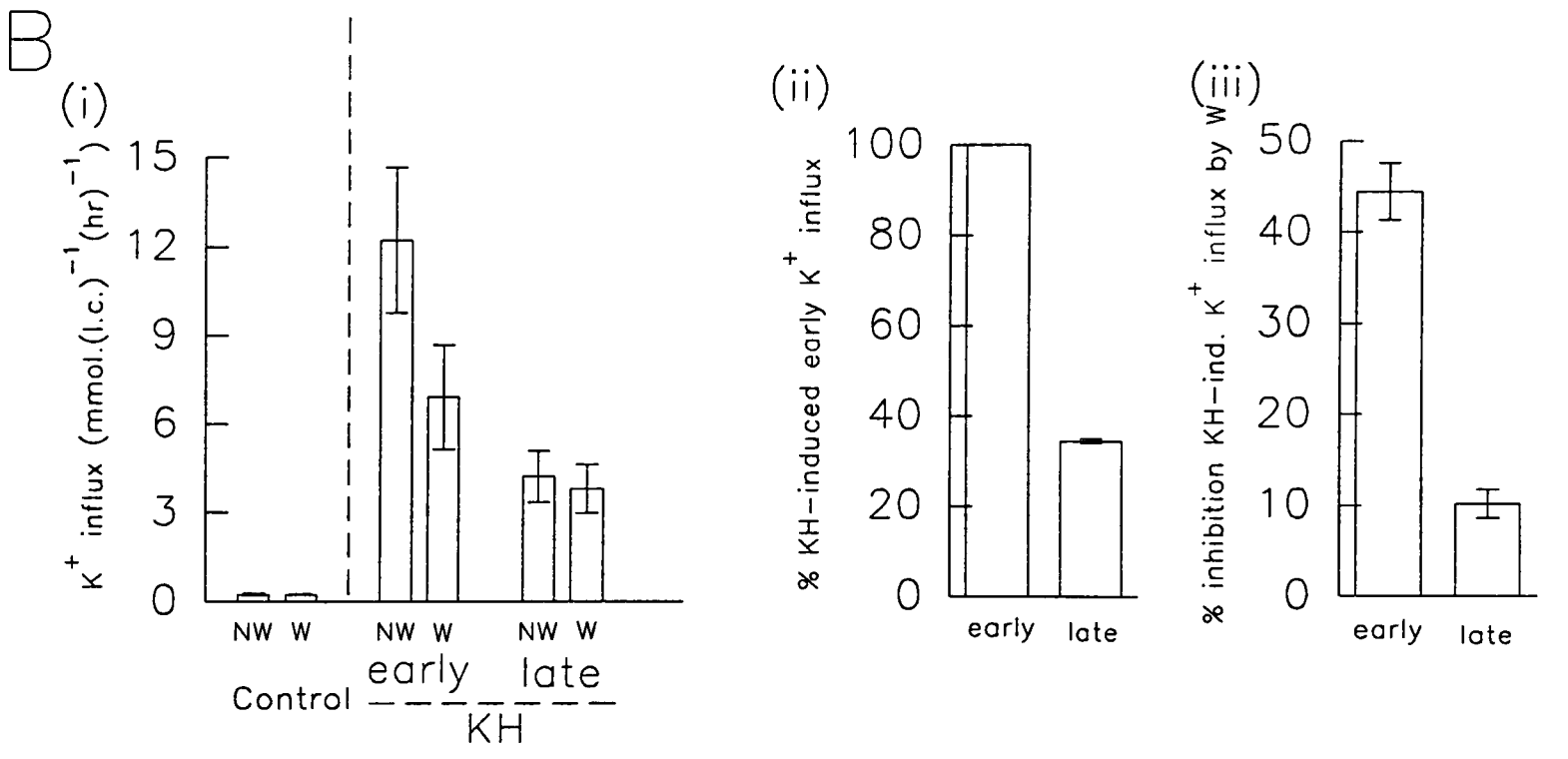


Figure 5.8 Sensitivities of KH-induced K^+ influx to washout. HRBC were suspended (Hct = 5 %) in MBS with sucrose (50 mM), ouabain, bumetanide and nitrendipine (each at 0.1 mM) and prewarmed to 37 °C, before addition (if appropriate) of KH (0.1 HU(ml)$^{-1}$) at time = zero. Aliquots (0.9 ml) were centrifuged (CP3) at 2 minutes (early) and 30 minutes (late) after KH addition; one preparation (W) had 0.8 ml supernatant removed and replaced with KH-free suspending solution (as above) whereas the NW preparation was not subjected to such treatment. The HRBC of these preparations were vortexed back into suspension and incubated at 37 °C. K^+ influx was measured over a ten minute flux period for which the median time was 11.10 and 39.25 minutes for the early and late preparations, respectively. Fluxes were initiated by the addition of 100 µl K(^{86}Rb)Cl (150 mM) ($(K^+)_o$ = 15 mM). W and NW = washed and not washed, respectively. (A) Representative results from one of three such experiments: (i) K^+ influx for untreated and KH-treated HRBC, (ii) K^+ influx inhibited by washout, and (iii) percentage inhibition of K^+ influx by washout. (B) Pooled (n = 3) results: (i) K^+ influx for untreated and KH-treated HRBC, (ii) relative magnitudes of (NW) early and late phase fluxes, and (iii) percentage inhibition of K^+ influx by washout.

t-test of normalised data; $P < 0.005$, $n = 3$) different for early and late phases of KH-induced K^+ influx, being 44.4 ± 3.09 and 10.2 ± 1.55 %, respectively (Figure 5.8B(iii)).

In light of data (Figures 5.2 and 5.4) indicating a non-constant (high to low transition) induced cation permeability, the effect of centrifugation and resuspension on haemolytic time courses was measured. Figure 5.9 shows data describing the lack of effect of a centrifugation (CP3) and resuspension cycle every ten minutes during the time course; in the presence of KH (1 $HU(ml)^{-1}$), H^{50} values were (mean ± S.E.M.) 110.7 ± 26.03 (control) and 111.8 ± 29.84 (successive CP3 + resuspension) minutes, suggesting that once bound TDH was not released by CP3.

It is clear from Figure 5.10 that replacement of Cl^- by CH_3SO_4 had no effect on KH-induced K^+ influx. KH (0.05 $HU(ml)^{-1}$) caused a significant (unpaired *t*-test; $P < 0.001$, $n = 3$) increase in K^+ influx from 2.69 ± 0.226 to 16.31 ± 0.240 $mmol.(l.c.)^{-1}(hr)^{-1}$ (in Cl^-) and 2.11 ± 0.403 to 15.96 ± 0.358 $mmol.(l.c.)^{-1}(hr)^{-1}$ (in $CH_3SO_4^-$). This result eliminates a possible role for Cl^--dependent transport processes in the response to KH (0.05 $HU(ml)^{-1}$), when measured in MBS with sucrose (36 mM; to prevent volume increases due to colloid osmosis) and EGTA (0.1 mM; to prevent Ca^{2+}-stimulated K^+ influx).

Figure 5.11 describes the lack of effect of KH on K^+ influx in hagfish (*Eptatretus stouti*) erythrocytes inhibited by ouabain and bumetanide (0.1 mM), using human red cells as a positive control. Hagfish erythrocytes were used because they have very low numbers of the band 3 anion transporter (Ellory & Wolowyk, 1991); aggregation of this protein has been proposed to underly generation of cation leaks by other leak-inducing agents (Clague & Cherry, 1989). No support for such an event in the generation of the KH-induced cation leak, was obtained for KH (0.17 $HU(ml)^{-1}$; a different batch to that employed for experiments given in Figures 5.1 - 5.8); there was no stimulation of K^+ influx into hagfish cells, even though the flux into human cells (positive control) was significantly (unpaired *t*-test; $P < 0.01$, $n = 3$) increased from 0.23 ± 0.009 to 12.00 ± 0.830 $mmol.(l.c.)^{-1}(hr)^{-1}$.

5.2.2 Effects of non-enzymic membrane-active agents on KH-induced K^+ influx

Figure 5.12 examines the effects of divalent cations on KH-induced K^+ influx in the presence of ouabain, bumetanide and nitrendipine. Addition of extracellular Mg^{2+} and Ca^{2+} (1 mM) caused a small decrease in residual flux in cells untreated by KH but had no significant effect on KH-induced flux. However, Zn^{2+} (250 μM) and Cd^{2+} (1 mM) caused a small increase in residual flux in untreated cells and a significant decrease in

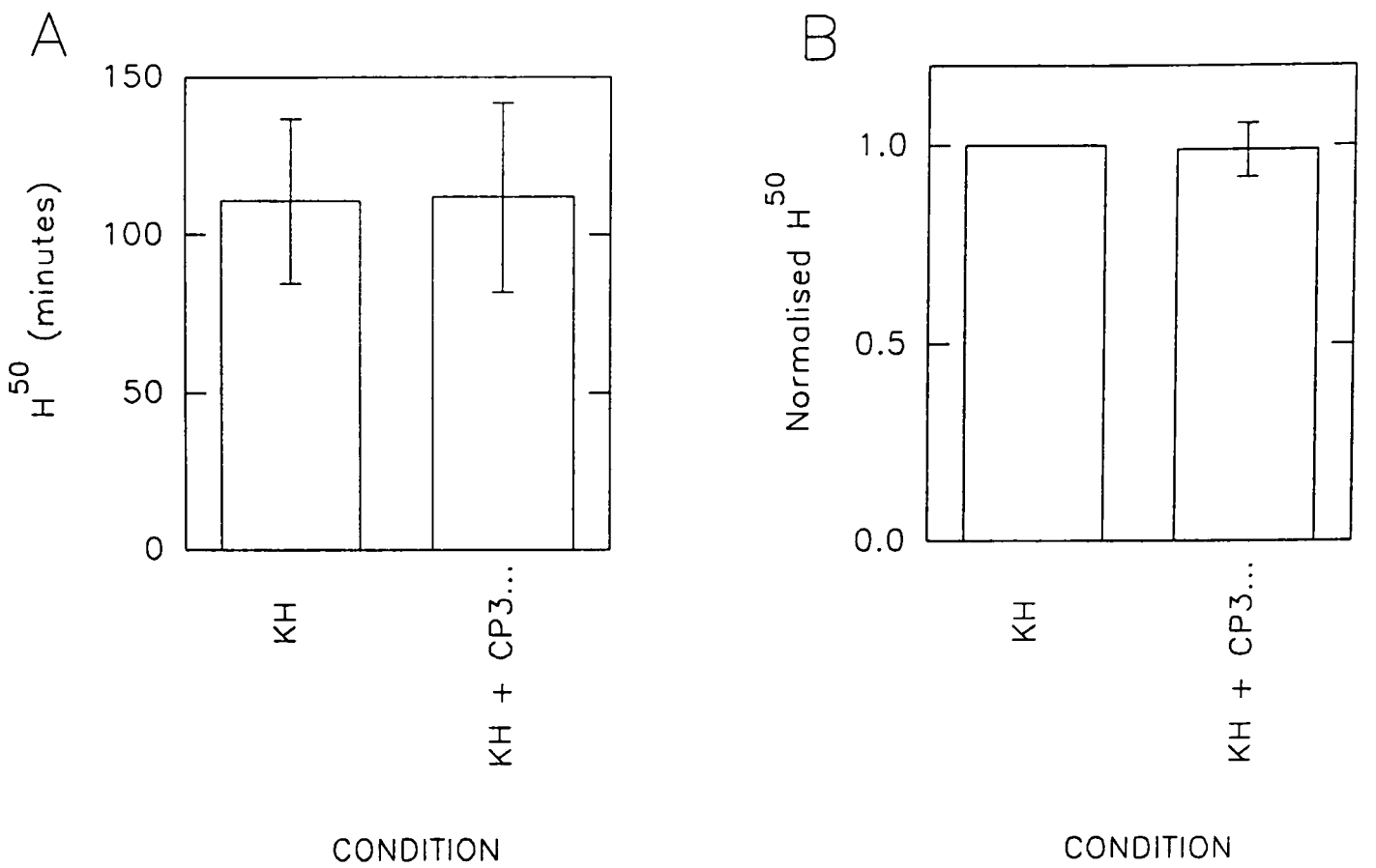


Figure 5.9 Lack of effect of successive CP3 + resuspension on KH-induced haemolysis. HRBC were suspended (Hct = 2 %; t = 0) in MBS with KH (1 $HU(ml)^{-1}$) and incubated in aliquots (Eppendorf; 0.7 ml) at 37 °C. One set of aliquots were centrifuged down (CP3) and resuspended every ten minutes. Time courses describing haemolysis (as % total) were measured, and H^{50} values calculated. Control haemolysis in MBS untreated by KH was < 2 % over the same time-course..

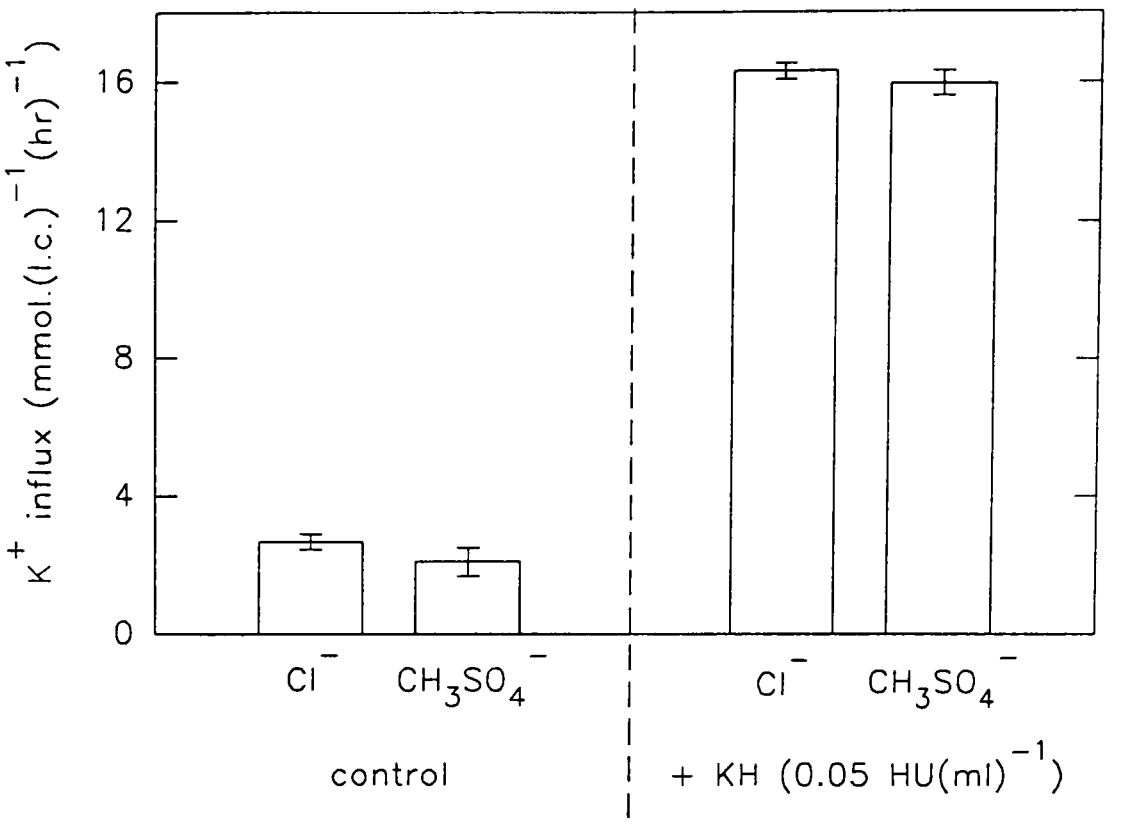


Figure 5.10 Lack of effect of anion substitution on KH-induced K^+ influx. HRBC were washed three times in either a chloride-based solution (MBS + sucrose (40 mM) + EGTA (0.1 mM)) or a chloride-substituted solution ($NaCH_3SO_4$ (145 mM) + MOPS (10 mM) + glucose (5 mM) + sucrose (40 mM) + EGTA (0.1 mM)) and left to stand at 37 °C for 5 minutes, before 2 final washes in the same solutions. $K(^{86}Rb)Cl$ or $K(^{86}Rb)CH_3SO_4$ (100 µl; 150 mM) was then added to cell suspensions as appropriate and in the presence and absence of KH (0.05 HU(ml)$^{-1}$), to instigate a ten minute flux period, in which $(K^+)_o$ = 15 mM, $(Na^+)_o$ = 135 mM and Hct = 2.5 %.

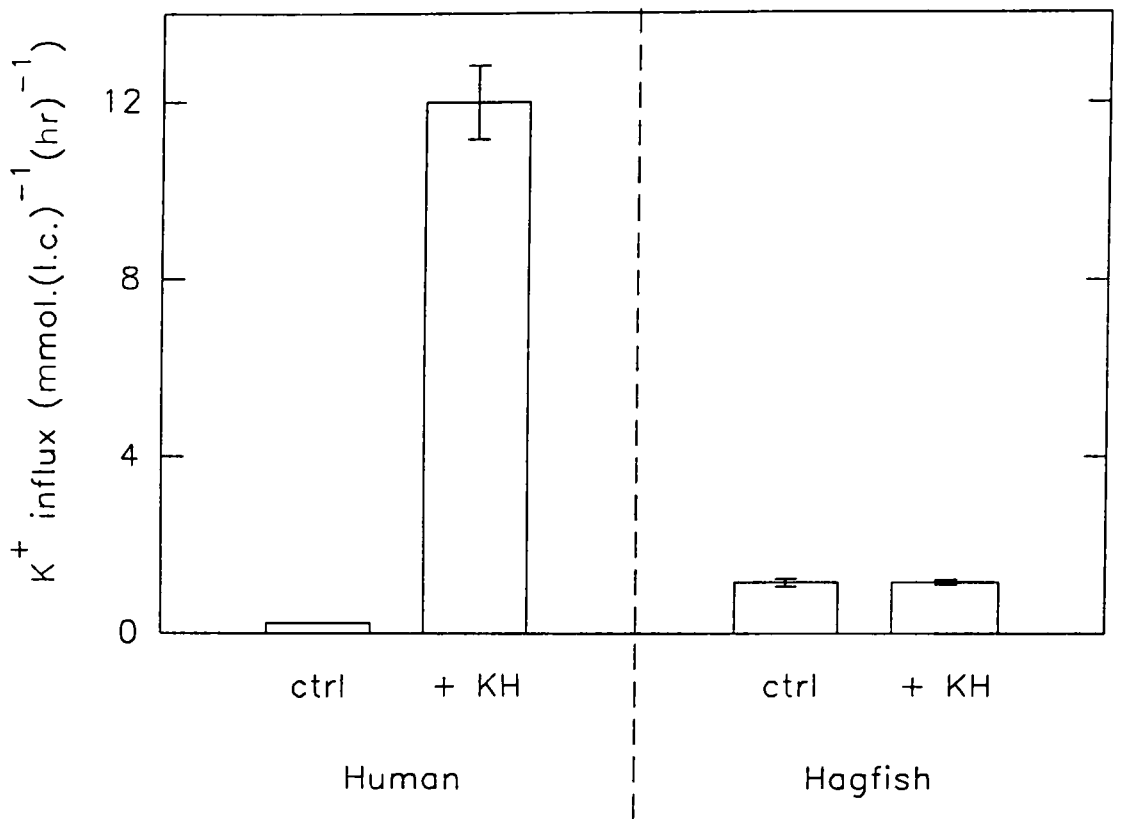


Figure 5.11 Lack of effect of KH on hagfish erythrocytes. Human and hagfish erythrocytes were suspended (Hct = 5 % and 3 %, respectively) in MBS and hagfish saline (NaCl (510 mM), MOPS (10 mM) and glucose (5 mM)), respectively, in the presence of ouabain and bumetanide (each at 0.1 mM). Aliquots (0.9 ml) of cell suspensions were subsequently incubated at 37 °C (human) and 22 °C (hagfish) and spiked with (^{86}Rb)KCl (100 µl; 150 mM (human) and 300 mM (hagfish)), such that $(K^+)_o$ = 15 mM (human) and 30 mM (hagfish). Flux periods measured were 15 minutes (human) and 60 minutes (hagfish).

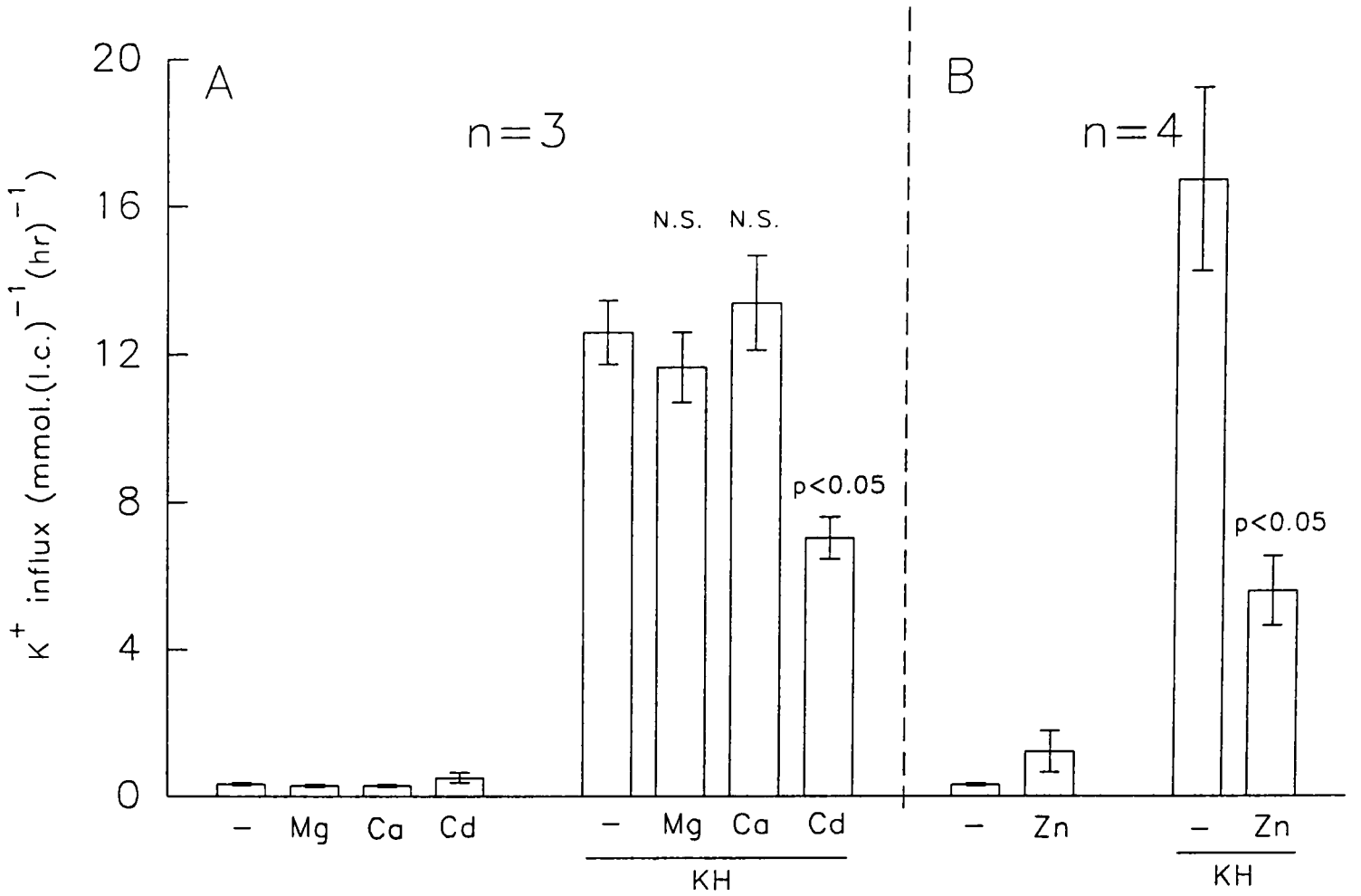


Figure 5.12 Effects of divalent cations on KH-induced K^+ influx. HRBC were suspended in MBS (Hct = 5 %) with sucrose (50 mM) and ouabain (100 μM), bumetanide (100 μM) and nitrendipine (10 μM). If appropriate, KH (0.1 $HU(ml)^{-1}$) was added and cells incubated at 37 °C for 5 minutes, before addition of divalent cation (10 μl; final concentration = 1 mM for Mg^{2+}, Ca^{2+} and Cd^{2+}, and 250 μM for Zn^{2+}). Suspensions (0.9 ml) were vortexed before addition of 100 μl K(^{86}Rb)Cl (150 mM) such that $(K^+)_o$ = 15 mM and $(Na^+)_o$ = 135 mM. Values are mean ± S.E.M. for *n* experiments.

KH-induced flux, a protective effect similar to that noted for other cytolytic agents (Bashford *et al.*, 1988a,b). Concentrations of Zn^{2+} in excess of 250 μM were noted to cause cell clumping.

Figure 5.13 shows data concerning the effects of phloretin (250 μM) and DIDS (20 μM) on KH-induced K^+ influx. HRBC (for DIDS experiments, cells were preincubated with DIDS for 1 hour) treated with ouabain, bumetanide and nitrendipine were exposed to KH (final concentration = 0.08 $HU(ml)^{-1}$) and incubated at 37 °C for five minutes prior to addition of (^{86}Rb)KCl. For experiments examining the effects of phloretin, phloretin (250 μM) was added as appropriate to the cell suspension which was then incubated for a further 5 minutes prior to spiking. Phloretin also caused an increase in residual leak of cells untreated by KH, consistent with a lowering of the endogenous dipole potential (Deuticke *et al.*, 1991). Neither phloretin nor DIDS had any significant effect on KH-induced K^+ flux.

Figure 5.14 shows the effect of vortexing a KH preparation (Sigma - a different batch from that used for the previous two experiments) vigorously with DBP for 1 minute before centrifugation by CP3 (as for Figure 4.13B). KH (0.075 $HU(ml)^{-1}$) caused an increase in K^+ influx into ouabain, bumetanide and nitrendpine treated HRBC from 0.26 ± 0.014 to 1.64 ± 0.039 $mmol.(l.c.)^{-1}(hr)^{-1}$ (Figure 5.14A). This increase was significantly (unpaired *t*-test of normalised data; $P < 0.005$, $n = 3$) less for KH pretreated by DBP, being reduced to 36.6 ± 2.60 % of the maximally stimulated condition (Figure 5.13B).

5.2.3 Effects of neuraminidase treatment of cells on subsequent KH action

As discussed in section 5.1, previous evidence suggests that TDH action is mediated by neuraminidase-sensitive gangliosides in the cell membrane (Takeda *et al.*, 1975, 1976). Neuraminidase treatment (Stein *et al.*, 1987; section 3.6.4) of HRBC was therefore examined as a prospectively protective treatment against KH action. Stein *et al.* (1987) used either a 1 or 3 hour neuraminidase incubation. The longer incubation period was adjudged too harsh for these experiments because haemolysis was substantial after the first wash (data not shown). However, after 1 hour incubation no haemolysis was visible.

In point of fact, neuraminidase pretreatment *increased* the rate of KH-induced haemolysis. The half-time of haemolysis (H^{50}) was decreased from 66.9 ± 12.46 to 43.4 ± 8.22 minutes (Figure 5.15B), these high S.E.M.s being attributable to the donor dependency of KH-induced haemolysis; normalised H^{50} (measured in MBS with KH (1

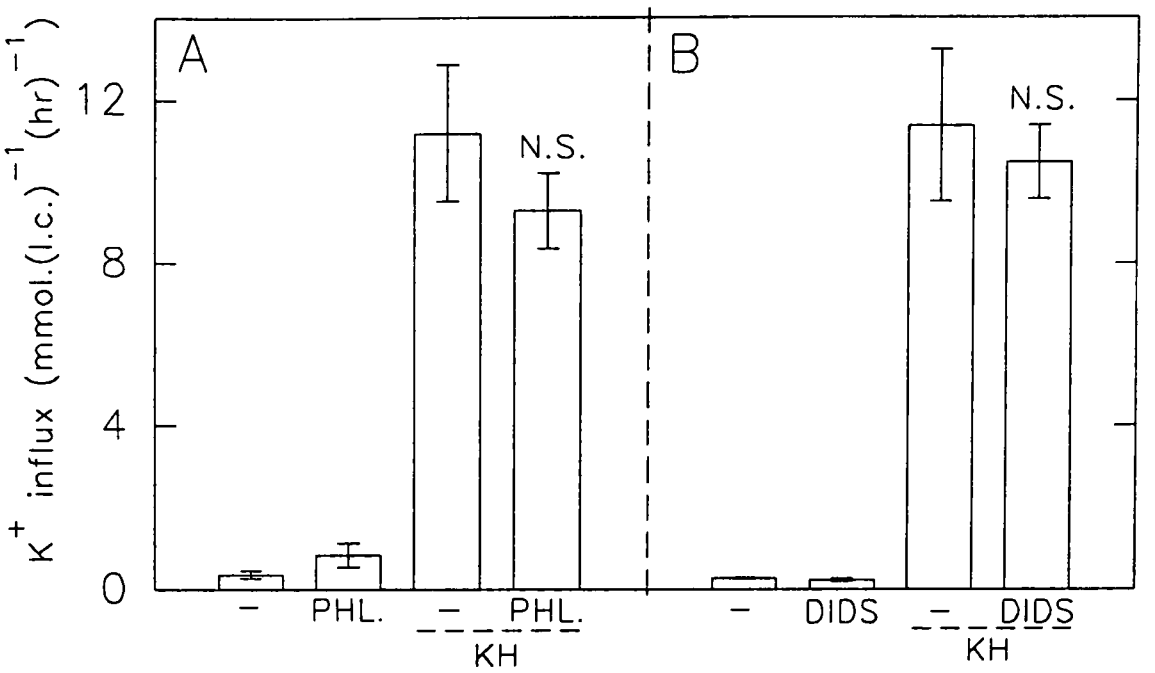


Figure 5.13 Lack of effects of phloretin and DIDS on the KH-induced K^+ influx. HRBC were suspended in MBS (Hct = 5 %) (for (B) cells (Hct = 5 %) were preincubated with DIDS (20 μM) at 37 °C, for 1 hour) with ouabain (100 μM), bumetanide (100 μM) and nitrendipine (10 μM). If appropriate KH (0.08 $HU(ml)^{-1}$) was added and cells incubated at 37 °C for 5 minutes. Cells of (A) were incubated with phloretin (250 μM) for an additional 5 minutes. Fluxes were then initiated by addition of 100 μl $K(^{86}Rb)Cl$ (150 mM) to cell suspension (0.9 ml) such that $(K^+)_o$ = 15 mM and $(Na^+)_o$ = 135 mM. $(K^+)_o$ = 15 mM, $(Na^+)_o$ = 135 mM,

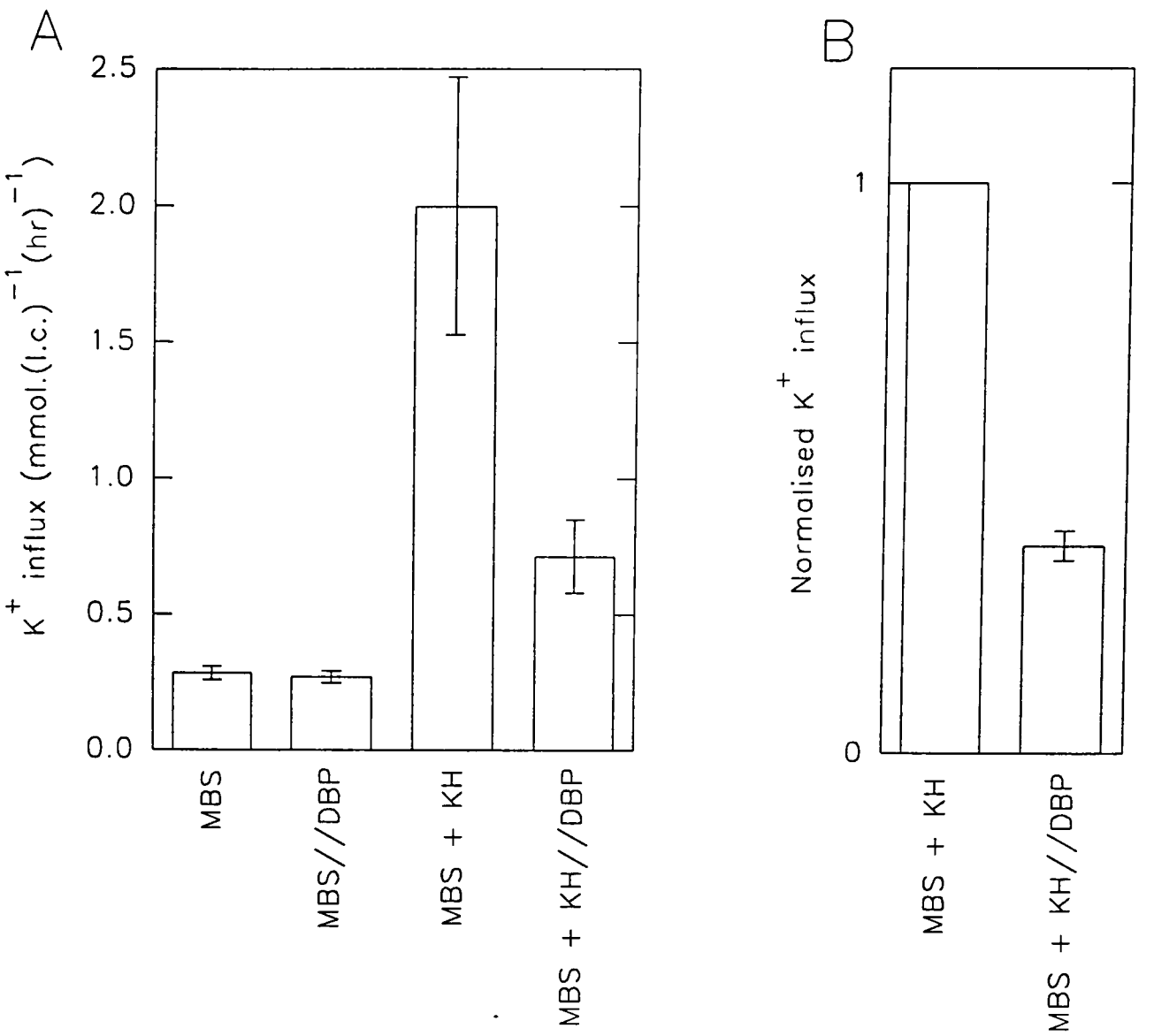


Figure 5.14 Effect of DBP on KH-induced K^+ influx. HRBC were suspended (Hct = 5 %) in MBS with sucrose (50 mM), and ouabain, bumetanide, and nitrendipine (each at 100 µM). Aliquots (0.89 ml) of this cell suspension were added to 100 µl K(^{86}Rb)Cl (150 mM) + 10 µl MBS with sucrose (50 mM) with and without KH (0.075 HU(ml)$^{-1}$) (prevortexed (or not) with DBP as for Figure 4.13B), to initiate ten minute flux periods. (A) Pooled results for three donors. (B) Pooled normalised data for three donors.

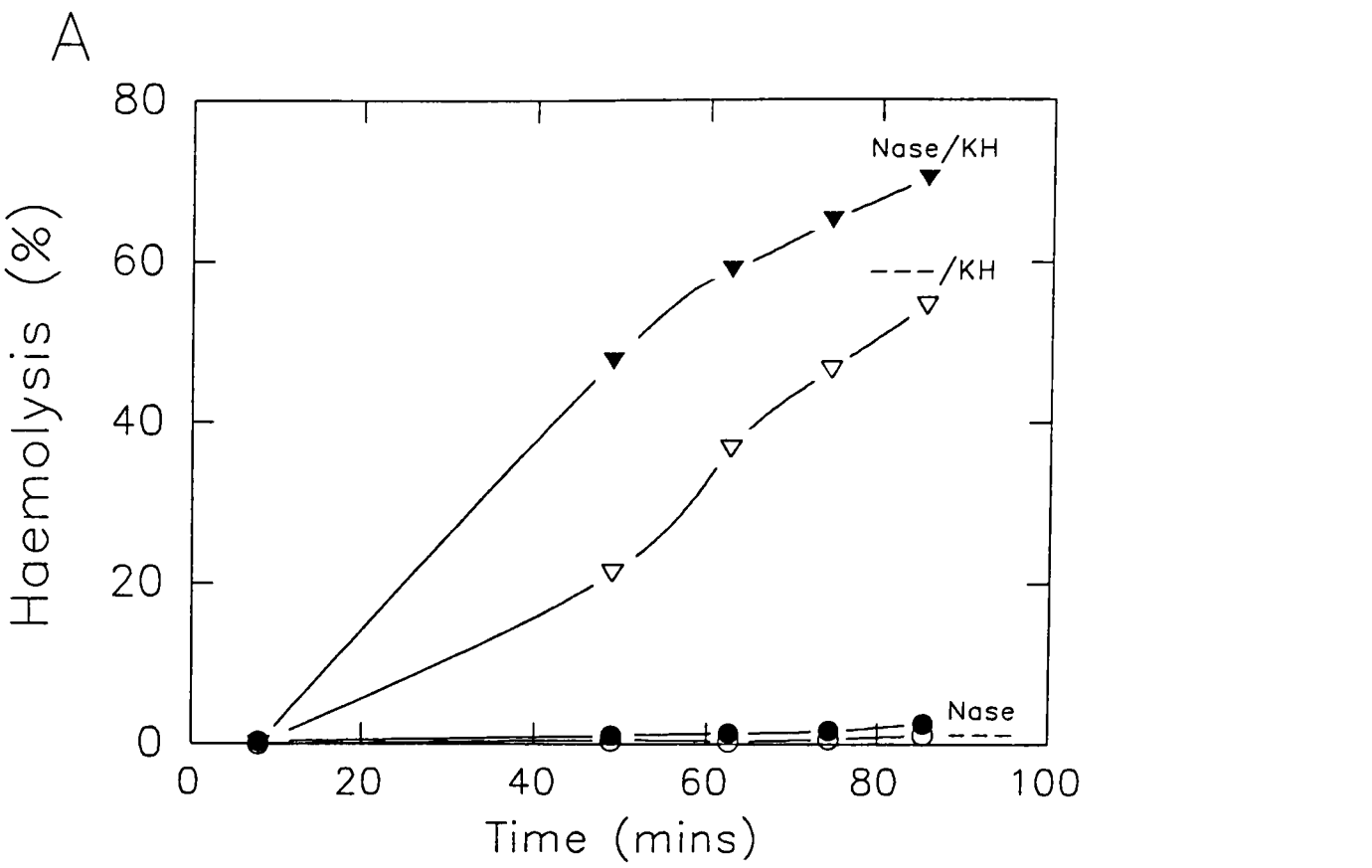


Figure 5.15 Effect of neuraminidase treatment on rate KH-induced haemolysis. Human erythrocytes were treated according to the protocol of Stein *et al.* (1987) (section 3.7.4). Haemolysis time-courses (at 37 °C) were followed for HRBC suspensions (initial Hct = 2 %) in MBS, in the presence or absence of KH (1 $HU(ml)^{-1}$). (A) Results from a single experiment representative of three such experiments. Triangles and circles represent haemolysis in the presence and absence of KH, respectively. Filled and empty symbols represent cells preincubated in the presence and absence of neuraminidase, respectively (B) Pooled H^{50} results from three experiments. (C) Pooled normalised H^{50} results from three experiments.

$HU(ml)^{-1}$) and Hct = 2 %) was significantly (unpaired *t*-test of normalised data; $P < 0.001$, $n = 3$) decreased to (mean ± S.E.M.) 64.8 ± 0.37 % of control values (Figure 5.15C).

Figure 5.16 shows experiments performed to investigate the effect of neuraminidase treatment on the osmotic fragility of HRBC. Saline solutions of different osmolality were constructed by combining a range of relative volumes of MBS (pH = 7.4) to a MOPS (10 mM; pH = 7.4) solution. The osmolalities of the resultant solutions were measured using a Wescor 5100C vapour pressure osmometer. The graphs 5.16A (untreated HRBC), B (preincubation: 1 hour at 30 % Hct and 37 °C, in MBS with BSA (0.5 % w/v) + $CaCl_2$ (8 mM)), and C (preincubation: 1 hour at 30 % Hct and 37 °C, in MBS with BSA (0.5 % w/v) + $CaCl_2$ (8 mM) + neuraminidase (0.42 $U(ml)^{-1}$)) show time courses of haemolysis for HRBC suspended in solutions of the given osmolalities. As expected in light of previous reports (Ponder, 1948; Sidel & Solomon, 1957; Rand, 1964; Jay & Rowlands, 1975; Anderson & Lovrien, 1977; Maeda *et al.*, 1977; Araki & Rifkind, 1981; Gibson *et al.*, 1993), the volume change of erythrocytes in response to such osmotic perturbation was rapid, and haemolysis (as % total) was essentially complete by the first time-point. In subsequent experiments (Figures 5.16D,E), haemolysis was measured ten minutes after addition of cells to saline solutions. Figure 5.16E shows pooled results for experiments comparing the osmotic fragilities of untreated and neuraminidase-treated cells, of which a representative set is given in Figure 5.16D. The osmolality just capable of causing 50 % haemolysis of HRBC suspended at 2 % Hct was 132.7 ± 1.30 and 132.3 ± 1.59 $mOsmoles(kg)^{-1}$, for cells preincubated as described in section 3.6.4, with and without neuraminidase, respectively. Thus neuraminidase treatment by this protocol did not significantly affect the osmotic fragility of the cells.

In a parallel protocol (Figure 5.17), selective inhibitors (ouabain, bumetanide and nitrendipine) were used to block endogenous K^+ transporters, allowing examination of the basal (ie inhibitor insensitive) leak, with properties determined solely by electrodiffusion. While neuraminidase pretreatment did not itself increase K^+ ($^{86}Rb^+$) influx into erythrocytes, KH-induced K^+ influx (in MBS with sucrose (45 mM), with [KH] = 0.05 $HU(ml)^{-1}$, $2.3 < Hct\ (\%) < 3.3$, and $[K^+]_o$ = 15 mM) was increased significantly by neuraminidase action ($P < 0.05$, $n = 3$) from (mean ± S.E.M.) 2.69 ± 0.225 (control; incubated similarly with bovine serum albumin + Ca^{2+} but no neuraminidase) to 4.86 ± 0.572 $mmol.(l.c.)^{-1}hr^{-1}$). This implies that the increased cation leak underlies the

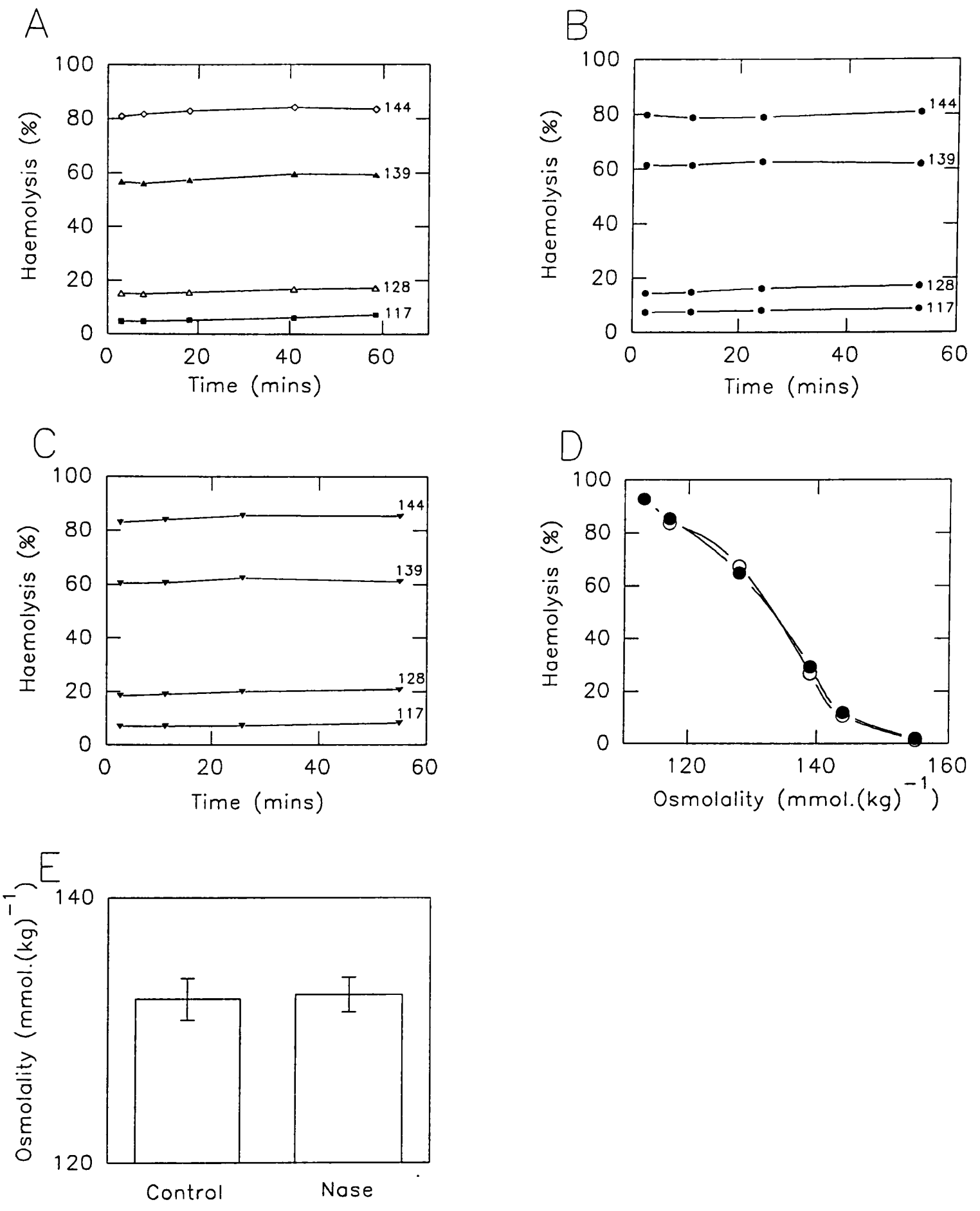


Figure 5.16 Lack of significant effect of neuraminidase treatment on osmotic fragility of human erythrocytes. Human erythrocytes were suspended (Hct = 2 %) in MOPS-buffered salines ((MOPS) = 10 mM, pH = 7.4) in which the (NaCl) (and osmolality) varied. For (A) untreated HRBC, (B) HRBC incubated with BSA + Ca^{2+} (see text), and (C) HRBC incubated with BSA + Ca^{2+} + neuraminidase (see text), haemolysis time courses are given for exposure to saline solutions of the indicated osmolality. In subsequent experiments (D, E) haemolysis was measured after ten minutes suspension. (D) Representative results for one of three such experiments. Closed and open circles represent HRBC treated/untreated by neuraminidase, respectively. (E) Pooled data indicating the osmolality that just causes 50 % haemolysis, for three such experiments.

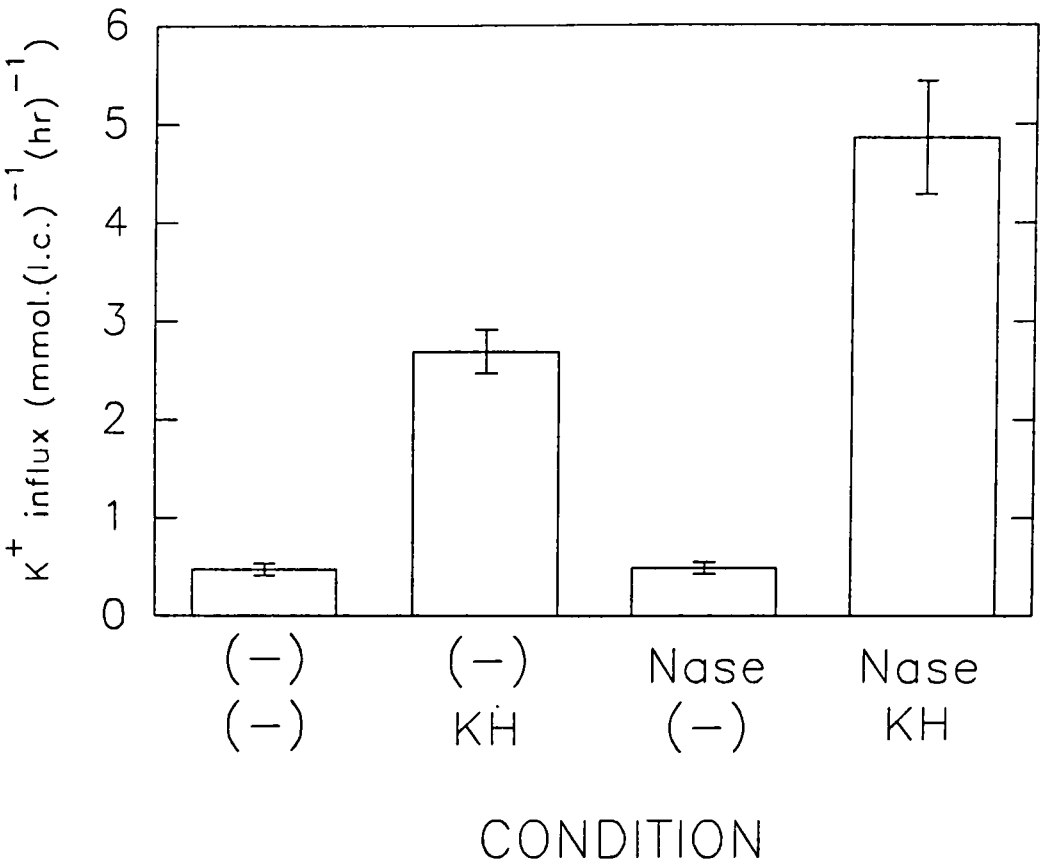


Figure 5.17 Effect of neuraminidase treatment on KH-induced K^+ influx. K^+ influx was measured in HRBC inhibited by ouabain, bumetanide and nitrendipine (each at 0.1 mM) and suspended ($2.3 \leq$ Hct (%) ≤ 3.3) in MBS with sucrose (45 mM). Erythrocytes were pretreated by 1 hour incubation with BSA and Ca^{2+} (see text) with/without neuraminidase. Fluxes were initiated, in the presence/absence of KH (0.05 HU(ml)$^{-1}$), by the addition of 100 µl K(^{86}Rb)Cl (150 mM) to HRBC suspensions ($(K^+)_o$ = 15 mM).

stimulation of KH-induced haemolysis by neuraminidase.

The monovalent cation selectivity of the KH (1 $HU(ml)^{-1}$) -induced lesion in cells either treated or not treated by neuraminidase was examined using the technique of isosmotic haemolysis (as for Figure 4.17). Although haemolytic rate was dramatically increased by neuraminidase pretreatment (Figure 5.18A), the selectivity series was the same for both preparations: $Cs^+ > Li^+ > K^+ > Rb^+ > Na^+$, an Eisenman VIII sequence (Eisenman & Horn, 1983) with a small K^+ anomaly (Figure 5.18B). This indicates that the *nature* of the lesion is itself unlikely to be affected by neuraminidase treatment.

5.2.4 Effects of KH on the activity of endogenous HRBC membrane transporters

KH has been noted to stimulate nitrendipine-sensitive K^+ influx even in nominally Ca^{2+} free MBS (Figure 5.1). The most likely explanation for this effect is an increase in $[Ca^{2+}]_i$ secondary to raised influx. This hypothesis was tested by raising extracellular Ca^{2+}, in the presence of KH (0.15 HU ml^{-1}) (Figure 5.19). This caused significant increases (unpaired *t*-tests; $P < 0.05$ for $[Ca^{2+}]_o$ = 0.34 and 0.67 mM; $P < 0.005$ for $[Ca^{2+}]$ = 1.0 mM; $n = 3$ for all conditions) in nitrendipine-sensitive K^+ influx from 1.35 ± 0.413 (nominally Ca^{2+} free) to 3.49 ± 0.355 ($[Ca^{2+}]_o$ = 0.34 mM), 5.25 ± 0.279 ($[Ca^{2+}]_o$ = 0.67 mM) and 6.03 ± 0.375 ($[Ca^{2+}]_o$ = 1 mM). Moreover, increased flux through this pathway appeared to account for the increase in total flux obtained with increased Ca^{2+}. Changes in the activities of both the Na^+ pump (ouabain-sensitive) and $Na^+/K^+/2Cl^-$ cotransporter (bumetanide-sensitive; high S.E.Ms and very small fluxes relative to other measurements; data not shown) were not significant.

No increase in nitrendipine-sensitive K^+ influx was observed in cells exposed to raised extracellular Mg^{2+} (0 - 6 mM; Figure 5.20) although there was a slight (but significant (unpaired *t*-test; $P < 0.01$, $n = 3$)) inhibition of bumetanide-sensitive K^+ influx from 1.05 ± 0.075 (in nominally Mg^{2+} free) to 0.45 ± 0.094 $mmoles(l.c.)^{-1}(hr)^{-1}$ (for $[Mg^{2+}]_o$ = 1.5 mM).

KH-induced Ca^{2+} influx was investigated directly (Figure 5.21) by measuring $^{45}Ca^{2+}$ influx into BAPTA loaded cells, using the dibutylphthalate multi-wash technique (section 3.5). KH (0.7 $HU(ml)^{-1}$ significantly (unpaired *t*-test; $P < 0.001$, $n = 3$) increased the Ca^{2+} influx into BAPTA loaded cells from (mean ± S.E.M.) 1.39 x 10^{-2} ± 8.047 x 10^{-3} to 3.98 ± 5.381 x 10^{-2} $mmoles(l.c.)^{-1}(hr)^{-1}$ (Figure 5.21B), with $[Ca^{2+}]_o$ = 0.5 mM. Time courses of KH-induced Ca^{2+} influx (Figure 5.21A) showed similar characteristics of rapid onset

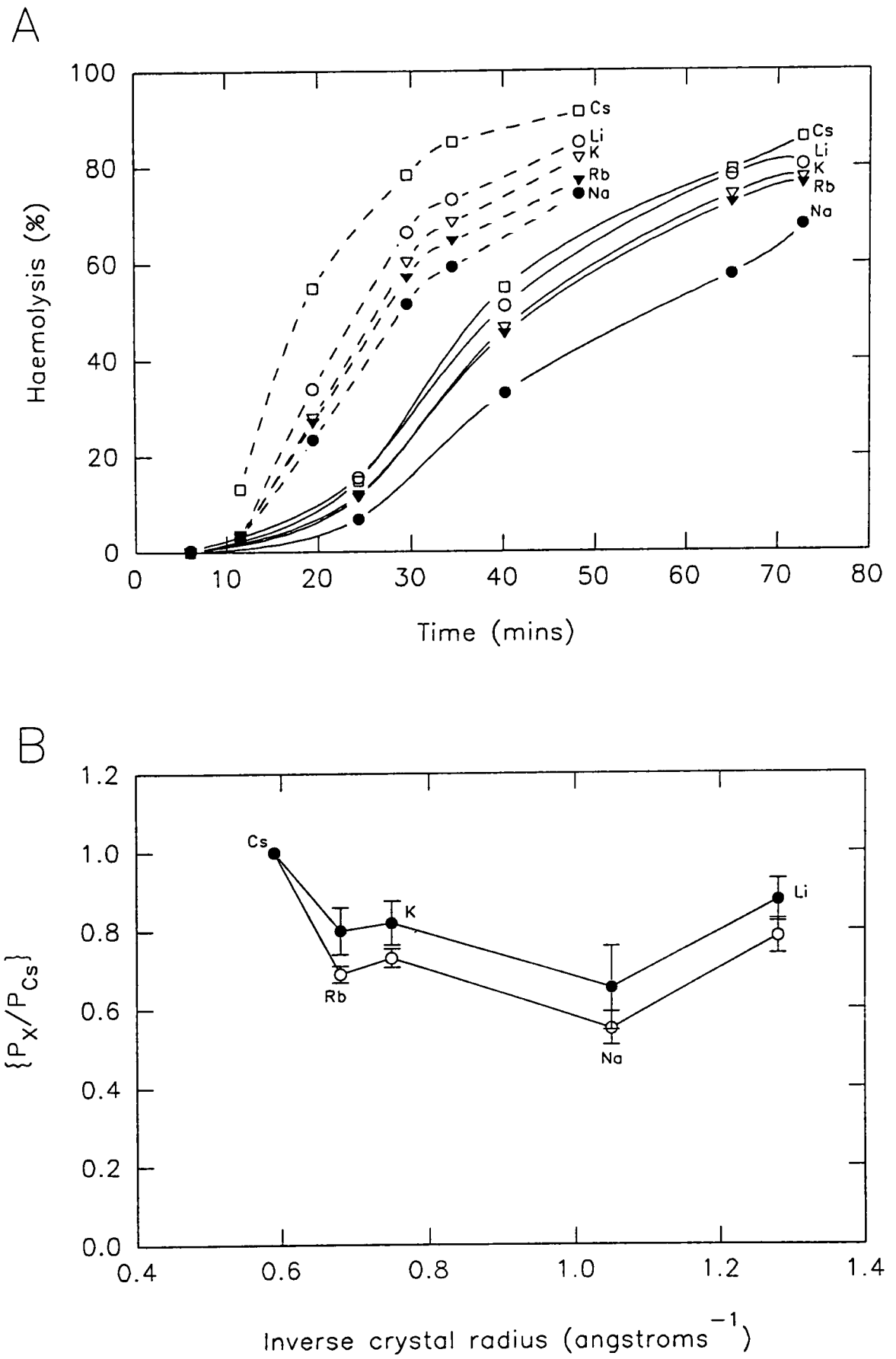


Figure 5.18 Lack of significant effect of neuraminidase-treatment on selectivity series for KH-induced haemolysis. (A) Typical time-courses of KH (1 $HU(ml)^{-1}$) -induced haemolysis in isosmotic solutions of monovalent cation chlorides. Solid lines depict data pertaining to cells untreated by neuraminidase, broken lines represent data from neuraminidase-treated cells. (B) Results are pooled and normalised data from three such experiments. Filled and open symbols represent cells treated/untreated by neuraminidase, respectively.

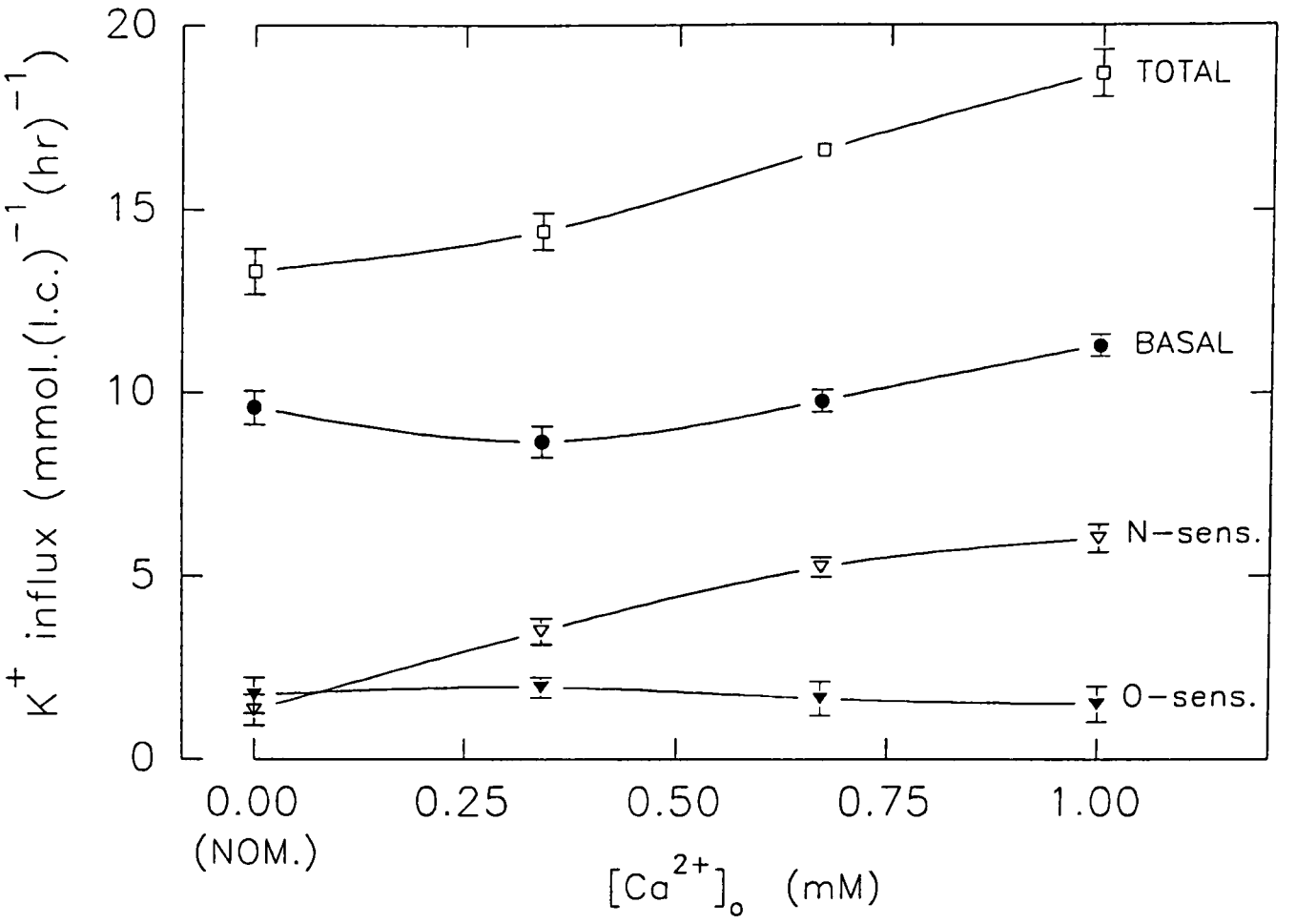


Figure 5.19 Effects of Ca^{2+} on ouabain-sensitive, nitrendipine-sensitive, and residual components of K^+ influx, in the presence of KH. KH (final (KH) = 0.15 HU(ml)$^{-1}$) was added to HRBC (Hct = 5 %) suspended in MBS with sucrose (40 mM). Aliquots (0.85 ml) of this cell suspension were added to Eppendorf tubes containing the relevant inhibitors and $CaCl_2$ (50 mM) in MBS (50 µl) with sucrose (40 mM). Fluxes were initiated by the addition of 100 µl K($^{86}Rb^+$)Cl (150 mM) to give $(K^+)_o$ = 15 mM.

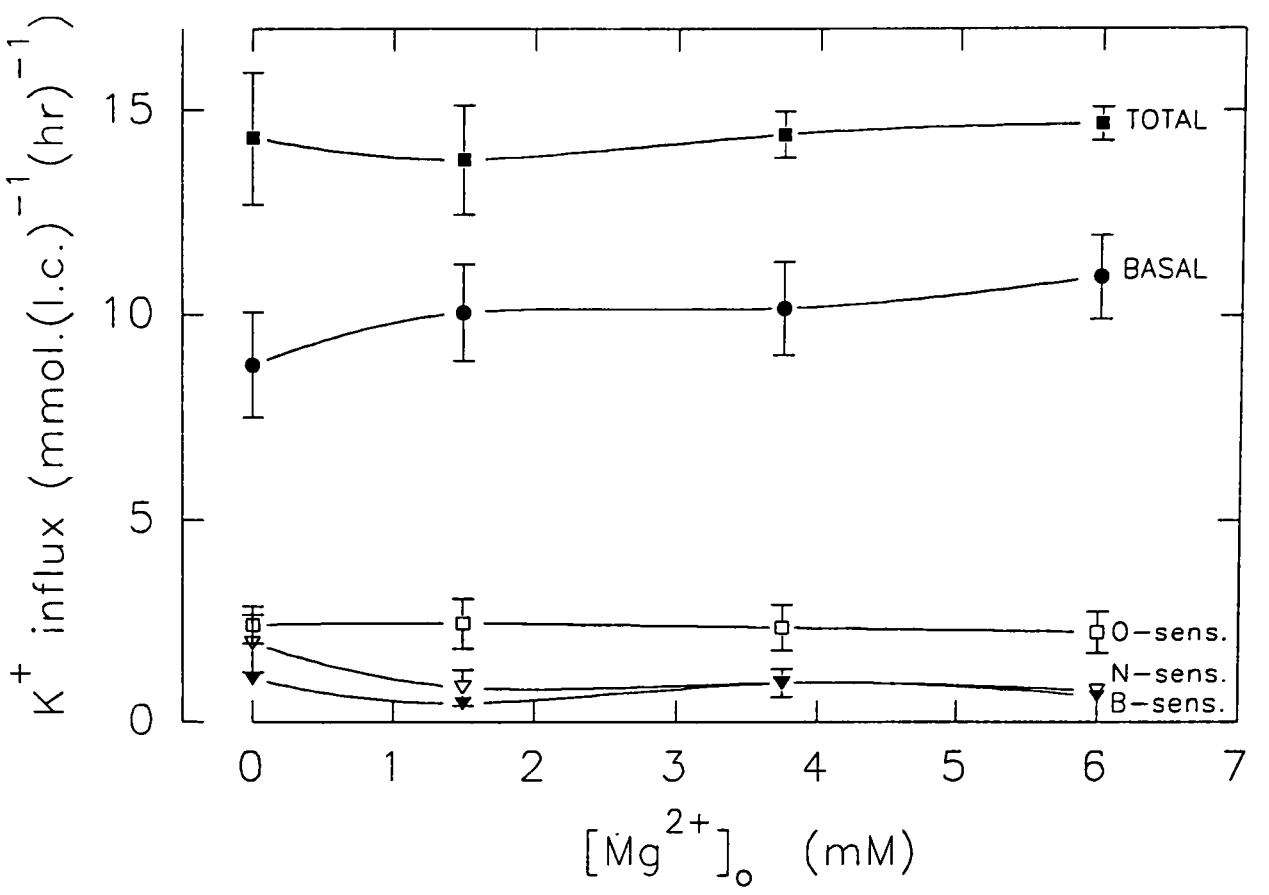
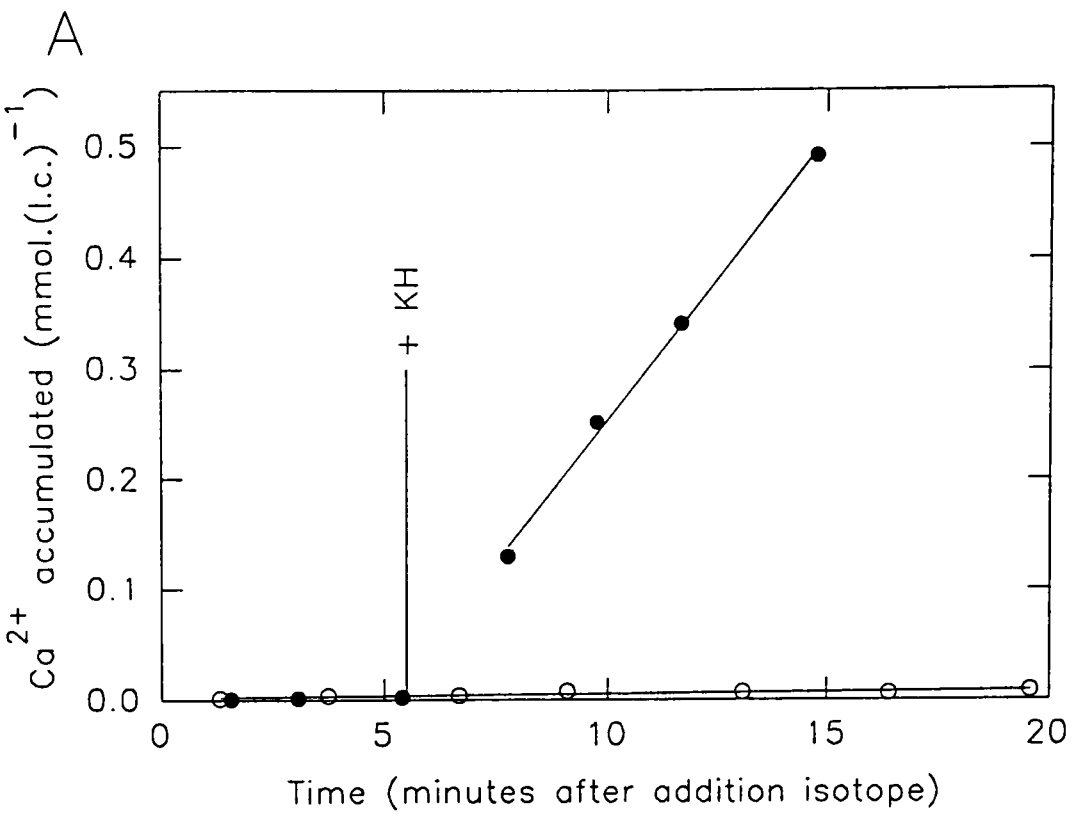


Figure 5.20 Effects of Mg^{2+} on ouabain-sensitive, bumetanide-sensitive, nitrendipine-sensitive, and residual components of K^+ influx, in the presence of KH. KH (final (KH) = 0.15 HU(ml)$^{-1}$) was added to HRBC (Hct = 5 %) suspended in MBS with sucrose (40 mM). Aliquots (0.83 ml) of this cell suspension were added to Eppendorf tubes containing relevant inhibitors and $MgCl_2$ (150 mM) in MBS (50 µl) with sucrose (40 mM). Fluxes were initiated by the addition of 100 µl K($^{86}Rb^+$)Cl (150 mM) to give $(K^+)_o$ = 15 mM.



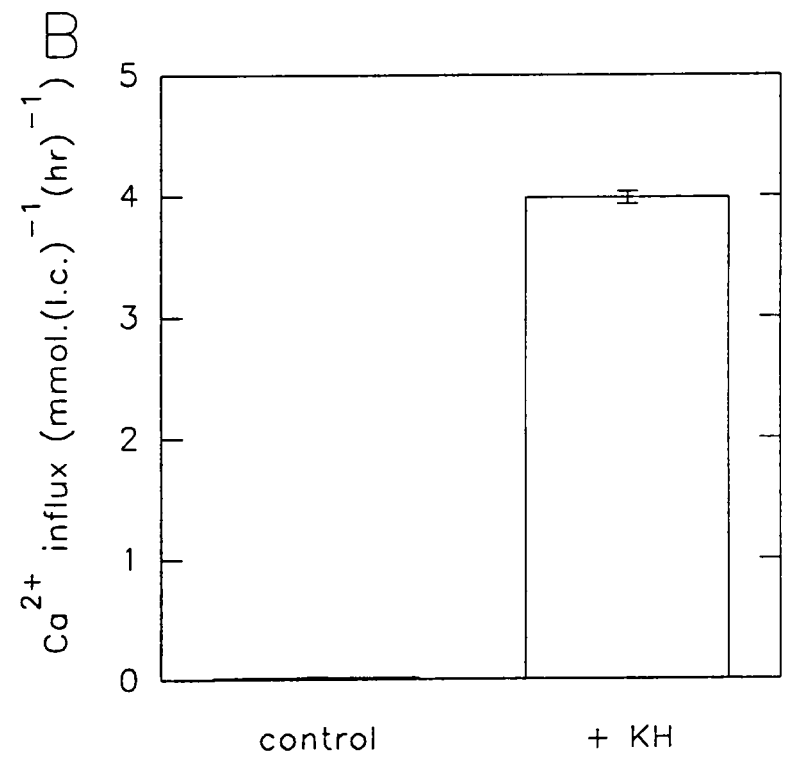


Figure 5.21 Effect of KH on Ca^{2+} influx. KH (5 µl; 100 HU(ml)$^{-1}$) was added to 700 µl BAPTA loaded (see section 3.6.2) HRBC were suspended (Hct = 5 %) in MBS with sucrose (50 mM). At time zero, an aliquot (50 µl) of ($^{45}Ca^{2+}$)$CaCl_2$ (10 mM) was added to 0.95 ml cell suspension ($(Ca^{2+})_o$ = 0.5 mM). Samples (100 µl) were taken in the presence or absence of KH (0.7 HU(ml)$^{-1}$) and Ca^{2+} accumulated calculated. (A) Time course of Ca^{2+} influx. Representative results of three such experiments. Open and filled circles represent cells to which KH was/was not added (at time indicated), respectively. Lines of best fit were calculated by linear regression. (B) Pooled data for 3 experiments showing Ca^{2+} influx in control conditions and when stimulated by KH.

(no definable lag phase) with apparent constancy of magnitude over the 8 minute period subsequent to KH addition, to those of the KH-induced inhibitor-insensitive K^+ leak observed earlier (Figure 4.6).

Data in Table 5.1 describe the effects of nitrendipine on K^+ influx induced in MBS-based media containing either Ca^{2+} (1 mM) or EGTA (0.15 mM; to chelate Ca^{2+} (Hall & Willis, 1986)), in the presence and absence of KH (0.075 $HU(ml)^{-1}$). Although KH induced a nitrendipine-sensitive K^+ flux (in Ca^{2+} media) for all 3 donors, it varied markedly between donors at this toxin dose (contrast to data for larger KH dose of Figure 5.19). However, this nitrendipine-sensitive K^+ influx was nearly abolished when fluxes were measured in EGTA, indicating a Ca^{2+}-dependency for this effect. The absence of an effect of nitrendipine in EGTA-containing media further suggests that nitrendipine does not inhibit the KH-induced pore directly.

Earlier experiments (Figures 5.1, 5.19 and 5.20) indicated no change in the magnitude of ouabain-sensitive (Na^+ pump-mediated) K^+ influx in response to toxin action. The ouabain-inhibitable K^+ influx in the presence or absence of KH was followed over several ten minute periods subsequent to KH (0.1 $HU(ml)^{-1}$) addition and incubation at 37 °C (Figure 5.22). KH did not appear to have a consistent effect on these ouabain-sensitive K^+ influxes (Figure 5.22B). Variations in this flux were noted for individual donors suggesting that several processes may be interacting in a complex and donor-dependent fashion (see section 5.3). Effects on total K^+ influx (Figure 5.22A) were quite reproducible and demonstrated a pattern of influx consistent with that shown earlier (Figures 5.2 and 5.8), given the contribution of other endogenous transporters.

5.3 DISCUSSION

The results presented show that KH increased the residual leak and nitrendipine-sensitive components of K^+ influx into human erythrocytes in MBS. Effects on other pathways were not significant (except inhibition of $Na^+/K^+/2Cl^-$ cotransporter activity in the presence of extracellular Mg^{2+}). The appearance of this large inhibitor-insensitive flux supports the contention that osmotically protectable KH-induced haemolysis occurs as a result of an increase in the cation permeability of the membrane. Therefore, the factor responsible for this leak may be the important virulence factor in *V. parahaemolyticus*-associated disease.

The line of experimentation given for Figure 5.1 was pursued with two aims: (i) to

containing:	Ca^{2+} (1 mM)	Ca^{2+} (1 mM)	EGTA (0.15 mM)	EGTA (0.15 mM)
? toxin	---	KH	---	KH
Donor 1	-0.027 ± 0.034	2.21 ± 0.37	-0.027 ± 0.0045	0.030 ± 0.100
Donor 2	0.017 ± 0.013	0.99 ± 0.46	0.016 ± 0.023	0.024 ± 0.028
Donor 3	0.014 ± 0.016	1.57 ± 0.49	0.018 ± 0.041	0.019 ± 0.073

Table 5.1 Effect of KH on nitrendipine-sensitive K^+ influx in MBS containing Ca^{2+} or EGTA. Cells were washed three times in MBS containing sucrose (50 mM) and then once, immediately prior to fluxing in the same solution but with either Ca^{2+} (1 mM) or EGTA (0.15 mM) before resuspension (Hct = 5 %) in the same. Fluxes ($(K^+)_o$ = 15 mM) were performed in the presence of ouabain and bumetanide and the presence/absence of nitrendipine (each drug at 100 μM). Where appropriate, (KH) = 0.075 HU(ml)$^{-1}$. Data are given in units of mmol.(l.c.)$^{-1}$(hr)$^{-1}$.

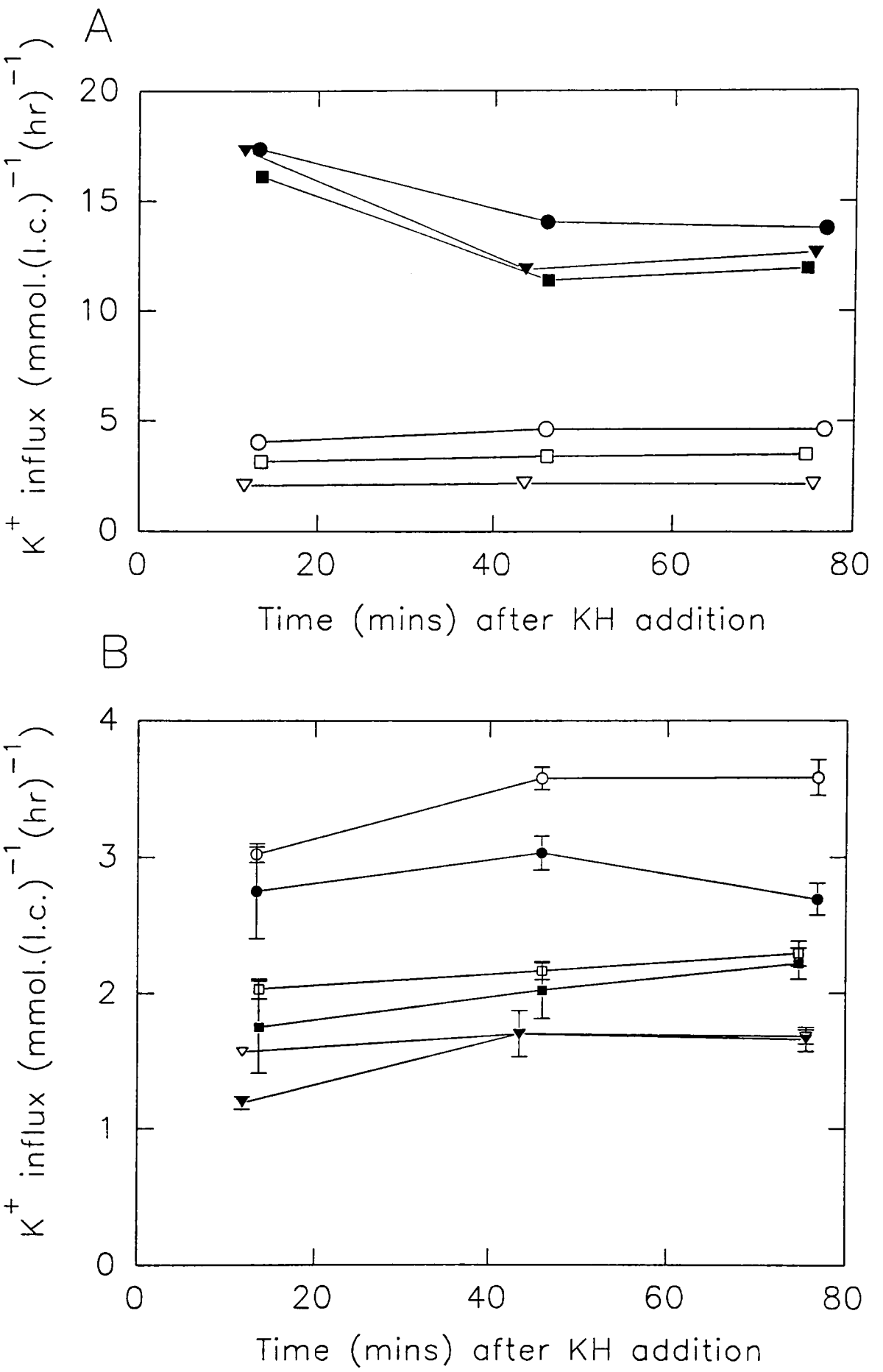


Figure 5.22 Time-dependency of ouabain-sensitive K^+ flux in the presence and absence of KH. HRBC suspended (Hct = 5 %) in MBS with sucrose (50 mM) were incubated at 37 °C in the presence/absence of KH (0.1 HU(ml)$^{-1}$), added (or not) at time = zero. Ouabain-sensitive K^+ influxes were measured for three ten minute periods (for which median times are given) by spiking aliquots (0.9 ml) of the cell suspensions, in the presence and absence of ouabain (100 µM), with 100 µl K(^{86}Rb$^+$)Cl (150 mM) to give $(K^+)_o$ = 15 mM. Filled and open symbols respresent data obtained in the absence and presence of toxin respectively. (A) Total K^+ influxes (in the absence of ouabain). (B) Ouabain-sensitive K^+ influxes.

examine the relation of the induced cation influx to endogenous membrane transporters, particularly in light of recent reports on the mechanism of action of palytoxin (Habermann, 1989; Tosteson *et al.*, 1991) which is inhibitable by ouabain and appears to act on the Na^+/K^+ ATPase, and (ii) to make a preliminary identification of effects on endogenous membrane transporters which might have pathogenic significance in terms of possible perturbed membrane function of other tissues.

Results given in Figure 5.2 show the pattern of KH-induced inhibitor-insensitive K^+ influx with time after toxin addition. It appears that the induced pathway is initially (c. first ten minutes) of high magnitude, but is subsequently decreased to a lower and relatively constant level (c. 20 - 60 minutes after KH addition). As noted elsewhere there was a profound donor dependence of effect, but the pattern of influx magnitude was the same for all donors.

Evidently, KH is capable of causing an increase in the cation permeability (documented by experiments measuring either isotope influx or efflux) of the erythrocyte membrane that results in net cation influx. Normally the HRBC membrane has a membrane potential of c. - 10 to - 14 mV (Stein, 1990). It is not inconceivable that the increased cation influx might cause a depolarisation of the membrane, thus lessening the electrical force favouring cation influx, and that it this phenomenon that underlies the observed dynamics of Figure 5.2. The dynamics of $^{86}Rb^+$ efflux, however, demonstrate similar characteristics, being large initially and subsequently reduced (Figure 5.4). This means that it is the cation permeability *per se* that is initially raised drastically, and remains increased subsequently but to a less marked extent. It is intriguing to speculate as to what might be occurring in physico-chemical terms of membrane structure and toxin-membrane interaction.

Recently, Honda *et al.* (1992) proposed that TDH was a pore-forming protein on the basis of electron micrographic and haemolytic evidence. However, pore (or channel) -like behaviour remained unsubstantiated in cell physiological terms, according to the features outlined in Table 2.3. However, Figure 4.17 does suggest that the lesion induced has only limited selectivity for monovalent cations (this behaviour is more channel-like than carrier-like). K^+ influx with respect to $[K^+]_o$ was examined (Figure 5.5), and not found to give any indication of saturation in the 0 - 30 mM range, further supporting the contention that the lesion is pore-like in nature.

Dose-response data measured over the first ten minutes after KH addition exhibited a lag phase with respect to KH concentration. A similar relation has been documented previously for the action of complement component C6 on Molt 4 cells and interpreted as implying a channel-forming process of multi-hit nature (Koski *et al.*, 1983). However to make this assertion, the dynamics of the higher to lower magnitude cation permeability transition, induced by KH (Figure 5.2), must be understood to be independent of the dose of toxin used. The shape of the dose-response relation could be accounted for by the higher to lower magnitude transition occuring faster (and < 10 minutes after KH addition) for lower doses of toxin. However data presented in Figure 5.4 do not support this alternative explanantion, the transition appearing to occur at the same time irrespective of KH concentration. It is hence possible that KH stimulates channel formation by a multi-hit mechanism. This suggestion was examined further (Figure 5.7) at a low dose of KH (0.06 $HU(ml)^{-1}$) by varying the concentration of target cell. The results are interpreted as follows: (1) over the initial haematocrit range (relatively constant high flux and increasing K^+ transit) the sites available for leak formation are close to saturation ie the amount of KH present in solution is greater than that required for generation of a leak pathway of maximum magnitude, and (2) several components of KH (as it exists in the aqueous phase) are required to form the leak pathway responsible for high K^+ influx. This is because not only flux but also K^+ transit (an index of total rather than fractional uptake of cation) decreases after reaching a maximum, rather than simply levelling out. Thus leak induction is a multi-hit process, in which KH components have a proportionately greater effect when they operate in unison as opposed to singly. Interestingly, K^+ transits at higher haematocrits are significantly higher than those corresponding to the basal leak of control cells. The increased K^+ transit, with increasing haematocrit (in this higher haematocrit range), may indicate that single components of KH are capable of raising the cation permeability of the membrane, although not as effectively as several components acting in concert. Previous multi-hit studies have used haemolysis (expressed both as percentage and total, whilst varying haematocrit) as their assay of toxin action (Inoue *et al.*, 1976; Johnson, 1972; Johnson & Boese-Marrazzo, 1980; Kothary & Kreger, 1985; Oberley & Duncan, 1971). However, the conclusions that can be drawn from such studies are limited in that they indicate only the number of hits required for *cell death*, rather than for *lesion formation* itself. The fact that in the lower haematocrit range there is a relative

constancy of K^+ influx may indicate a saturation of the pore-formation process during the first ten minutes after toxin addition, mediated by receptor sites on the erythrocyte membrane. Such a process of leak formation may corroborate previous evidence (Takeda *et al.*, 1975, 1976) which suggests the presence of a neuraminidase-sensitive ganglioside receptor (G_{T1} or G_{D1} rather than G_{M1} or G_{M2}) necessary for TDH action. The involvement of such receptors in KH action was investigated further in section 5.2.3. These multi-hit findings at low toxin dose are especially interesting in light of the non-multi-hit relation observed for KH-induced haemolysis at rather higher doses (Figure 4.1).

The nature of the different phases of the dynamics of the KH-induced K^+ influx (Figures 5.2 and 5.4) was examined further by investigating the effects of washout (CP3). As noted previously, the late phase (median time = 39.25 minutes after KH addition) was of a markedly less magnitude than the early phase (median time = 11.1 minutes after KH addition). Furthermore, a much higher percentage of the early phase flux was inhibited by washout. It has been proposed previously that toxin binding occurs over a rather protracted period (section 4.3), despite the magnitude of influx being (apparently) constant for the first ten minutes after KH addition (Figure 4.6). It is likely therefore that the increased fraction of influx susceptible to washout in the early phase is due to washout not of membrane-bound entities but rather of aqueous KH. Given that it is a permeability change rather than a membrane potential effect that underlies the dynamics of change between early and late phases, and that TDH is still binding during the early phase, then it is tempting to speculate that TDH causes the production of a larger magnitude pathway during some transitory phase necessary for the production of the relatively stable and constant magnitude 'pores' observed in the later phase (Figure 5.3).

Sakurai *et al.* (1975) speculated that the high pressures encountered in centrifugation (eg 10,000 g in CP3) might cause bound TDH to be released from the membrane. Evidence obtained earlier (see section 4.3) tends to suggest that this is not the case; however, because the pattern of KH-induced K^+ influx is such that it is higher initially and lower subsequently (Figures 5.2 and 5.4), a simple haemolysis experiment was performed in which the effect of a centrifugation (CP3) and resuspension cycle was determined. If bound TDH was released from the membrane then it would be expected to instigate a lesion of initial phase properties (ie mediating a larger cation flux than that occurring during the later phase (Figures 5.2 and 5.8)). However this treatment had no effect on the

rate of KH-induced haemolysis (Figure 5.9), suggesting that CP3 was unlikely to cause release of bound entities. This further strengthens the previous assertation (section 4.3) that the binding phase is longer than 1 - 2 minutes, because it contraindicates the possible alternative explanation that haemolytic activity recovered in the aqueous portion from a centrifuged KH-treated suspension (Figures 4.12 and 4.14) was of membrane-bound origin.

Data in Figure 5.1 showed that the predominant effect of KH was on the inhibitor-insensitive component of K^+ influx. A volume-sensitive KCl cotransport system has been described in human erythrocytes that is quiescent under normal conditions but may be stimulated by NEM treatment, hydrostatic pressure, cell swelling and acid pH (Ellory *et al.*, 1987; Ellory & Hall, 1988). No specific inhibitor was used in experiments detailed in Figure 5.1 to rule out a contribution from this cotransporter to the induced influx, although volume changes were inhibited by the addition of sucrose to raise the extracellular osmolarity. On the basis of the Cl^--dependency of the KCl cotransport system, experiments were performed in an anion-substituted medium (Ellory & Hall, 1988; Bernhardt *et al.*, 1991; Gibson *et al.*, 1993) to see if Cl^- replacement significantly reduced KH-induced K^+ influx. Cl^- replacement (by $CH_3SO_4^-$) had no such effect (Figure 5.10), indicating that, under these conditions, there is no role for Cl^--dependent processes in the cation flux induced by KH.

The opinion of Bhakdi *et al.* (1985a), that aggregation of endogenous membrane protein(s) is an unlikely mechanism for pore-forming bacterial toxins, is far from proven. The anion (Cl^-/HCO_3^-) exchanger (named Band III, after its postion in SDS PAGE gels) is the major membrane protein of human (and other species) erythrocytes, there being in excess of 1 million copies of this protein per red cell (Stein, 1990); on the basis of the hydropathy plot and chemical evidence, this protein has 14 putative membrane-spanning domains and a glycosyl chain attached the largest extracellular domain. Bjerrum *et al.* (1980) found that low pH (5.0) could induce aggregation of peripheral and intrinsic proteins in resealed erythrocyte ghosts, this aggregation developing slowly over a 24 hour period. Their studies further showed that concomitant to the aggregation phenomena, there was a permeability increase towards small polar molecules. The permeability increase could be decreased by about 80 % by exposure to DIDS (0.5 mM) and could be almost completely reversed (together with the state of aggregation) by retitration to pH

7.2 followed by heating to 38 °C. Benz *et al.* (1984) documented the effect of incorporating solubilised Band 3 protein from human erythrocytes into lipid bilayers. Band 3 protein increased the membrane conductance by a factor related to the fourth power of protein concentration, and this was held to suggest that four Band 3 subunits form an ion permeable pathway in the lipid bilayer system. Protein preincubated for ≥ 30 minutes in an H_2DIDS solution (10 μM) was unable to increase membrane conductance. Clague and Cherry (1989) used laser flash-induced transient dichroism to document changes in the rotational diffusion of eosin-labelled Band 3 proteins in erythrocyte ghosts, in response to melittin, polylysine and Zn^{2+}. The observed retardation of Band 3 mobility was interpreted as reflecting aggregation or 'clustering' of the protein in the plane of the membrane. In the present study, hagfish erythrocytes were used in an attempt to preclude Band 3 aggregation as a potential mechanism of pore formation as proposed for other agents (Clague & Cherry, 1989). These cells were insensitive to KH action (Figure 5.11), in a manner similar to that of horse cells. Such insensitivity does not support the contention that Band 3 aggregation is involved. Rather hagfish erythrocytes can simply be added to the list of cells insensitive to KH - this insensitivity need not be due to a lack of Band 3 because the insusceptible horse cells have this protein in abundance. Furthermore, DIDS (20 μM) had no significant effect on the KH-induced leak (Figure 5.13).

A functional characterisation of the KH-induced leak was performed by examining its sensitivity to several other membrane-active agents. The induced inhibitor-insensitive leak was unaffected by Ca^{2+} and Mg^{2+} (1 mM), though it was inhibited by Cd^{2+} (1 mM) and Zn^{2+} (250 μM), an effect shown for many other leak-inducing agents (Bashford *et al.*, 1988a,b; Pasternak, 1991; Pasternak *et al.*, 1992). Divalent cations have long been known to decrease leakage of ions and other water-soluble molecules across membranes (Frankenhauser & Hodgkin, 1957; Wyn-Jones & Lunt, 1967; Woodhull, 1973; Hille, 1984; Behrens *et al.*, 1989), whether such leakage is through endogenous ion channels (Gilly & Armstrong, 1982a,b; Wolosin & Forte, 1985; Smart, 1990) or through channels formed by membrane-inserting peptides such as bacterial toxins (Bashford *et al.*, 1986, 1988a; Menestrina, 1986; Miyake *et al.*, 1989; Menestrina *et al.*, 1990) or molecules of the immune system (Bashford *et al.*, 1988b). Divalent cations may exert effects at a number of temporally distinct sites, which can be considered as belonging to one of three classes:

(i) channel induction. Divalent cations may have either inhibitory or stimulatory effects on this process, depending on the ionic species and protein studied. For example the action of lymphocyte cytolysin requires Ca^{2+} in both cell membranes (Henkart, 1985) and lipid bilayers (Young *et al.*, 1986c; Bashford *et al.*, 1988b). Zinc ions however, have the opposite effect on this protein as they do for aerolysin (Wilmsen *et al.*, 1990). The induction of *S. aureus* α-toxin is promoted by both Ca^{2+} and Zn^{2+} (Pasternak *et al.*, 1991).
(ii) channel size. Similar effects (ie stimulatory or inhibitory) are recorded to those of divalent cations on channel induction (see above). Conductances through single channels formed by *S. aureus* α-toxin and sea anenome cytolysin are increased and decreased by divalent cations, respectively (Pasternak, 1991).
(iii) channel closure. Divalent cations tend to promote closure of pores (ie decrease open time), often in a voltage-dependent manner (Bashford *et al.*, 1986, 1988b; Menestrina, 1986; Menestrina *et al.*, 1990; Pasternak, 1991) with increased likelihood of closure at higher voltages. The relative efficacy of cations in promoting closure is the same for all proteins so far examined (Zn^{2+} (and Cd^{2+}) > Ca^{2+} > Mg^{2+}).

Sakurai *et al.* (1975) found that TDH-induced haemolysis was stimulated by Ca^{2+} or Mn^{2+} compared with Mg^{2+} (25 mM). However they make no reference to osmotic control aside from this; Mg^{2+} was also deemed as stimulatory to haemolysis in reference to their 'control'. Furthermore, their report makes no attempt to discriminate between an effect on toxin activity itself or a stimulation of events leading to haemolysis, subsequent to toxin action. For example, it is possible that these results reflect the effects of raised intracellular divalent cation concentrations (subsequent to influx through the TDH-induced pore) on cytosketal elements and their interaction with the cell membrane.

Miyake *et al.* (1989) have documented the effects of divalent cations on haemolysis induced by *V. metschnikovii* cytolysin. This cytolysin is functionally distinct from TDH as it gives (in contrast to KH - see section 4.2.1, Figure 4.1) multi-hit haemolytic characteristics (Miyake *et al.*, 1988). However certain 'heavy' divalent cations (Cu^{2+}, Sn^{2+}, Zn^{2+}, Cd^{2+}, Ni^{2+}) were found to be inhibitory to haemolysis at low concentrations (ID_{50} values in the range 0.06 - 4.5 mM). It is worth mentioning that the ID_{50} values in their study were calculated from doses at which % haemolysis was halved at a given time, rather than using a more direct indication of rate.

From the data of Figure 5.12, indicating Zn^{2+} and Cd^{2+} inhibition of KH-induced

$K^+(^{86}Rb^+)$ influx, it is not possible to ascertain which of the above three processes (channel induction, channel size or channel closure) is affected. This line of experimentation could be pursued in the future using either lipid bilayer or patch-clamp methodology (see Chapter 10). An assessment of the effects of divalent cations on channel conductances due to many pore-forming toxins in lipid bilayers is difficult because of the heterogeneity of channel sizes encountered for progressive barrel-stave pore-forming toxins. This should not, however, be the case for TDH, for which pore size appears to be a fixed parameter with respect to TDH concentration. Therefore a lipid bilayer model would be an appropriate direction to take.

Phloretin is a potent inhibitor of several energy-independent transport processes, including sugar transport, anion exchange and urea transport (Jennings & Solomon, 1976 and references therein). However it binds to several sites in the erythrocyte membrane, with different affinities (Jennings & Solomon, 1976), has effects on red cell non-electrolyte permeability (Owen *et al.*, 1974) and respectively causes increases and decreases in the cation and anion conductances of lipid bilayers (Andersen *et al.*, 1976; Melnik *et al.*, 1976). Furthermore phloretin has been described as an inhibitor ($ID_{50} \leq 250$ μM) of leaks induced by oxidative damage from agents such as diamide, periodate and O_2-derived species which are thought to involve the lipid domain (Deuticke *et al.*, 1991). The lack of a significant effect of phloretin on the KH-induced leak (Figure 5.13A), and the lack of an inhibitory effect of Zn^{2+} on the leak induced by oxidative damage (Deuticke *et al.*, 1991) point to distinct sites for these two leaks.

Previous results (section 4.2.5; Figures 4.12 and 4.13) indicated that vigorous vortexing of KH with DBP dramatically reduced its subsequent haemolytic activity. On this basis, the effect of similar treatment on the flux inducing potential of KH was tested (Figure 5.14) and a similar reduction found. This confirms the action of DBP on KH and further supports the contention that the induced cation flux underlies haemolysis. However, although this inhibitory action suggests a lipid-protein interaction for KH, it is not clear whether KH is (i) denatured by DBP, (ii) absorbed to the lipid water interface, or (iii) partitioned into the lipid. These data mirror previous experiments (Takeda *et al.*, 1975, 1976; see section 2.6.4 and 5.1) in which incubation of TDH with ganglioside preparations decreased haemolytic activity. If the ganglioside mixture itself was pretreated by neuraminidase then its protective effect was abolished. Further experiments on purified

ganglioside preparations implicated the neuraminidase-sensitive gangliosides G_{D1a} and G_{T1} as the TDH-binding entities important in the TDH:cell interaction. Therefore the effect of neuraminidase treatment of target cell (HRBC) on subsequent susceptibility to KH action was investigated (Figures 5.15 - 5.18). The rate of KH-induced haemolysis was increased rather than decreased by this treatment and was due to an increase in the induced cation leak, rather than an effect on osmotic fragility. Neuraminidase incubation did not itself cause an increase in cation leak; rather it enhanced that observed as a result of KH action (Figure 5.17), and the nature of the leak did not appear to be altered (Figure 5.18). These results indicate the presence of a class of receptor that can be unmasked by neuraminidase action and furthermore suggest that an increase in the amount of such an endogenous membrane component can augment KH action at a particular toxin concentration.

If *V. parahaemolyticus* were able to elaborate a protein with neuraminidase-like activity, then leak generation would be an interesting point of synergy in the pathogenic process. However, for 16 pathogenic strains of *V. parahaemolyticus* tested, all were neuraminidase-negative (Kabir *et al.*, 1984). There is thus no evidence to suggest that *V. parahaemolyticus* produces neuraminidase and TDH in concert. Neuraminidase is produced by certain strains of *V. cholerae* and may have a role in pathogenesis by converting higher order gangliosides into G_{M1}, the receptor for cholera toxin (Galen, 1991; Galen *et al.*, 1992). Often in diarrhoeal disease, several different organisms are co-isolated (Hoge *et al.*, 1989), so neuraminidase action may still be pertinent to pathogenesis associated with this toxin. Whether neuraminidase sensitisation of target cell membranes to TDH action is relevant to the pathophysiology of diarrhoea associated with *Vibrio* infection, it nevertheless indicates a class of 'receptor' that can be unmasked by neuraminidase action. An increase in the number of receptors augments KH-induced K^+ influx at a given toxin concentration, although the nature of the lesion itself appears to be unaffected. This is an important mechanistic aspect in the emerging detail of the TDH:membrane interaction. The red cell is a practical system for further assessment of the putative roles of endogenous membrane proteins and gycolipids as receptor molecules (Takeda *et al.*, 1975, 1976) in TDH action. This important area may be investigated by attempting to confer susceptibility to TDH action on the insensitive horse erythrocytes. Indeed, the erythrocyte facilitates such studies, having the ability to accumulate

gangliosides from an experimental medium (the methodology for such accumulation being well established for human erythrocytes; Lazarovici & Yavin, 1985a), and being devoid of endocytic activity (Ellory & Tucker, 1983). The studies of Lazarovici and Yavin (1985a,b) used radio-iodinated tetanus toxin as the measure of incorporated gangliosides G_{T1b} and G_{D1b} although induced K^+ influx would be a simpler method to measure conferred sensitivity to TDH. Other studies have evaluated the role of glycolipids as toxin receptors by inserting the glycolipid into an otherwise insusceptible cell type, for example insertion of ganglioside G_{M1} into rat glioma C6 cells (Spiegel, 1988), and the neutral glycolipid G_{b3} into Daudi human lymphoma cells (liposomal fusion method; Waddell *et al.*, 1990).

In contrast to the lack of effect of low concentration (1 mM) Mg^{2+} or Ca^{2+} on the KH-induced inhibitor-insensitive flux, these divalent ions had significant effects on endogenous K^+-transporting systems in the presence of KH (Figures 5.19 and 5.20). The effect of KH on the (nitrendipine-sensitive) Ca^{2+}-activated K^+ channel (Figures 5.1 and 5.19) is likely to result from an increase in intracellular Ca^{2+} (secondary to a KH-induced Ca^{2+} leak) rather than either a mechanosensitive stimulation (secondary to a volume increase; since such a response would be limited by the extracellular sucrose) or a direct effect (because the magnitude of the Gardos channel flux was dependent on the $[Ca^{2+}]_o$; Figure 5.19). The possibility that the increase in nitrendipine-sensitive K^+ influx was due to (partial) nitrendipine inhibition of KH-induced K^+ influx, independent of the sodium pump, $Na^+/K^+/2Cl^-$ cotransporter and Gardos channel was examined: (i) in the presence of the calcium chelator EGTA (0.15 mM) (Table 5.1), and (ii) in varied $[Mg^{2+}]_o$. Results from Table 5.1 suggest that the KH-induced lesion is unlikely to be affected directly by nitrendipine because in the presence of EGTA, nitrendipine showed no inhibitory effect on KH-induced K^+ influx into ouabain and bumetanide-inhibited cells. Under conditions of increasing $[Mg^{2+}]_o$, there was no effect on nitrendipine-sensitive influx analagous to that obtained in the presence of extracellular Ca^{2+}. However, bumetanide-sensitive K^+ influx was reduced in the presence of extracellular Mg^{2+} (1.5 mM). These results indicate that at physiological levels of the extracellular divalent cations Ca^{2+} and Mg^{2+}, pore-forming toxins can perturb endogenous membrane function. It is worth emphasising that the effects on nitrendipine-sensitive and bumetanide-sensitive K^+ influx detailed above, occur within the first ten minutes after toxin addition. Such effects are likely to be even more

pronounced subsequently, when more marked alterations of intracellular Ca^{2+} and Mg^{2+} may have arisen.

KH action was shown to be capable of mediating a large Ca^{2+} influx, albeit at a large toxin dose ([KH] = 0.7 $HU(ml)^{-1}$; Figure 5.21); the rapidity of onset and constancy of flux magnitude in the eight minutes after toxin addition were similar to those previously noted for inhibitor-insensitive K^+ influx, suggesting that KH causes increased $[Ca^{2+}]_i$ by allowing Ca^{2+} influx through the same lesion. A KH-induced change in $[Ca^{2+}]_i$ may underly pathophysiological events associated with *V. parahaemolyticus*. Schanne *et al.* (1979) have documented the dependence of cell death by many toxic agents on the presence of extracellular Ca^{2+}. Furthermore, Goshima *et al.* (1978), reported that extracellular Ca^{2+} was required for the degeneration of cardiac myocytes induced by TDH. Results given in section 5.2.4 are interesting in that they suggest that alterations in $[Ca^{2+}]_i$ arising from the effect of TDH at physiological $[Ca^{2+}]_o$ levels can greatly perturb the function of certain endogenous membrane transporters under conditions that do not cause cell lysis. Investigation of the role of Ca^{2+} in KH-induced perturbations of *enterocyte* function is an important next step (see Chapters 9 and 10).

Evidence described in Figure 5.1 shows that KH (0.1 $HU(ml)^{-1}$) did not stimulate ouabain-sensitive K^+ influx in the first ten minutes after KH addition, although an increase in nitrendipine-sensitive K^+ influx was noted and the onset of the early phase (high magnitude) cation permeability has been shown to be extremely rapid (Figures 4.6 and 5.2). Furthermore, in the first ten minutes after KH addition, endogenous membrane transport function is sensitive to changes in extracellular divalent cation concentrations (Figures 5.19 and 5.20). However, although intracellular Na^+ is presumed to rise after KH addition, Na^+/K^+ ATPase activity (ouabain-sensitive K^+ influx) does not rise. There are several possible explanations for this lack of effect:

(i) the Na^+/K^+ ATPase may be saturated with respect to $[Na^+]_i$, operating at its V_{max}. Although $[Na^+]_i$ increases, this is beyond the capacity of the pump.

(ii) an increase in $[Na^+]_i$ may cause an increase in Na^+ pump activity but this may be offset by other factors; for example the Na^+/K^+ ATPase may be inhibited by: (a) an increase in $[Ca^{2+}]_i$, through a calnektin or calmodulin mediated effect (Yingst, 1988), and/or (b) an increased ADP/ATP ratio (Kennedy *et al.*, 1986) after either leakage of ATP or increased operation of the Ca^{2+} ATPase (Carafoli, 1987).

(iii) the $[Na^+]_i$ is not sufficiently raised over this time course to cause a measurable increase in Na^+/K^+ ATPase activity.

This final possibility was assessed (Figure 5.22) by examining ouabain-sensitive K^+ influx over several ten minute periods following various intervals of incubation with KH at 37 °C. No consistent effect was observed, leaving this area open to further investigation. However, perturbations of transport by this pathway were noted for individual donors, suggesting that several processes may be occurring in response to KH action, with some donor-dependent variability. As indicated above, a number of factors may be involved, but it is likely that increases in Na^+ pump activity are limited by the presence of sucrose (40 mM) which has the effect of slightly shrinking the cells, hence raising $[Na^+]_i$ and shifting the activity of the pump towards V_{max}.

It is noteworthy that the effects of El Tor haemolysin (ETH) of *V. cholerae* on ion transport into human erythrocytes has been assessed by similar experiments to those presented here (Hall *et al.*, 1986). These workers also found that incubation of toxin with red cells did not increase ouabain-sensitive K^+ influx (in fact there was a slight decrease), although the Na^+ permeability was dramatically enhanced.

Figure 5.23 is a working scheme summarising much of the data of this chapter together with that of previous chapters. This scheme indicates plausible sites for, and sequelae to, KH action considered from the perspective of *V. parahaemolyticus* pathogenicity.

The next chapter describes microbiological investigations and protein purification procedures used to: (i) obtain highly purified TDH to verify that features of membrane attack instigated by KH are indeed attributable to TDH (Chapter Seven), (ii) obtain purifed ETH for investigation by analagous lines of experimentation to those already given for KH (Chapter Eight), and (iii) search for the action of - as yet - unidentified toxins produced by *V. cholerae* non-O1. Having identified plausible derangements of membrane function by TDH that might be important in a diarrhetic effect, preliminary experiments concerning KH action on the more pathogenically relevant target of isolated enterocytes are given in Chapter Nine.

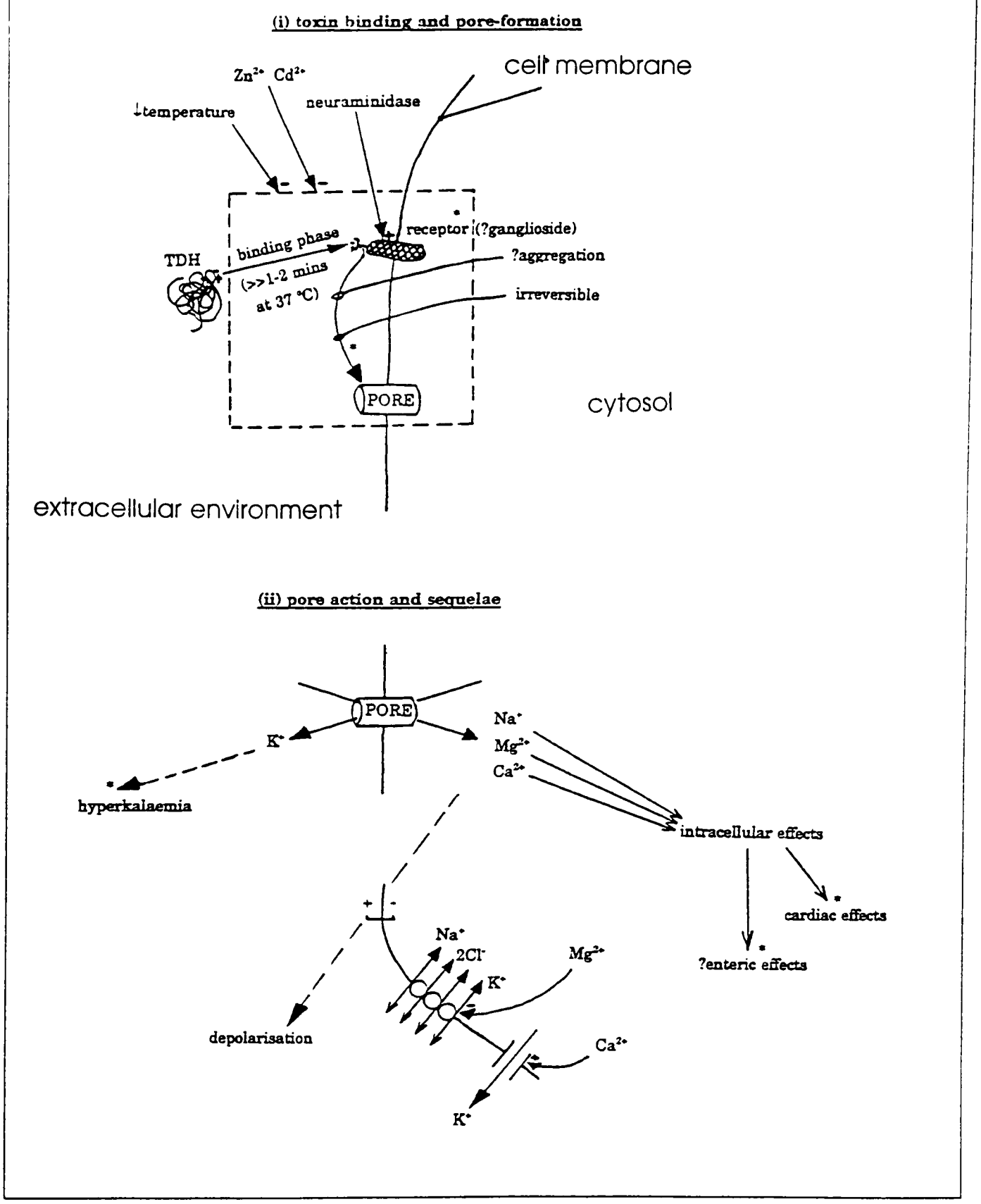


Figure 5.23 Working scheme of plausible pathogenic role of TDH. The information available in the literature, together with evidence presented in Chapters 4 and 5, provide the basis for this speculative diagram. Key points of uncertainty for future investigation are marked by an asterisk.

CHAPTER 6

Microbiological enquiries and the isolation of *Vibrio* exotoxins

'Let's purge this choler without letting blood...'
William Shakespeare, *in* Richard II, I.i.153

6.1 INRODUCTION

V. parahaemolyticus and *V. cholerae* El Tor each produce a major exoprotein capable of causing haemolysis, designated the thermostable direct haemolysin (TDH) and the El Tor haemolysin (ETH), respectively (sections 2.6.4 and 2.7.4). Several reports detail culture conditions and subsequent purification procedures for TDH (see section 2.6.4*(i)*; Obara, 1971; Zen-Yoji *et al.*, 1971, 1974, 1975; Honda *et al.*, 1976c; Miyamoto *et al.*, 1980; Cherwonogrodzky & Clark, 1981, 1982a,b; Takeda, 1988; Douet *et al.*, 1992; Nishibuchi *et al.*, 1992) and ETH (Honda & Finkelstein, 1979; Sutton *et al.*, 1985; Hall & Drasar, 1990). TDH is commercially available as a one-step purified preparation (ion exchange (0 - 1 M NaCl) on a Sephadex-DEAE A-50 column (Pharmacia)), termed Kanagawa haemolysin (Sigma; Cherwonogrodzky & Clark, 1982a; section 3.2.1), and it is this preparation that has been characterised in Chapters 4 and 5.

In the present investigation no thermolabile activity could be detected for KH (Table 4.1). Furthermore, horse red cells were insensitive to KH, as has been noted previously for highly purified TDH preparations (Takeda, 1983), leading to the suggestion that activity could be accounted for by TDH alone (section 4.3). However, on SDS-PAGE with silver staining, protein present in the KH preparation did not appear as a homogeneous band (Douet *et al.*, 1992). It was therefore important to obtain a highly purified form of TDH, to investigate if the features of membrane damage described for KH in Chapters 4 and 5 could indeed be ascribed to TDH (Chapter 7).

The aim of procedures described in this chapter has been to isolate TDH and ETH in pure form and in sufficient quantities to allow study of their effects on target membranes. In addition, the effects of culture filtrates of other *V. cholerae* non-O1 strains on the morphology of Chinese hamster ovary (CHO) cells were examined, with a view to the identification of other (as yet unknown) factors capable of perturbing cellular physiology.

The principles and methods employed in this chapter have been described in sections 3.7 to 3.10. The results (section 6.2) are classed in three subsections:

(i) Data pertaining to V. parahaemolyticus and TDH isolation. The strain used was *V. parahaemolyticus* AQ 3817, previously used by Nishibuchi *et al.* (1992) to determine the importance of TDH in changing the I_{SC} across rabbit ileal tissue in Ussing chamber experiments (section 2.6.4).

(ii) Data pertaining to V. cholerae non-01 strain and ETH isolation. Strain 8731, of environmental origin, was used as it has been shown to be a good source of ETH (Hall & Drasar, 1990).

(iii) Data pertaining to the demonstration of a factor(s), present in supernatants of hlyA-disrupted CT⁻ V. cholera strains, capable of causing CHO cell elongation. A series of strains developed in the quest for a live oral vaccine (Levine *et al.*, 1988a,b) were investigated.

6.2 RESULTS

6.2.1 *V. parahaemolyticus* and TDH

(i) Purification of TDH from culture supernatant. As has already been described (section 2.6.4), growth conditions of cultures can greatly affect the production of extracellular proteins (Cherwonogrodzky & Clark, 1981, 1982a,b, 1984). Several different media (see Table 3.4) were tested for their abilities to promote (i) bacterial growth, and (ii) TDH production (Figure 6.1). Furthermore, because initial medium pH has been shown to be an important determinant of TDH production (Clark & Cherwonogrodzky, 1981), a preliminary investigation was made into the effects of altering initial medium pH.

Cultures were grown in media (1 l) in loosely capped Fernback flasks (2.8 l) for 16 hours at 37 °C (shaking speed = 100 r.p.m.), after innoculation with LB1 starter culture (1 % v/v). Initial and final media pH values and optical densities were recorded (Figure 6.1A,B). Culture supernatants were harvested after centrifugation (CP7), by precipitation with ammonium sulphate (351 $g(l)^{-1}$). The precipitate was resuspended in Tris-HCl buffer (20 mM; pH = 7.5; volume = 3 ml, for each system) containing protease inhibitors, and dialysed exhaustively against the same (section 3.8.1). After clarification of the exoprotein concentrate

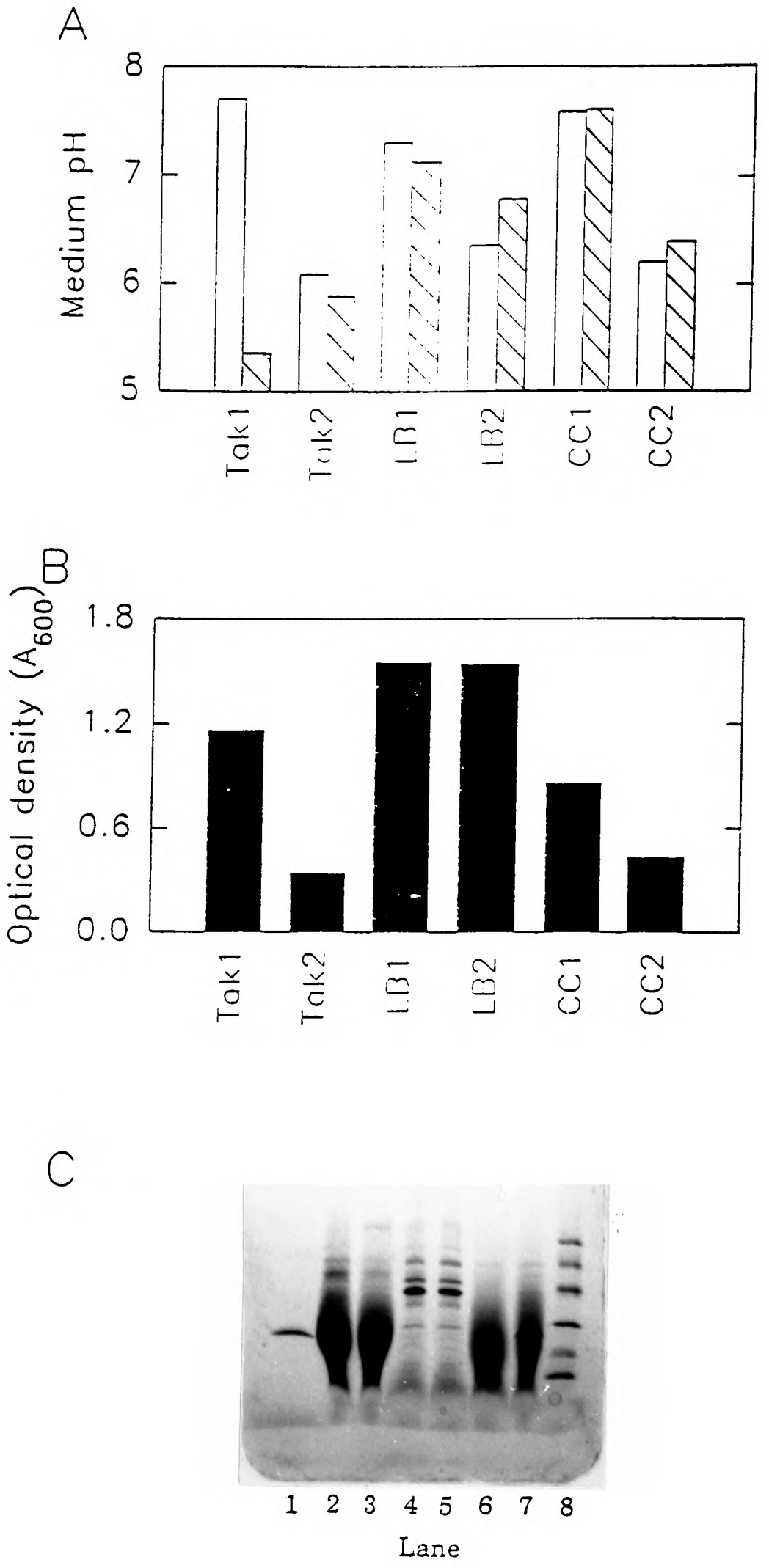


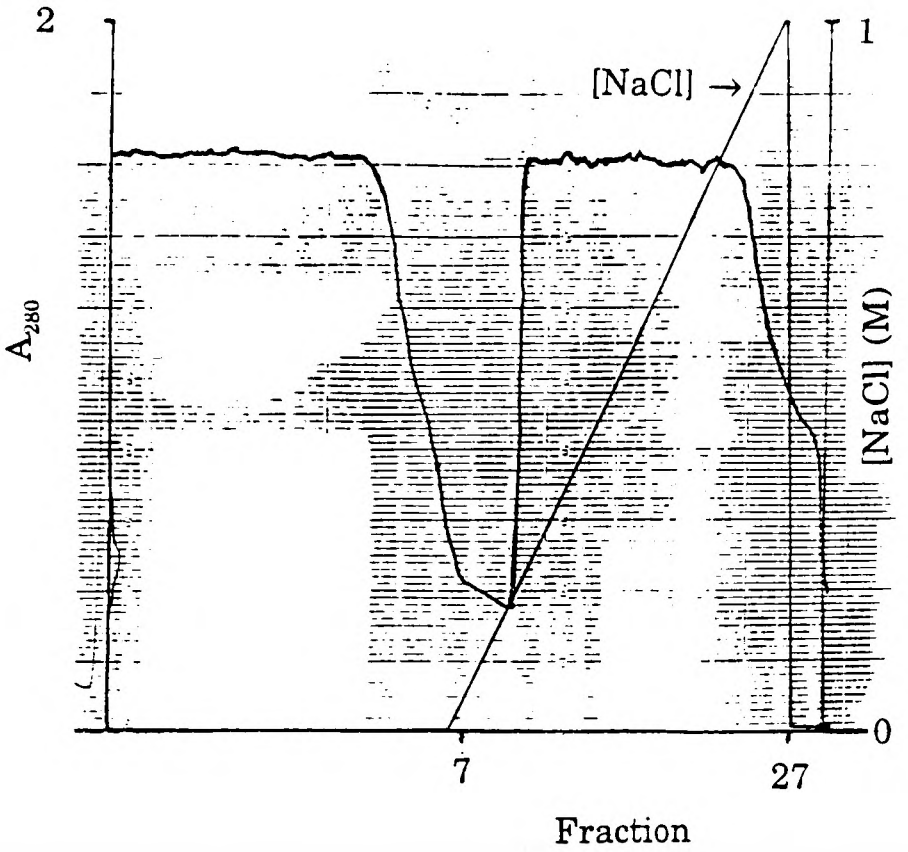
Figure 6.1 Growth conditions for *V. parahaemolyticus*. Cultures were grown (see text) in six media (Table 3.4): Tak1 (lane 7 in ©), Tak2 (lane 6 in ©), LB1 (lane 5 in ©), LB2 (lane 4 in ©), CC1 (lane 3 in ©), CC2 (lane 2 in ©). Initial (open bars) and final (shaded bars) media pH values (A), and final (shaded) Absorbance (600 nm) values (B) were measured. © Exoprotein concentrates from each of the media (see above for lane designation) were analysed by SDS-PAGE on gradient gels (8 to 25 % total acrylamide). Lane 8 contained molecular weight markers in descending order of molecular weight: phosphorylase *b* (97.4 kd), BSA (66.2 kd), ovalbumin (45.0 kd), carbonic anhydrase (31.0 kd), soybean trypsin inhibitor (21.5 kd), and lysozyme (14.4 kd). Lane 1 was loaded with purified TDH.

by CP10, samples (20 µl) were added to an equal volume of SDS-boiling buffer, boiled for 3 minutes, and aliquots (1 µl) run on an 8 - 25 % gradient gel, bands being stained by Coomassie Blue (section 3.9; Figure 6.1C). TDH was recognised by M_w (22.8), and correspondence to a previously purified (and amino-terminal sequenced) TDH preparation (lane 8; courtesy of Dr. V. Sathyamoorthy (Sathyamoorthy, 1994)). Interestingly, although LB (independent of starting pH) was the optimal medium for growth, it was comparatively poor for sustaining TDH production, although other exoproteins were produced in abundance (Figure 6.1C - lanes 4 and 5 are LB1 and 2 respectively). Bacterial growth in the medium of Cherwonogrodzky & Clark (designated CC1; Cherwonogrodzky & Clark, 1981, 1982a,b) was poor, as has been noted for another *V. parahaemolyticus* strain by Douet *et al.* (1992). In LB media, pH appeared to have little effect on either production of exoproteins, or bacterial growth; however, in Tak and CC media, a decreased initial media pH caused a decrease in the final measured optical density of the culture. On the basis of these data, Tak1 medium was selected as the medium of choice for the production of TDH.

Samples (lanes 2 - 7) were pooled and purified to homogeneity (ie migration as a single band on SDS-PAGE) by sequential steps of ion exchange (MonoQ, using a NaCl gradient of 0 - 1 M) and gel filtration (Superose12, of peak fractions assayed by SDS-PAGE), described in sections 3.8.4 and 3.8.5. The elution patterns (with attendant SDS-PAGE) are shown in Figures 6.2A,B. In order to maximise purity in as few steps as possible, only sample fractions (12 and 13), achieving good differentiation between TDH and other exoproteins, were selected after anion exchange (Figure 6.2A), for centricon concentration and resolution by gel filtration. After gel filtration (Figure 6.2B), samples 32 and 33 were pooled as pure TDH, again recognised by molecular weight on SDS-PAGE (see Figure 6.1), and its correspondence to previously purified TDH (courtesy of Dr. V. Sathyamoorthy). To positively identify the purified protein, a small sample was subjected to amino-terminal sequencing (section 3.10; Moos *et al.*, 1988), and the sequence obtained was found to correspond to established data (Nishibuchi & Kaper, 1985; first 12 residues). These data illustrate that good purification of TDH (M_w = c. 22.8 kd, by Ferguson plot analysis) is possible by a two step procedure (ion exchange followed by gel filtration) if care is taken to eliminate those samples with contaminating protein (even if they contain some TDH). This

A

(i)



(ii)

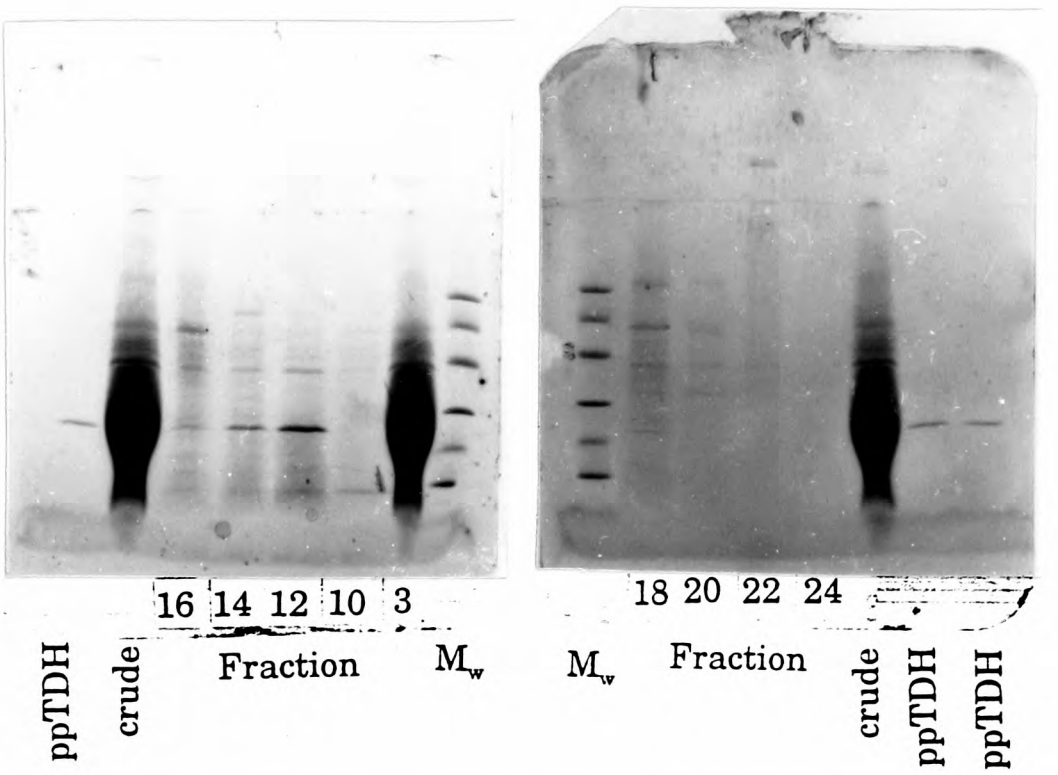
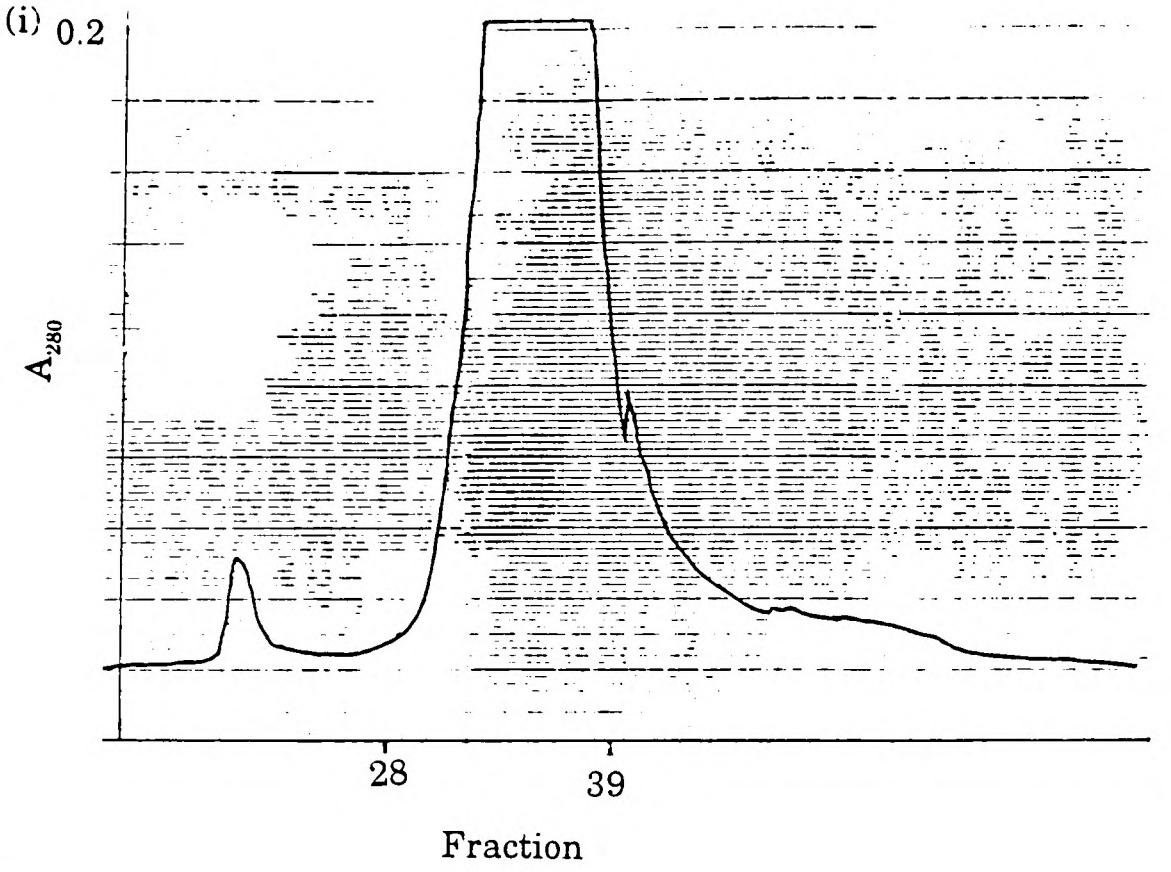
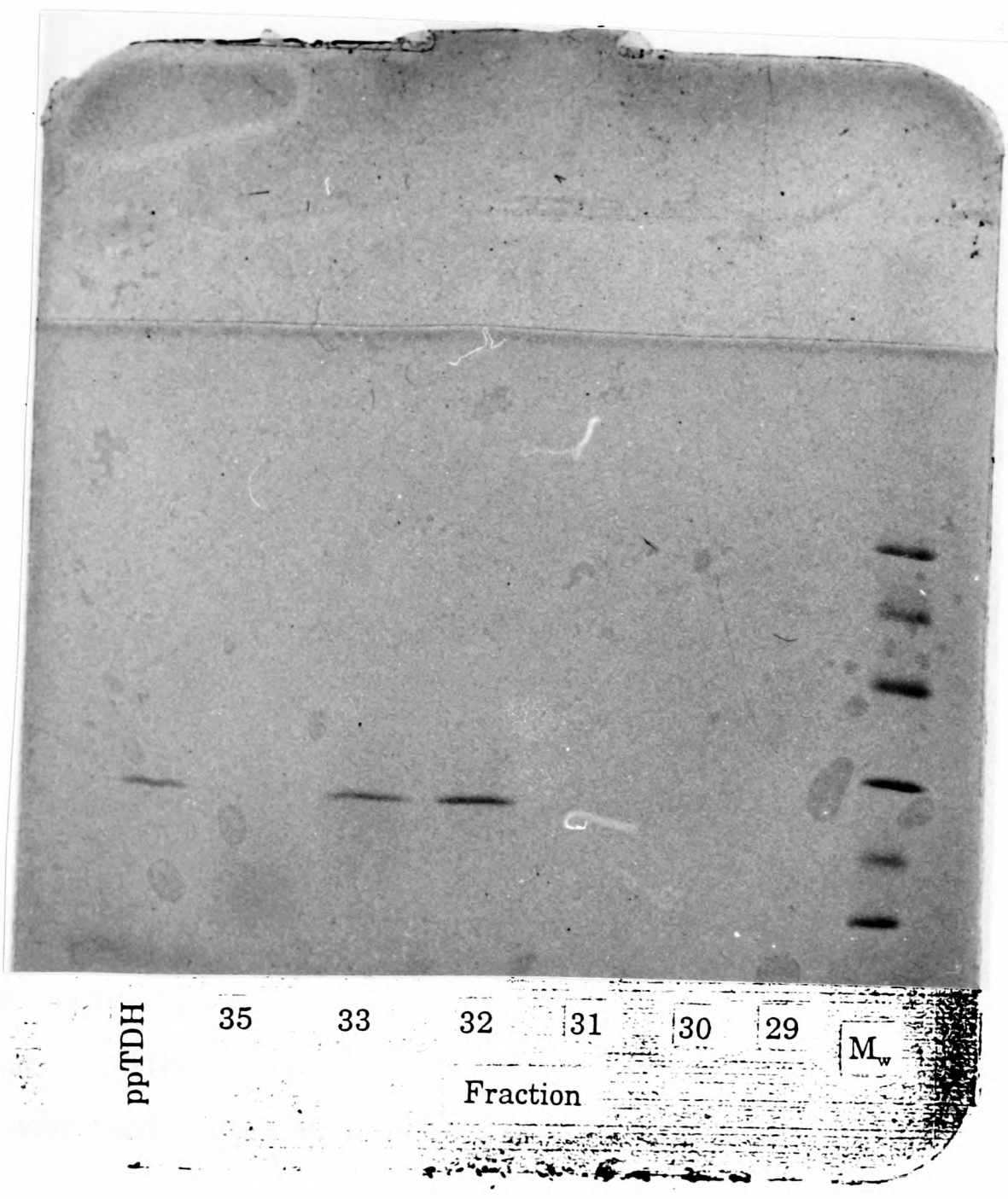


Figure 6.2 Purification of TDH from pooled culture supernatants. Elution profiles and SDS-PAGE analyses obtained in the preparation of pure TDH. For gel lanes, loads are as indicated; M_w = molecular weight markers (as for Figures 3.10 and 6.1), ppTDH = previously purified TDH (courtesy of Dr. V. Sathyamoorthy), and crude = preparation prior to fractionation. (A) (i) MonoQ (anion exchange) elution pattern (flow rate = 1 ml(min)$^{-1}$; fraction size = 1 ml), and (ii) attendant SDS-PAGE of fractions. (B) - see next page - (i) Superose12 (gel filtration) elution pattern (flow rate = 0.7 ml(min)$^{-1}$; fraction size = 1 ml) of peak fractions (12 and 13, from (A)), and (ii) SDS-PAGE of fractions obtained (fraction 32 taken as pure TDH).

B



(ii)



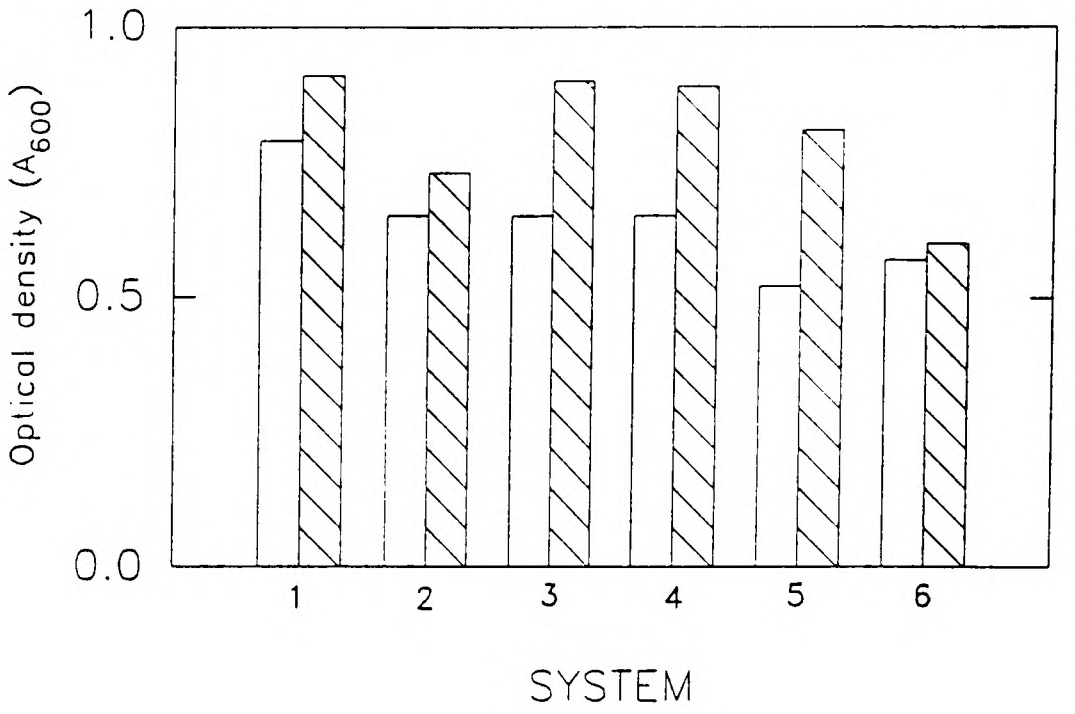
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shows the advantage of fraction analysis by SDS-PAGE, rather than an assay of activity (eg haemolytic activity), which may allow the copurification of other factors, (Honda *et al.*, 1976c).

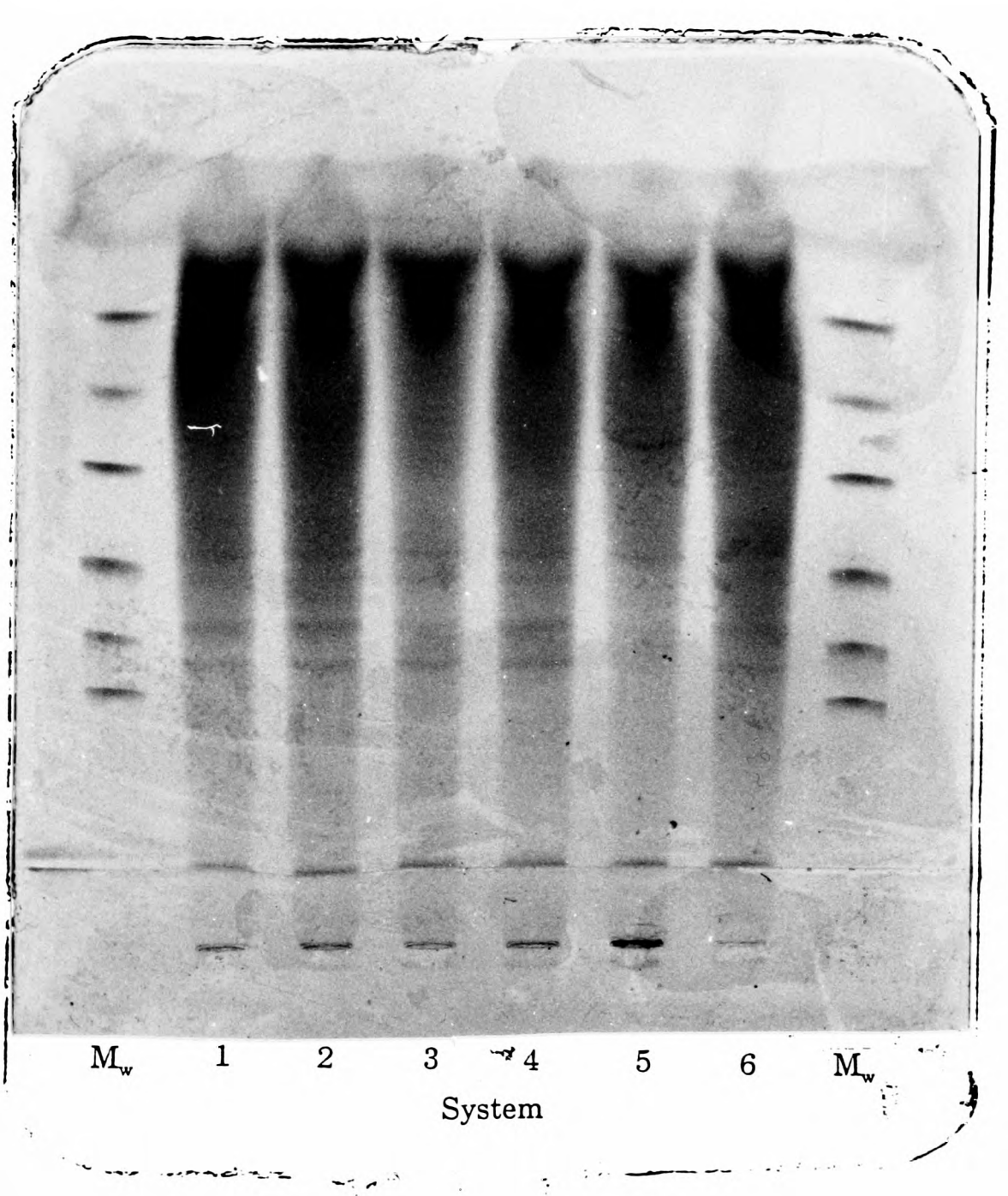
It seemed that the ability of media to support *V. parahaemolyticus* growth and to induce TDH expression might be inversely related (Figure 6.1; Clark & Cherwonogrodzky, 1984). The possibility of using a two stage method of bacterial culture to produce TDH was investgated. Reports have shown that osmolality changes can trigger virulence factor production by *V. cholerae* (Mekalanos, 1992), and that iron limitation can increase TDH production by *V. parahaemolyticus* though it decreases bacterial growth (Dai *et al.*, 1992). In order to assess the possibility of manipulating one of these variables to enhance TDH production by *V. parahaemolyticus* AQ 3817, the composition of some of the second phase media were altered from LB1, either by decreasing $[Na^+]$ or limiting iron available in the medium by the addition of 2,2'-dipyridyl (200 μM) (Dai *et al.*, 1992). Compositions of the second phase media are given in the legend to Figure 6.3. In the first stage, *V. parahaemolyticus* was cultured in LB1 (0.8 l), which has been shown to support good bacterial growth (Figure 6.1) for 20 hours (after LB1 starter culture innoculation (1 % v/v)), when A_{600} = 1.3. Bacteria were lightly centrifuged (CP6) and resuspended in fresh LB1 (75 ml). Aliquots (10 ml) of this thickened culture were added to six fresh media systems (150 ml each, in GSA centrifuge bottles). These cultures were grown for 7 hours, initial and final A_{600} values being recorded (Figure 6.3A), before harvesting by centrifugation (CP7) and ammonium sulphate precipitation (as above). Exoprotein precipitates were resuspended in Tris-HCl (20 mM, pH = 7.5; volume = 3 ml, for each 150 ml culture) buffer with protease inhibitors, and were then dialysed extensively against the same. Each system was concentrated tenfold using TCA precipitation (section 3.8.3) and run on an 8 - 25 % gradient gel (Figure 6.3B). These data confirm Tak1 medium as being optimal for TDH expression, and suggest that the two stage method for bacterial culture and toxin production may be a viable approach in future.

Subsequently, TDH purification was attempted on a larger scale. Forty litres of Tak1 medium were used to grow *V. parahaemolyticus* AQ 3817 as outlined above. Final culture

A


1.0
0.5
0.0
Optical density (A_{600})
1
2
3
4
5
6
SYSTEM

B


M_w
1
2
3
4
5
6
M_w
System

previous page:
Figure 6.3 TDH production in two phase cultures of *V. parahaemolyticus*. Bacteria were cultured as described in text. Six media were used for the second phase of culture: (1) LB1, (2) LB1 (but pH = 6.7), (3) LB1 (but (NaCl) = 1.5 g(l^{-1}), as opposed to 5 g(l^{-1})), (4) LB1 + 2,2'-dipyridyl (200 μM), (5) Tak1, and (6) CC2. After 7 hours growth, the supernatants were harvested and concentrated as described in text. Initial (open bars) and final (shaded bars) A_{600} values for the second phase culture were recorded (A). In (B) each of these samples were run on an 8 - 25 % total acrylamide gel. Lanes 1 and 8 contained molecular weight markers in ascending order of molecular weight: phosphorylase *b* (97.4 kd), BSA (66.2 kd), ovalbumin (45.0 kd), carbonic anhydrase (31.0 kd), soybean trypsin inhibitor (21.5 kd), and lysozyme (14.4 kd). Tak1 (Lane 6; system 5) yielded a pronounced band correspondent to TDH. The other media did not produce such yields.

A_{600} values were less than those observed previously (Figure 6.1), being 0.32 ± 0.03 (n = 4). The final yield of TDH after analagous purification to that outlined in Figure 6.2, although not outstanding (estimated from Coomassie-stained SDS-PAGE to be c. 6 mg) would have been adequate for the purposes envisaged - analysis of the effects of TDH on (i) erythrocyte membranes, and (ii) mounted rabbit ileal tissue in Ussing Chambers (Hall *et al.*, 1994), using the methodology outlined by Nishibuchi *et al.* (1992). Unfortunately, an extra band (c. 20 kd) and a brown pigment co-eluted with TDH in this preparation. Because of time limitations, KH was used as the starting preparation from which to produce highly purified TDH (see (ii) below).

(ii) Purification of TDH from the KH (Sigma) preparation. Highly purified TDH, for further studies (see Chapter 7), was purified from the commercially available KH by Dr. V. Sathyamoorthy, following the procedure outlined above (in *(i)*). KH (1000 HU(ml)$^{-1}$) was reconstituted in Tris-HCl buffer (20 mM, pH 7.5; volume = 1 ml) and subjected to ion exchange chromatography (MonoQ) and gel filtration (Superose12), as described above, and in sections 3.8.4 and 3.8.5 (see Figure 6.4). The identity of this protein was confirmed by amino-terminal sequencing (twenty residues (Nishibuchi & Kaper, 1985; for methodology, see section 3.10; Moos *et al.*, 1988). The sample was then lyophilised by speedvacuum. Reconstitution was by addition of 50 µl doubly distilled water and then 3.95 ml MBS; aliquots (0.33 ml) were stored at - 22 °C and thawed prior to use. Two such preparations were made: for one, the mass of protein purified was assayed (Sathyamoorthy, 1994) using the method elaborated by Bradford (1976), allowing later estimation of the number of molecules interacting with target membranes (see Chapter 7).

6.2.2 *Vibrio cholerae* El Tor non-O1 8731 and ETH

V. cholerae 8731 is an environmental *ctx*A$^-$ strain, with high constitutive expression of ETH (Hall & Drasar, 1990). LB1 was used as the medium of choice for optimal expression of ETH (Hall, 1994). LB1 (15 l) was innoculated with *V. cholerae* 8731 (1 % v/v) starter culture (LB1; 16 hours, 37 °C, shaking speed = 100 r.p.m.) and cultured (section 3.7) for 24 hours (culture conditions as above; final O.D. = 1.25) before harvesting of exoproteins by centrifugation (CP7) and precipitation with ammonium sulphate (313 g(l)$^{-1}$)). The precipitate

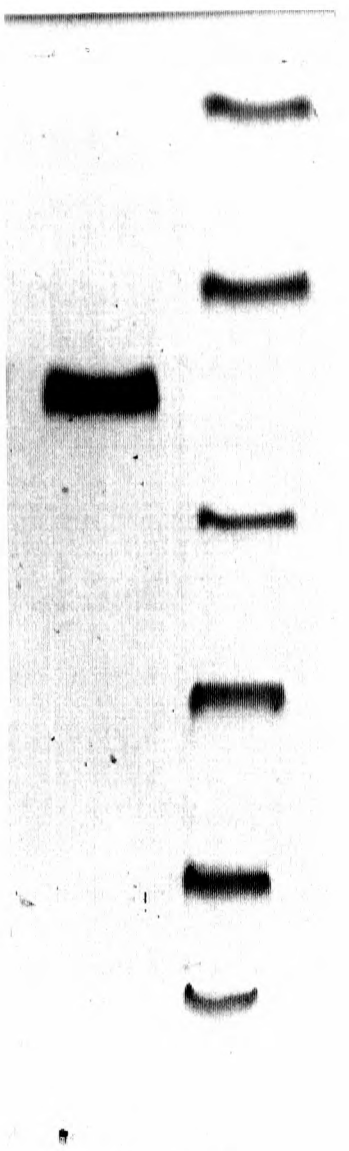


Figure 6.4 Purity of TDH purified from KH. This was determined by SDS-PAGE in gradient gels containing 10 - 20 % total acrylamide, with subsequent staining by Coomassie Blue. Track A was loaded with 10 μg purified TDH. Track B contained molecular weight markers in ascending order of molecular weight: phosphorylase *b* (97.4 kd), BSA (66.2 kd), ovalbumin (45.0 kd), carbonic anhydrase (31.0 kd), soybean trypsin inhibitor (21.5 kd), and lysozyme (14.4 kd).

was resuspended in *bis*-Tris-HCl buffer (25 mM; pH = 7.1; c. 150 ml) containing peptidase inhibitors and dialysed exhaustively against the same (section 3.8.1).

ETH was purified to homogeneity (ie migration as a single band on SDS-PAGE) by sequential steps of chromatofocussing (MonoP, using a starting buffer of *bis*-Tris-HCl (25 mM; pH = 7.1) and an eluent of polybuffer 74/HCl (pH = 4.0)) and gel filtration (Superose12, of peak fractions), described in sections 3.8.4 and 3.8.5. The elution patterns are shown in Figures 6.5A,B with attendant SDS-PAGE. Sample fractions 24 to 32 were selected after chromatofocussing (Figure 6.5A) for centricon concentration and resolution by gel filtration. After gel filtration (Figure 6.5B), samples 39 to 42 were pooled as pure ETH which was then freeze-dried. ETH was recognised by molecular weight on SDS-PAGE, and after amino-terminal sequencing (section 3.10; Moos *et al.*, 1988). Analysis of the first 12 residues indicated ETH to be the 68.6 kd form, as given by Hall and Drasar (1990).

6.2.3 CT⁻ *Vibrio cholerae* and CHO cell morphology

Following an observation that even CT⁻ *V. cholerae* produced a factor that was capable of elongating CHO cells (Kothary, 1994), *V. cholerae* 8731 was examined for this activity. The bacteria were grown (20 hours; final A_{600} value = 1.2) in CYE (4 l), after innoculation (1 % v/v) from LB1 starter culture, and the exoproteins harvested by ammonium sulphate (313 g per l) precipitation. After resuspension in buffer (Tris-HCl (20 mM); pH = 7.5; volume = 5 ml) and exhaustive dialysis against the same, the sample was filter sterilised (section 3.8.2) and applied to a MonoQ column before elution by a 0 - 1 M NaCl gradient (Figure 6.6A). Each of the resultant fractions were tested on CHO cells (Table 6.1; section 3.3.3); samples (20 µl) of each fraction were added to wells of 120 µl capacity (CHO cell suspension volume added = 100 µl). Each successive row (Tables 6.1 and 6.2) contained a x 2 dilution of sample. Both cytotoxic (attributable to ETH) and cytotonic activities were discernible. The fractions with cytotonic activity were concentrated (10 x) by TCA precipitation (section 3.8.3) and run on SDS-PAGE (8 - 25 % total acrylamide), before silver staining (Figure 6.6B; section 3.9.1). A number of bands were observed, and it was not possible to ascribe the cytotonic activity to any in particular.

Other *V. cholerae* strains (Table 3.3) were also cultured in CYE (20 hours) after

A

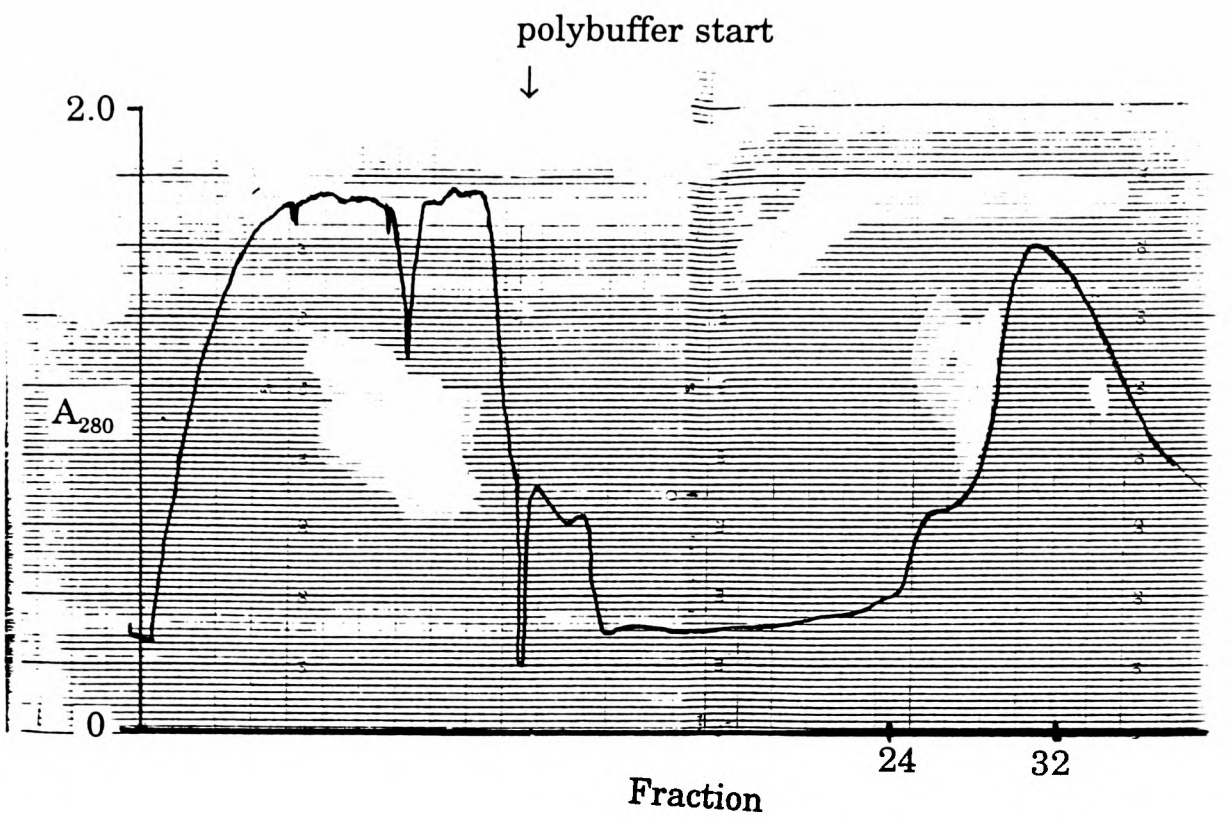
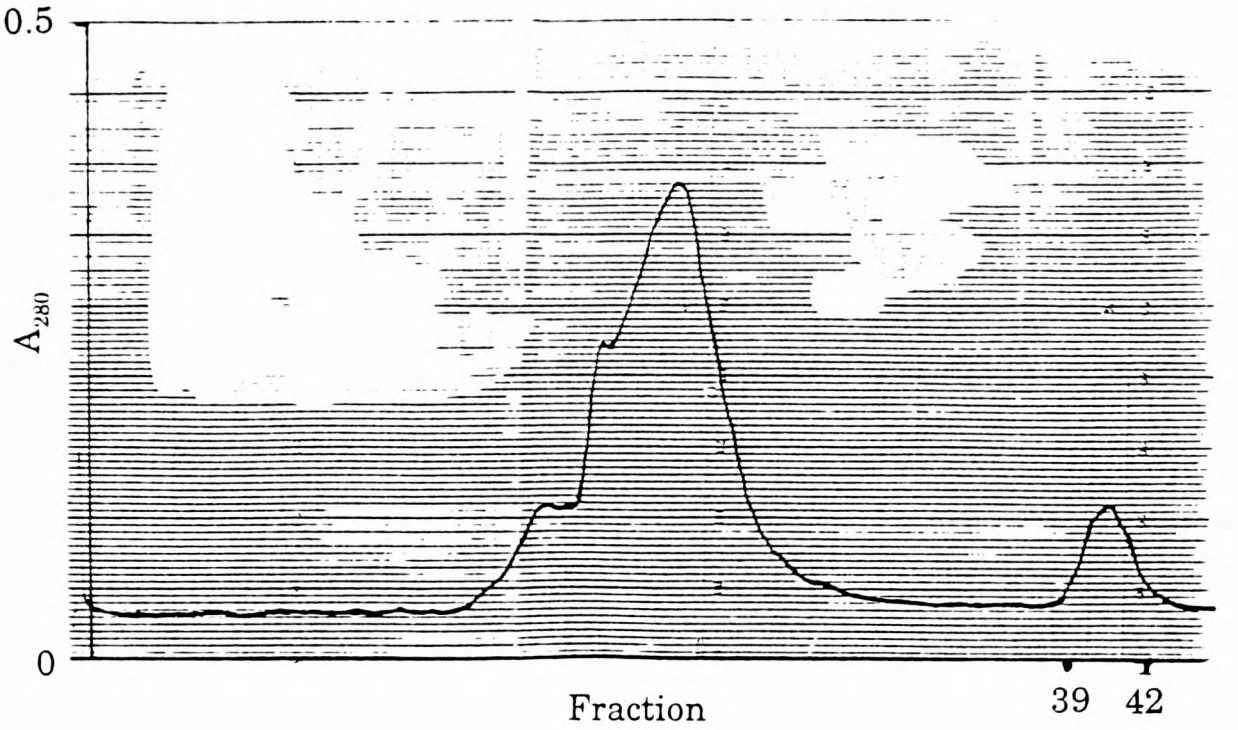
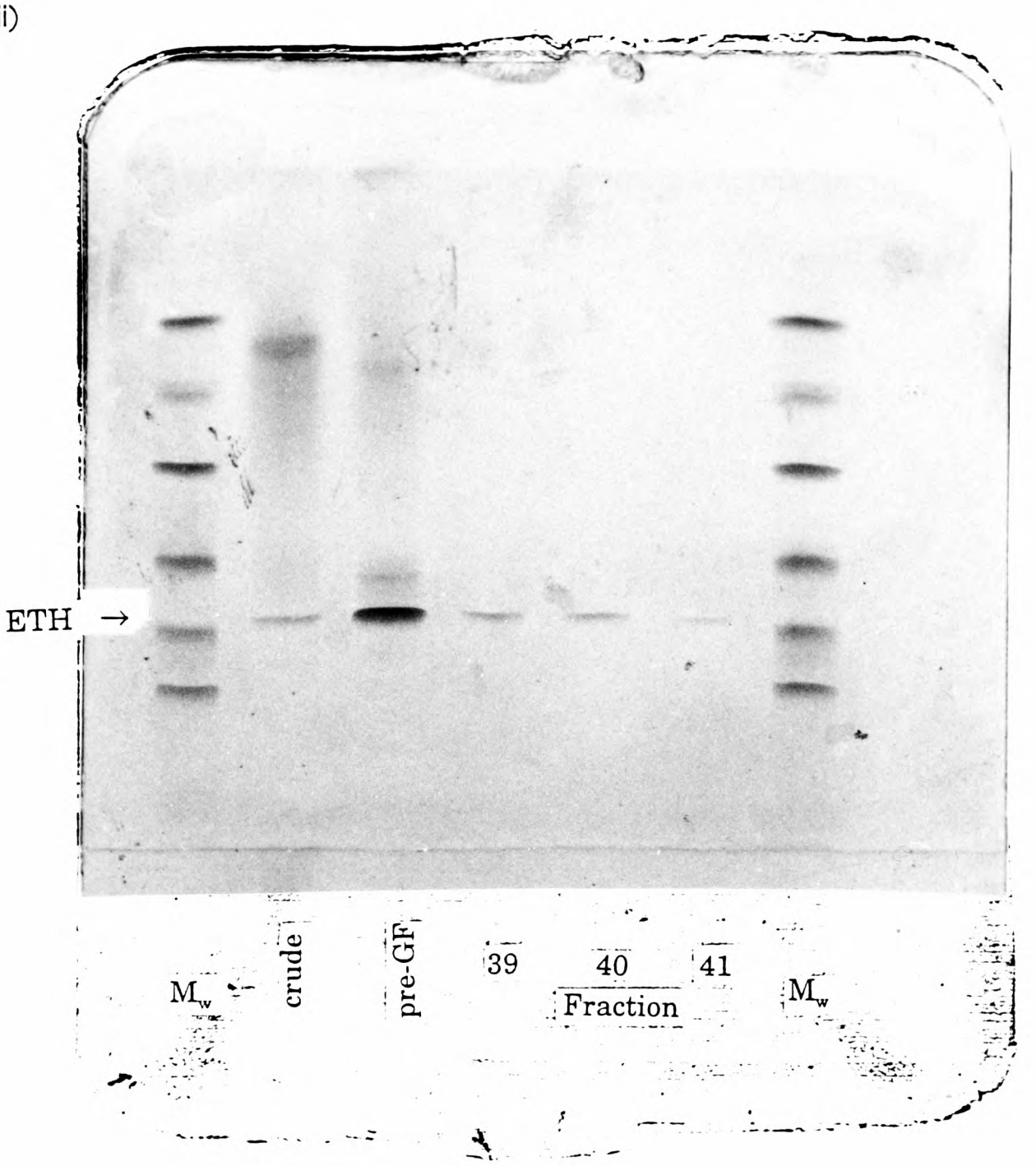


Figure 6.5 Purification of ETH. (A) MonoP (chromatofocussing) elution profile of the dialysed ammonium sulphate fraction (flow rate = 1 ml(min)$^{-1}$; fraction size = 1 ml); the start buffer was *bis*-Tris-HCl (25 mM; pH = 7.1) and the eluent was polybuffer 74/HCl (pH = 4.0). Sample fractions 24 - 32 were pooled and concentrated prior to gel filtration. (B) (i) Superose12 (gel filtration) elution profile (flow rate = 0.7 ml(min)$^{-1}$; fraction size = 1.5 ml) of sample obtained from (A). (ii) Fractions were analysed by SDS-PAGE on gradient gels containing 8 to 25 % total acrylamide. Samples 39 - 42 were pooled as purified ETH. Lanes 1 and 8 contained molecular weight (M_w) markers in ascending order of molecular weight: phosphorylase *b* (97.4 kd), BSA (66.2 kd), ovalbumin (45.0 kd), carbonic anhydrase (31.0 kd), soybean trypsin inhibitor (21.5 kd), and lysozyme (14.4 kd). Other lanes are as indicated: crude = exoprotein mixture applied to MonoP column; pre-GF = exoprotein mixture applied to Superose12 column. Fractions are after gel filtration.

(i)

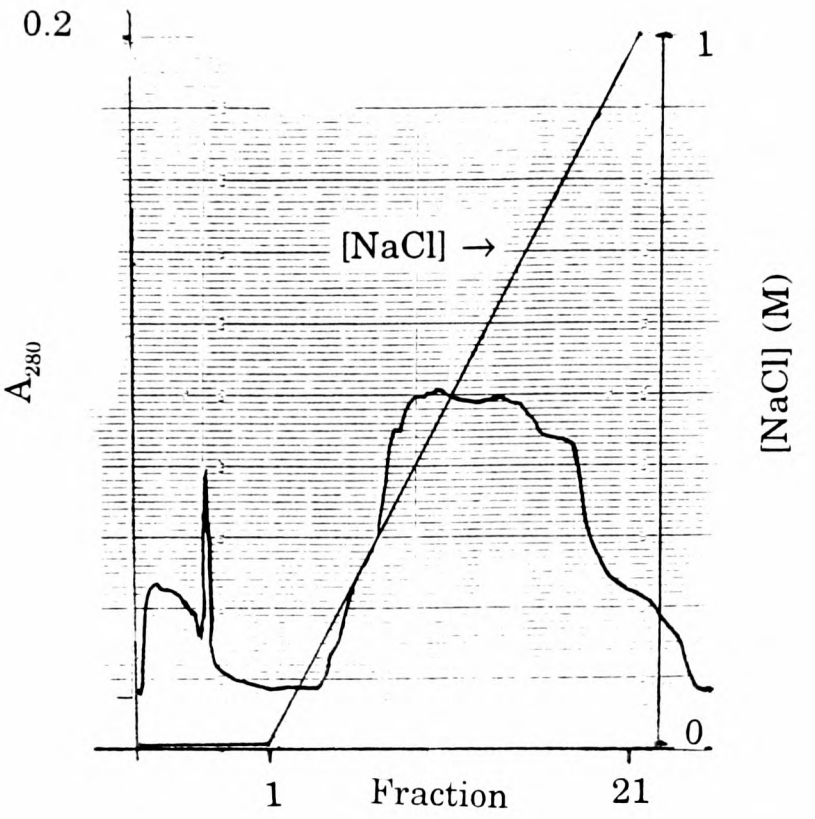


(ii)



see previous page for legend

A



B

Figure 6.6 Fractionation of exoproteins from *V. cholerae* 8731. (A) MonoQ (anion exchange) elution pattern (flow rate = 1 ml(min)$^{-1}$; fraction size = 1 ml) of exoprotein concentrate from *V. cholerae* 8731 (see text). (B) SDS-PAGE (with silver staining); lanes 2 to 7 contained the indicated fraction concentrated 10 times using TCA precipitation (samples were then heated with an equal volume of SDS boiling buffer (section 3.9) and 1 µl samples were used). Lane 1 contained molecular weight markers in ascending order of molecular weight: phosphorylase *b* (97.4 kd), BSA (66.2 kd), ovalbumin (45.0 kd), carbonic anhydrase (31.0 kd), soybean trypsin inhibitor (21.5 kd), and lysozyme (14.4 kd). Refer to Table 6.1 for CHO cell activity of these fractions.

Fraction	-	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	PQ	FT
1/12	-	L	E	L	E	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
1/24	-	L	E	E	E	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
1/48	-	E	E	E	E	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	-
1/96	-	-	E	E	E	E	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	-
1/192	-	-	E	E	E	E	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	-
1/384	-	-	E	-	E	E	-	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	-
1/768	-	-	-	-	-	-	-	L	L	L	L	L	L	L	L	L	L	L	L	-	-	L	-
1/1536	-	-	-	-	-	-	-	L	L	L	L	L	L	L	L	L	L	L	-	-	-	L	-

Table 6.1 CHO cell assay of fractions derived from the supernatant of *V. cholerae* 8731. The assay was performed as described in section 3.3.3. The first column shows the titre (volume:volume) of the eluted fractions (obtained as described in accompanying text; see Figure 6.6) added to the assay medium. Two types of toxic activity were identified: (i) cytotoxic (causing lysis (L = 100 % lysis)), and (ii) cytotonic (causing cell elongation (E = > 50 % cells elongated)). See Figure 6.6B for SDS-PAGE of fractions shown to have cytotonic activity. PQ is the crude preparation prior to MonoQ treatment; FT (flow-through) is the sample obtained prior to elution with the NaCl gradient.

V. cholerae strain:	395 N1	CVD 101	CVD 103	CVD 103-HgR	CVD 103-HgR2	buffer solution
1/12	lysed 100 %	lysed 100 %	lysed 100 %	lysed 100 %	lysed 100 %	-
1/24	lysed 100 %	lysed 100 %	lysed 100 %	lysed 100 %	lysed 100 %	
1/48	lysed 100 %	lysed 100 %	lysed 100 %	lysed 100 %	>50 % elong.	
1/96	>50 % elong.	lysed 100 %	lysed 100 %	lysed 100 %	>50 % elong.	-
1/192	>50 % elong.	lysed 100 %	lysed 100 %	lysed 100 %	>50 % elong.	
1/384	>50 % elong.	>50 % elong.	lysed 100 %		>50 % elong.	-
1/768	-		-		>50 % elong.	
1/1536	-		-			-

Table 6.2 CHO cell assay of crude exoprotein mixtures derived from the supernatant of CT⁻ *hly*A-disrupted *V. cholerae*. The assay was performed as described in section 3.3.3. The first column shows the titre (volume:volume) of samples (obtained as described in text) added to the assay medium. Two types of toxic activity were identified: (i) cytotoxic (causing lysis (lysed 100 %)), and (ii) cytotonic (causing cell elongation (> 50 % cells elongated)).

innoculation by LB1 starter culture (1 % v/v). Final A_{600} values were in the range 1.11 - 1.51. Supernatants were concentrated c. 200 x by ammonium sulphate precipitation (313 g per l), with resuspension of protein in Tris-HCl buffer (20 mM; pH = 7.5; 3 ml volume each), and exhaustive dialysis against the same buffer. Samples (20 µl) of these exoprotein concentrates were added to wells of 120 µl capacity (CHO cell suspension volume added = 100 µl). Each successive row (Table 6.2) contained a x 2 dilution of exoprotein concentrate. Both cytotoxic (lytic) and cytotonic (cell elongating) activites were noted over a range of titres; cytotonic activities were especially apparent for *V. cholerae* strains 395 N1 and CVD 103-HgR2. The resuspending-buffer solution was ineffective (negative control).

6.3 DISCUSSION

The data presented in this chapter show the microbiological basis underlying, and purification protocols used for, the production of bacterial toxins in pure form and sufficient quantities for subsequent study (Chapters 7 and 8). Also evident are the problems that may arise in such investigations, including the copurification of a separate (but close to TDH in M_w) exoprotein.

Douet *et al.* (1992) purified TDH which they identified on the basis of thermostability, insensitivity of equine erythrocytes, and peptide mapping on SDS-PAGE. Under denaturing conditions, TDH showed as a band of M_w 29 kd, higher than that previously shown (Honda *et al.*, 1976c; Takeda *et al.*, 1983; section 2.6.4). In addition, TDH gave a band of c. 29 kd under non-denaturing conditions, suggesting it to be a monomeric protein. This contrasts with other studies which have suggested TDH to exist in dimeric form (Zen-Yoji *et al.*, 1974; Miyamoto *et al.*, 1980). As indicated above (section 6.1), Douet *et al.* (1992) also reported the non-homogeneity of KH, finding it to give an extra band at c. 32 kd, and it was therefore important to obtain a highly purified preparation to confirm that the activity characterised in Chapters 4 and 5 could indeed be attributed to TDH (Chapter 7).

V. parahaemolyticus produced a variety of exoproteins, dependent on the growth conditions (Figure 6.1). Interestingly, although Luria Broth media (designated LB1 in the present investigation) used to culture *V. parahaemolyticus* in the study of Nishibuchi *et al.* (1992)

was found to be the best for bacterial growth (Figure 6.1B), it was the poorest of the media tested for TDH production (Figure 6.1C). Tak1 media (see Table 3.4) was optimal for TDH production and hence selected as the medium of choice.

Sakazaki *et al.* (1974) examined the enteropathogenic activity of various strains of *V. parahaemolyticus* using ligated loops of rabbit gut. These workers found that culture filtrates of bacteria caused fluid accumulation irrespective of KP, and that the factor was heat labile. A TDH preparation did not produce comparable effects. However, when innoculi of live bacteria were used in the gut loop model, KP^+ strains were significantly more reactogenic than KP^- ones. These results were held to suggest that it is the ability of *V. parahaemolyticus* to multiply within the intestine (which they found to be generally associated with KP) which is the essential factor governing enteropathogenicity, and that an enterotoxigenic factor, produced by the bacteria irrespective of KP, is the major virulence factor.

If, however, TDH is an important enterotoxin, as implied by other studies (sections 2.6.3 and 2.6.4; Obara *et al.*, 1974; Nishibuchi *et al.*, 1992; Douet *et al.*, 1992), then it may be a limitation on bacterial growth that acts to trigger toxin production and disease (Dai *et al.*, 1992). Data shown in Figure 6.1A are in accord with this hypothesis, TDH production being minimal in the LB media that was optimal for bacterial growth. In addition, Cherwonogrodzky and Clark (1981, 1982a,b) showed that TDH was produced by *V. parahaemolyticus* throughout the growth cycle but especially when culture medium pH was in the range 6.0 - 6.5, when growth was relatively limited. Clark and Cherwonogrodzky (1984) observed that continued growth of *V. parahaemolyticus* could result in the culture converting to a non-haemolytic form, and further that there are two genetically distinct forms of non-haemolytic bacteria, one of which carries the information for TDH synthesis in a silent manner.

TDH purified to homogeneity from the first culture batch (Figure 6.2) was donated to Dr. R. H. Hall to produce anti-TDH antibodies (Hall, 1994), for use in a bead-based immunoaffinity column for subsequent purification of TDH, as has been reported by Honda *et al.* (1986b). Good yields were also obtained from a two-stage culture, in which *V. parahaemolyticus* was grown in LB1 before resuspension in second phase media (Figure 6.3),

and this may be a strategy for future use. Furthermore, Tak1 was confirmed as the media of choice for TDH production. However, subsequent attempts to purify large amounts of TDH from culture supernatants by the protocol given in Figure 6.2 were not successful, with the co-elution of a protein indicated by a band of c. 20 kd. The reason for the differences between these two preparations remains unclear.

Using a starting preparation of KH, it was possible to produce highly purified TDH by an analagous protocol (Figure 6.4). The properties of this TDH preparation have been investigated by experiments described in Chapter 7. The homogeneous ETH preparation obtained (Figure 6.5; Hall & Drasar, 1990; Hall, 1994) was freeze-dried for transit; samples were reconstituted by addition of the appropriate volume doubly distilled water (20 - 40 µl range) and MBS (see Chapter 8).

A potential vaccine strain, *V. cholerae* JBK 70 has been observed to produce a factor in its periplasmic space that is capable of altering CHO cell morphology (Kothary, 1994). Similar observations were made in the present study for *V. cholerae* 8731 (Table 6.1; Figure 6.6A), and a range of CT$^-$ *hly*A-disrupted *V. cholerae* strains (Levine *et al.*, 1988a,b; Tables 3.3 and 6.2). Work in progress is aimed at identification of both cytotonic and cytotoxic factors (Hall, 1994).

Data presented in Chapters 4 and 5 have comprehensively characterised the effects of Kanagawa haemolysin (as obtained from Sigma, purified according to the protocol outlined by Cherwonogrodzky & Clark, 1982a) on erythrocytes. This chapter has described work performed to produce highly purified preparations of TDH and ETH. The mechanisms of action of these toxins are examined in the two chapters which follow.

CHAPTER 7

Studies on the lesion induced in human erythrocyte membranes by the thermostable direct haemolysin

'We must now find a sanguine owner...'
Alistair Sampson, *in* Horrortorio

7.1 INTRODUCTION

Work described in Chapters 4 and 5 outlined the seminal features of membrane attack by Kanagawa haemolysin (KH), the commercially available preparation of the thermostable direct haemolysin (TDH) of *Vibrio parahaemolyticus*. As previously addressed (sections 4.1 and 6.1), the purity of this preparation has been questioned on the basis of silver staining after SDS-PAGE (Douet *et al.*, 1992). In Chapter 4 (see section 4.3), no evidence was obtained for the action of accessory factors in this preparation. In particular, haemolytic activity was unaffected by heat treatment (100 °C for 10 minutes) and horse cells were insensitive to toxin action. However, *V. parahaemolyticus* is known to express several putative exotoxins in addition to TDH (section 2.6.3 and Table 2.8) including a phospholipase A (Yanagase *et al.*, 1970), a lysophospholipase (Yanagase *et al.*, 1970), and a TDH-related haemolysin (TRH) (Nishibuchi *et al.*, 1989). Furthermore, thermostable factors other than TDH have been reported (Taniguchi *et al.*, 1990; Honda & Iida, 1993). A thermostable toxin reported by Taniguchi *et al.* (1990), designated δ VPH, was found in all 121 *V. parahaemolyticus* strains tested, irrespective of $KP^{+/-}$. This toxin is genetically and antigenically distinct from TDH, and is capable of lysing human, rabbit, sheep and horse erythrocytes with approximately equal potency. The insensitivity of horse cells to KH (section 4.2.3) suggests that δ VPH is unlikely to make a major contribution to the haemolytic activity of KH.

Results obtained in Chapters 4 and 5 are compatible with other published data, although the conclusions do differ in one important respect from those given by Honda *et al.* (1992). The reasons for the shortcomings of this and other reports have already been discussed (section 4.3). Nevertheless, it is important to exclude the possibility of an action by a toxic entity

other than TDH having influence in the haemolytic action of KH.

This chapter seeks to affirm that the features of KH action already described (Chapters 4 and 5) are attributable to TDH *per se*. The strategy employed was to use a highly purified TDH preparation derived from KH by ion exchange and gel filtration (section 6.2.1), and to re-examine key features of the haemolytic (Chapter 4) and isotope flux (Chapter 5) responses to KH action. Chapter 6 describes the use of microbiological and protein purification techniques to obtain high yields of uncontaminated toxins. For the purified preparation, protein appeared as a single band on SDS-PAGE when stained by Coomassie blue. This was recognised as TDH by molecular weight (c. 21 kd; Douet *et al.*, 1992), the identity being confirmed by amino-terminal sequencing (section 6.2.1).

Two such TDH preparations were produced. For the first, the mass of the preparation was determined and hence TDH concentration (for Figures 7.1- 7.3, 7.6) is given as $\mu g(ml)^{-1}$. The other was similarly purified from 1000 HU KH, but the mass of protein was not determined. This preparation was reconstituted in a total volume of 4 ml to make a stock TDH solution, the concentration of TDH used in subsequent experiments (Figures 7.4, 7.5, 7.7 - 7.10) being given as µl stock solution $(ml)^{-1}$.

Once again, the pharmacological inhibitors of endogenous cation transport pathways, ouabain (Bernhardt *et al.*, 1991), bumetanide (Bernhardt *et al.*, 1991) and nitrendipine (Ellory *et al.*, 1992) were used to block K^+ influx by the Na^+ pump, $Na^+/K^+/2Cl^-$ cotransporter and Gardos channel respectively (section 3.6.1).

7.2 RESULTS

7.2.1 Thermostability of TDH

Stability of haemolytic activity attributable to TDH was confirmed by experiments analagous to those shown for KH in section 4.2.3. Heat treatment (100 °C for 10 minutes) of TDH had no subsequent effect on haemolytic activity (Table 7.1), again suggesting that thermostable factors alone account for the haemolytic activity of this preparation.

7.2.2 Fundamental kinetic characteristics of TDH action

TDH action was investigated by haemolysis experiments analagous to those performed on

	H^{50} (mins)		standardised H^{50} (%)	
[TDH]	untreated	treated	untreated	treated
0.654 μg.(ml)$^{-1}$	39.8 ± 7.48	39.4 ± 7.19	100 (st.)	99.9 ± 4.42
0.327 μg.(ml)$^{-1}$	74.7 ± 18.09	71.1 ± 14.07	100 (st.)	98.4 ± 7.31

Table 7.1 Thermostability of haemolytic activity of TDH. Haemolysis time courses were initiated by addition of packed cells (100 μl) to a solution (5 ml total) comprised of CsCl (150 mM), HEPES (10 mM), ouabain (0.1 mM), bumetanide (0.1 mM), and nitrendipine (0.01 mM), at pH = 7.4, with TDH at the indicated concentration. For 'treated' preparations, the TDH preparation was heated at 100 °C for 10 minutes, and allowed to cool to room temperature before addition to the solution.

KH (section 4.2.1). Haemolytic rate was (similarly) strongly dependent on TDH concentration (Figure 7.1A). Haemolysis was also measured in the same haemolytic range ([TDH] = 0.654 $\mu g(ml)^{-1}$), but with varied haematocrit. Standardised $\{(H^{50})^{-1}\}$ was plotted against haematocrit (Figure 7.1B), and the lines of best fit calculated by least squares regression. The mean gradient of these relations (n = 3) is 0.0271 ± 0.00950 $(Hct\ (\%))^{-1}$, which is not significantly different from zero at the five percent significance level (unpaired t test). Thus, the rate of haemolysis (expressed as percentage of total) was essentially independent of target cell concentration ($1 \leq Hct\ (\%) \leq 6$).

Figure 7.2 shows dose-response data for TDH-induced haemolysis, a measure of haemolytic rate being given by the H^{50} index. As noted previously (section 4.2.1), the effect was profoundly donor dependent (Figure 7.2A). Normalisation of H^{50} values showed that the *relative* effects of given toxin doses did not vary greatly - if at all - between donors (Figure 7.2B). Furthermore, the parameter $\{(H^{50})^{-1}\}$ showed an apparently linear relation with TDH concentration (Figure 7.2C) in this range of TDH concentrations ($0.327 \leq [TDH]\ (\mu g.(ml)^{-1}) \leq 1.635$). Linear regression analysis of these data (all donors included) gives a line $y\ (\{(H^{50})^{-1}\}) = m.x([TDH]) + c$, where m = 1.183 ± 0.0969 $ml.(\mu g)^{-1}$ and c = - 0.1280 ± 0.08998 (dashed line in Figure 7.2C; correlation coefficient = 0.9649). It was possible to estimate the number of TDH molecules/HRBC. This estimate was obtained by substituting known values into the variables of the following equations:

$$\mathbf{h = (N \times m)/M} \quad ...(1)$$

wherein **h** is the number of haemolysin (TDH) molecules, **N** is Avogadro's number (6.02×10^{23} $particles(mol.)^{-1}$ (Stein, 1990)), **m** is the mass (μg) of haemolysin present and **M** is the mass (μg) of 1 mole of haemolysin (calculated from known M_w for TDH (21 kd; Takeda, 1983).

$$\mathbf{b = (V \times Hct)/a} \quad ...(2)$$

wherein **b** is the number of HRBC, **V** is the volume of the cell suspension (μm^3), **Hct** is a proportionate value and **a** is the average cell volume (μm^3).

For the TDH concentration taken as standard in Figure 7.2 (0.981 $\mu g(ml)^{-1}$), and taking the average initial HRBC volume to be 90 μm^3 (Stein, 1990), these calculations indicate that there

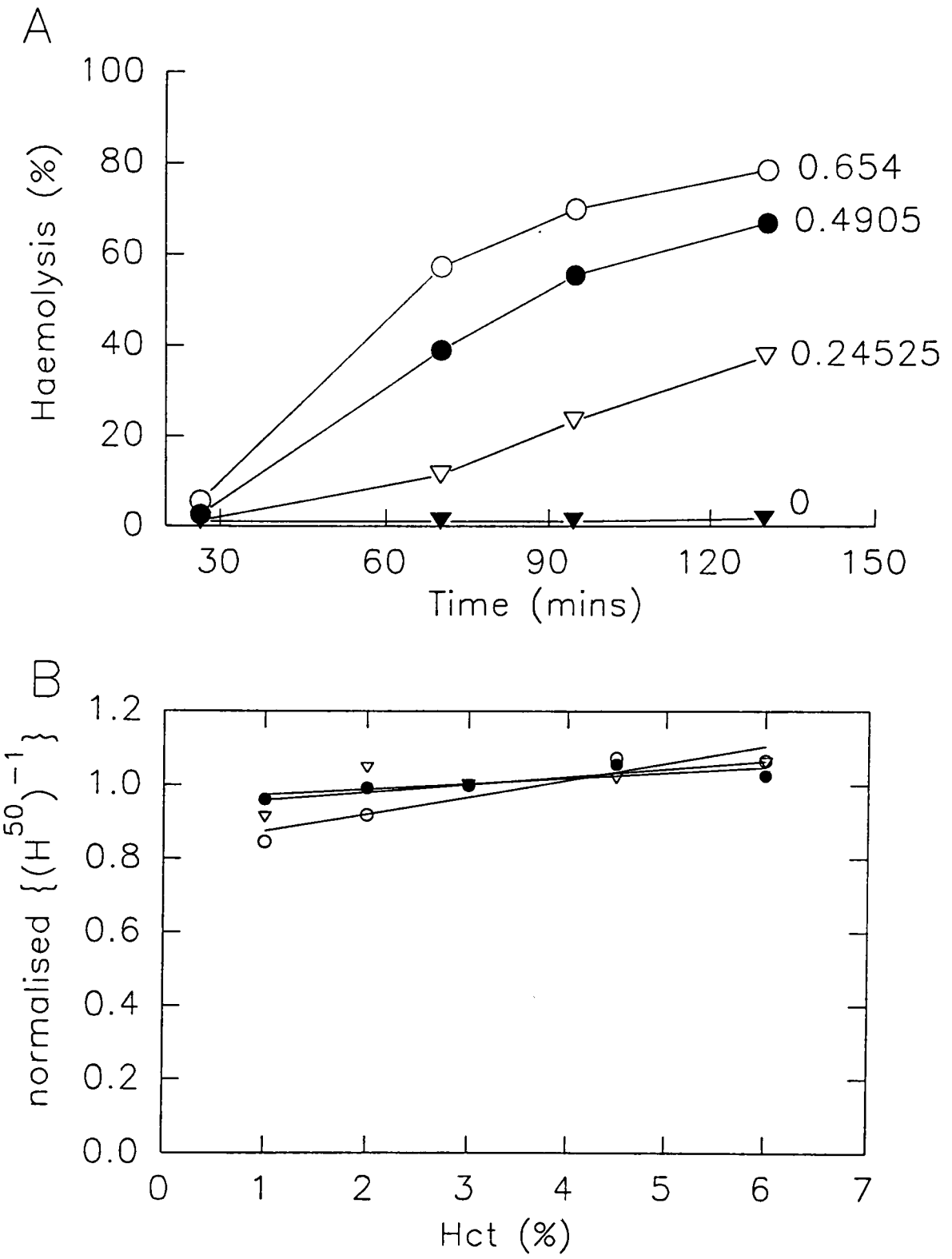


Figure 7.1 Kinetics of TDH-induced haemolysis. Time-courses of haemolysis were measured after the addition of packed cells to MBS containing ouabain, bumetanide and nitrendipine (each at 0.1 mM) and TDH. (A) Typical results from one of six experiments showing the dose-response relation for TDH-induced haemolysis time-courses. Experiments were initiated by the addition of 150 µl packed cells to MBS + indicated (TDH) ($\mu g(ml)^{-1}$); initial total volume = 5 ml. (B) Pooled standardised $\{(H^{50})^{-1}\}$ results from three experiments (each paired with (A) and on HRBC from a different donor - indicated by symbol type). Hct was varied whilst using a constant total reaction volume (initially 5 ml) and (TDH) = 0.654 $\mu g(ml)^{-1}$.

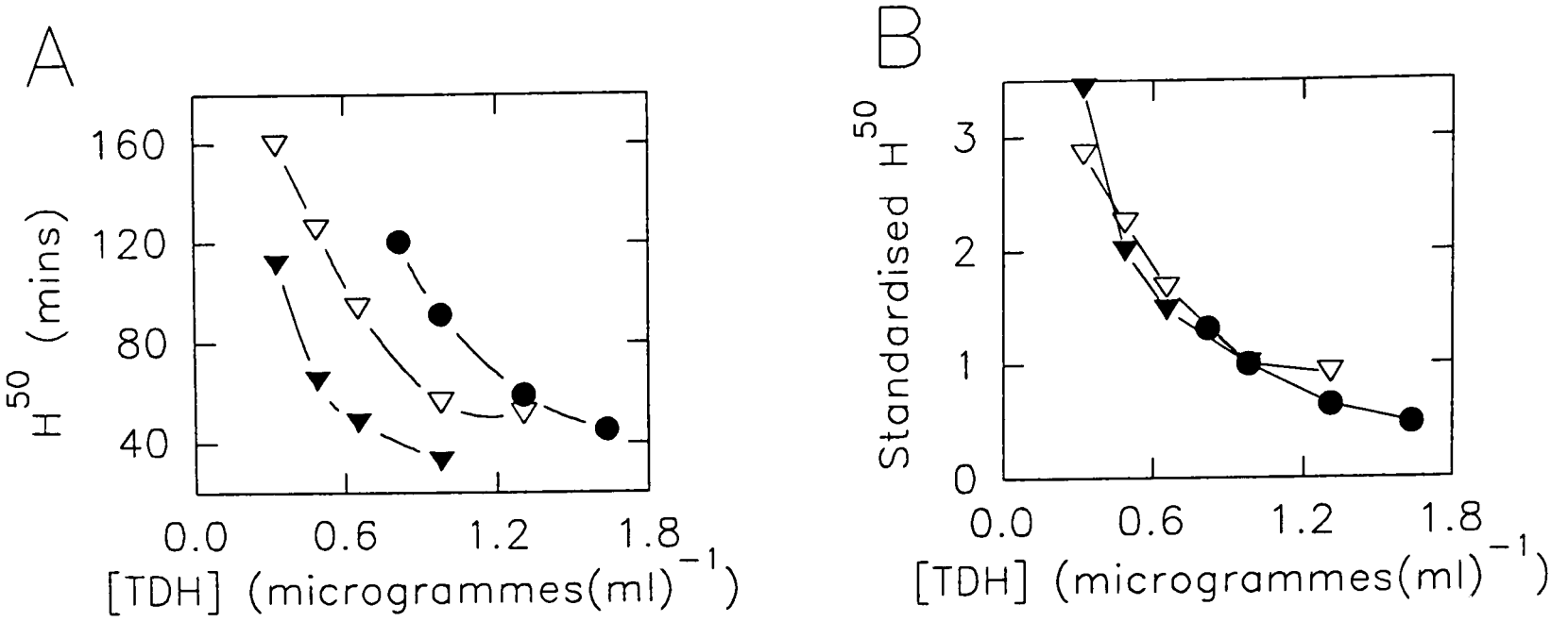


Figure 7.2 Dose-response of TDH-induced haemolysis. H^{50} values were obtained from time-courses of haemolysis, measured after the addition of TDH to an HRBC suspension (Hct = 2.2 %) in MBS containing ouabain, bumetanide and nitrendipine (each at 0.1 mM). (A) Results from HRBC of 3 donors (each indicated by symbol type). (B) Normalised H^{50} values with respect to that obtained for [TDH] = 0.981 $\mu g(ml)^{-1}$. (C) Normalised $\{(H^{50})^{-1}\}$ values with respect to that obtained for [TDH] = 0.981 $\mu g(ml)^{-1}$. Dashed line is line of best fit for all data.

are c. 1.1 x 10^5 molecules of TDH per erythrocyte.

The effect of extracellular volume on the rate of TDH-induced haemolysis was assessed (Figure 7.3) in a manner similar to that previously performed on KH (section 4.2.1), either allowing TDH concentration to vary with extracellular volume (A) or having an unvaried initial extracellular TDH concentration (0.49 $\mu g.(ml)^{-1}$) (B,C). The inhibitory effect of increasing extracellular volume (and decreasing TDH concentration) was seen as for the KH preparation. Figure 7.3B similarly exhibits the donor dependency described earlier, but further shows a lack of change in H^{50} according to the extracellular volume, when initial TDH concentration does not vary with volume. In Figure 7.3C these data are expressed as normalised $\{(H^{50})^{-1}\}$ with respect to data obtained at an initial extracellular volume of 8 ml. Lines of best fit were calculated by linear regression analysis of data obtained for each of the three donors. The gradient showed a very small, but significant (unpaired *t* test; $P < 0.05$, $n = 3$), deviance from zero, being 0.0271 ± 0.0040 ml^{-1}. Thus $\{(H^{50})^{-1}\}$ increased slightly with increasing extracellular volume, which can probably be explained by the existence of a small extracellular volume in the added packed cells, as postulated for the experiments using KH (section 4.2.1); extracellular volume itself exerted no protective effect.

Taken together, the data given in Figures 7.1 - 7.3, confirm the findings of section 4.2.1, in that haemolysin concentration and donor are the important determinants of haemolytic rate, and the fundamental kinetic characteristics of KH can be ascribed solely to the action of TDH. In addition, these data confirm the sensitivity of H^{50} as a measure of haemolytic rate for haemolysis of this type.

7.2.3 Underlying mechanism of TDH-induced haemolysis

Addition of an osmolyte (sorbitol at 23.5 mM) to the extracellular solution significantly (unpaired *t* test, $P < 0.05$, $n = 3$) inhibited TDH-induced haemolysis (representative time courses are given in Figure 7.4A), increasing the H^{50} by 18.7 ± 2.4 % (Figure 7.4B). As has been previously discussed for KH (section 4.3), the protective effect of increased extracellular osmotic pressure suggests that haemoglobin is released to the extracellular solution after membrane rupture due to volume expansion (because of osmosis secondary to an increased net cation influx). Such a lytic process depends on an increase in membrane cation permeability. This was documented using the DBP multiple-wash technique for K^+ ($^{86}Rb^+$)

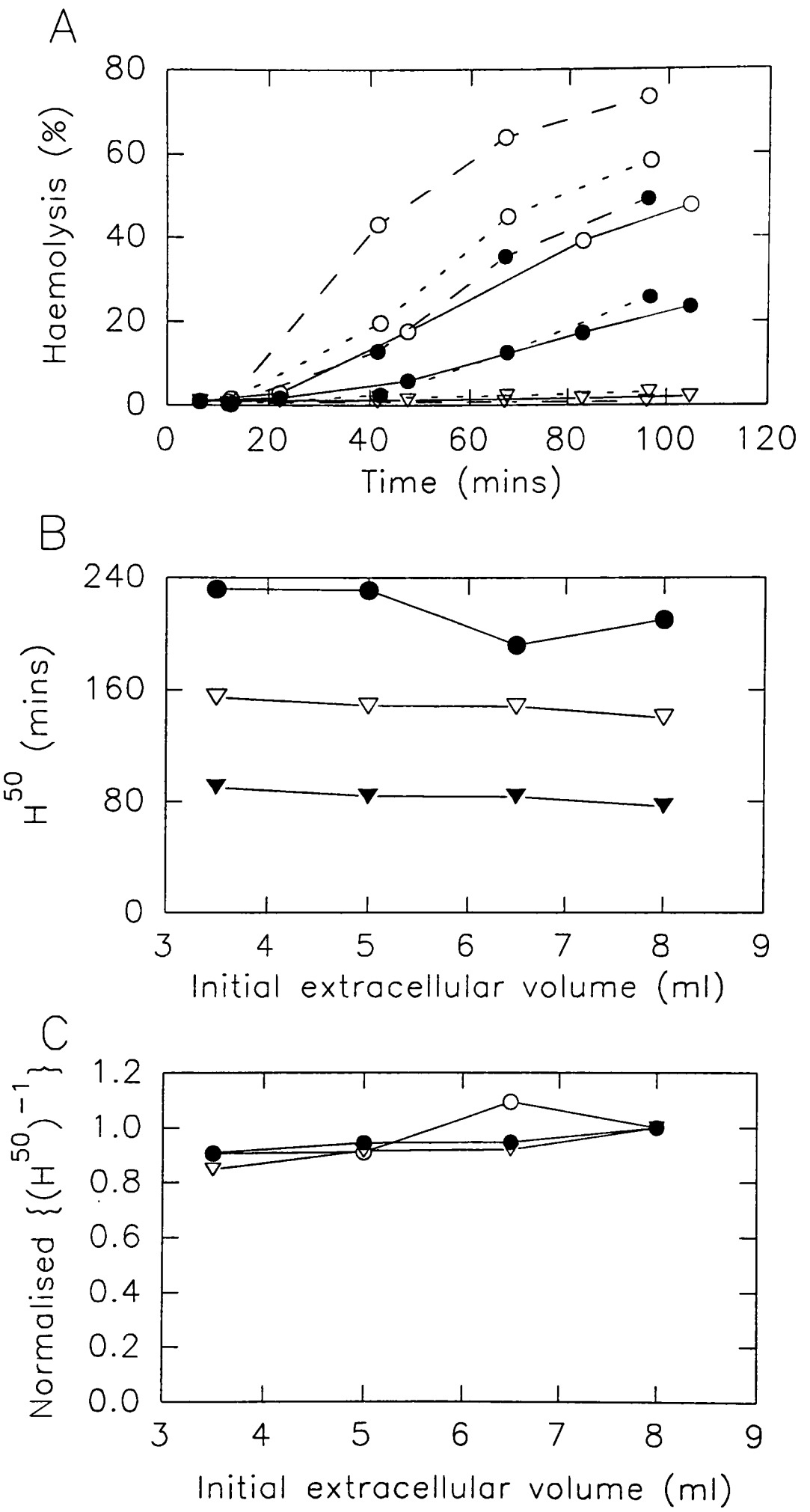
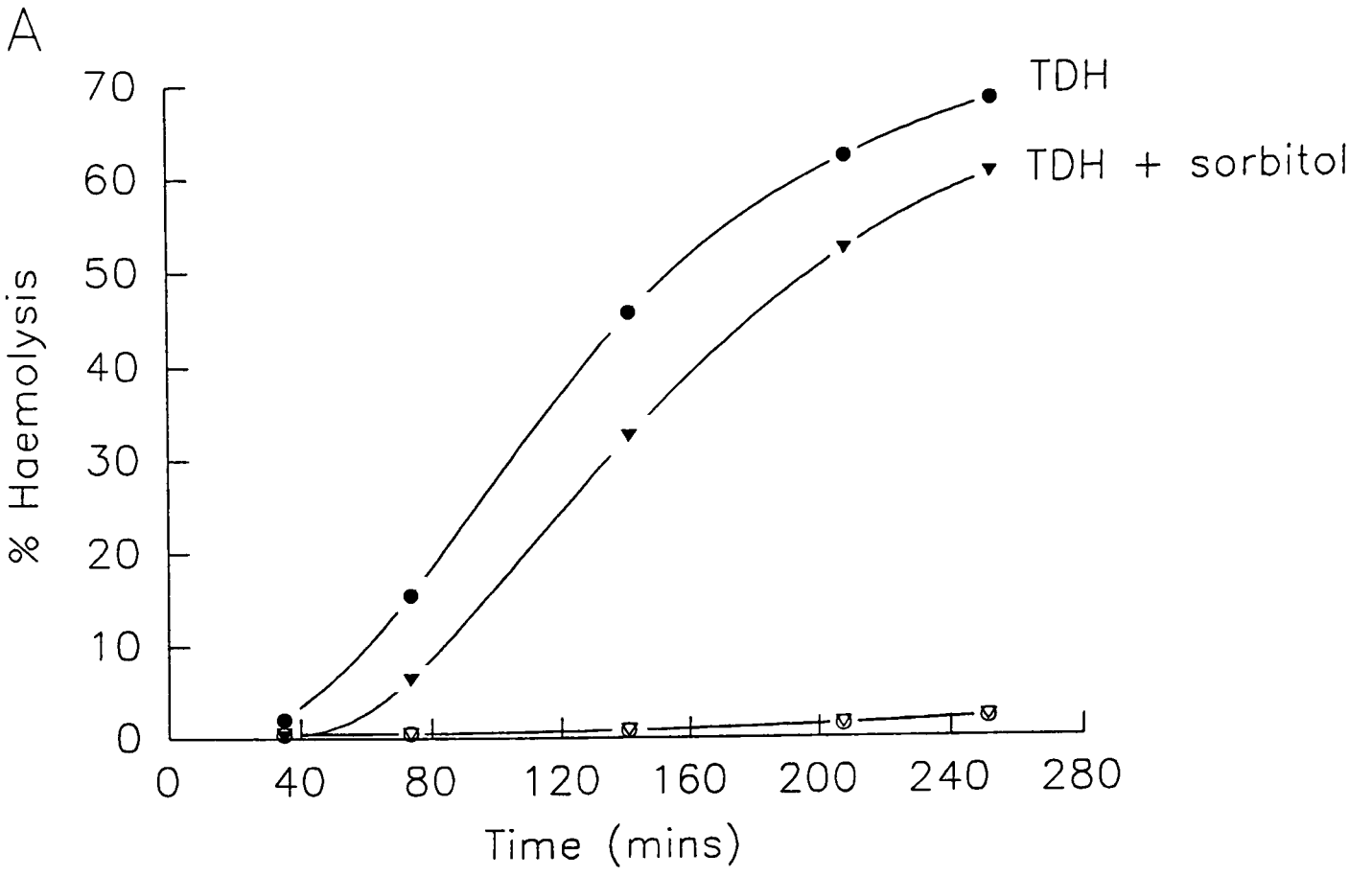


Figure 7.3 Effect of reaction volume on TDH-induced haemolysis. The effect was tested at either a constant KH:HRBC ratio (A) or an unchanged (TDH) (B,C). (A) Packed HRBC (100 µl) were added to either 4.9 (open circles) or 9.9 (closed circles) ml MBS containing TDH at 0.8175 and 0.4088 $\mu g(ml)^{-1}$ respectively, and ouabain, bumetanide and nitrendipine (each at 0.1 mM). Data given as closed inverted triangles are a negative control, performed in the absence of TDH. Each donor is accorded a line-type. (B) Packed HRBC (100 µl) were added to different volumes of MBS (3.5 - 8 ml) each containing TDH at 0.4905 $\mu g(ml)^{-1}$. Results from a particular donor are accorded a symbol type. (C) Because of the large donor variation evinced in (B), the data have been expressed as normalised $\{(H^{50})^{-1}\}$ (an index of relative permeability), with respect to that obtained for the 8 ml solution.



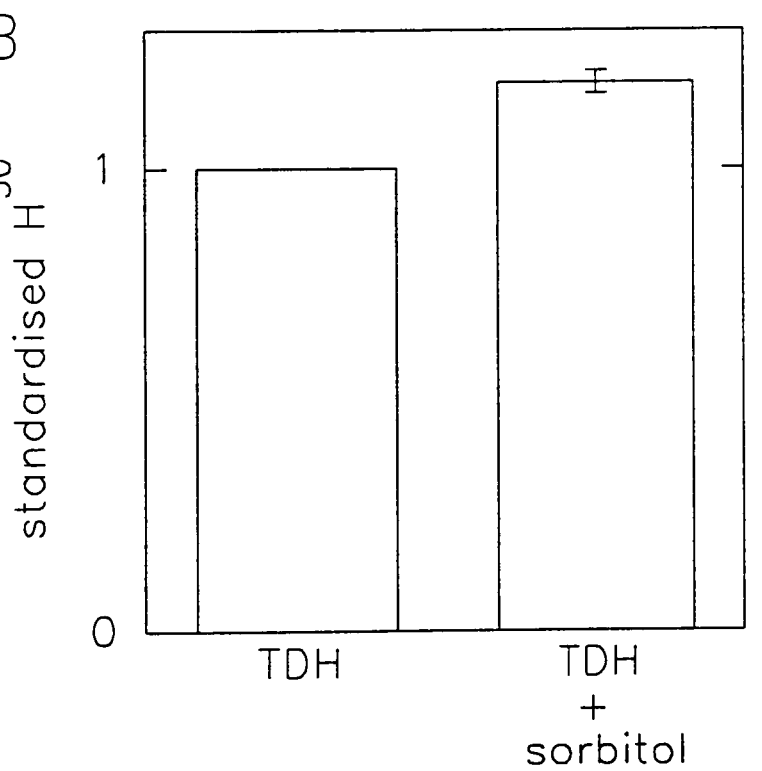


Figure 7.4 Osmotic attenuation of TDH-induced haemolysis. Packed human erythrocytes were added to MBS containing ouabain, bumetanide and nitrendipine (each at 0.1 mM). (A) Typical results from one of three such experiments. Open symbols indicate control results for which no TDH was present; filled symbols represent results obtained in the presence of TDH (10 µl *stock* $(ml)^{-1}$). Triangular symbols indicate the presence of D-sorbitol (23.5 mM). (B) Pooled results for standardised H^{50} values (from $n = 3$).

influx into ouabain, bumetanide and nitrendipine-inhibited cells to delineate the dynamics of the putative cation leak induced by TDH. Addition of TDH (3.4 $\mu l(ml)^{-1}$) caused a significant (unpaired *t* test, $P < 0.05$, $n = 3$) increase in K^+ influx from 0.20 ± 0.185 to 14.56 ± 2.786 $mmol.(l.c.)^{-1}(hr)^{-1}$ (Figure 7.5A,B). As shown for KH in Figure 4.6, this increase in flux was rapid in onset and constant in flux magnitude over the nine minute time course tested. The extent of elevation of flux varied according to donor; 2 donors showed a large increase in K^+ influx of similar magnitude after TDH addition - however the third donor was less responsive. This donor dependence of induced flux is also in accord with previously described data (sections 4.2.2 and 5.2.1).

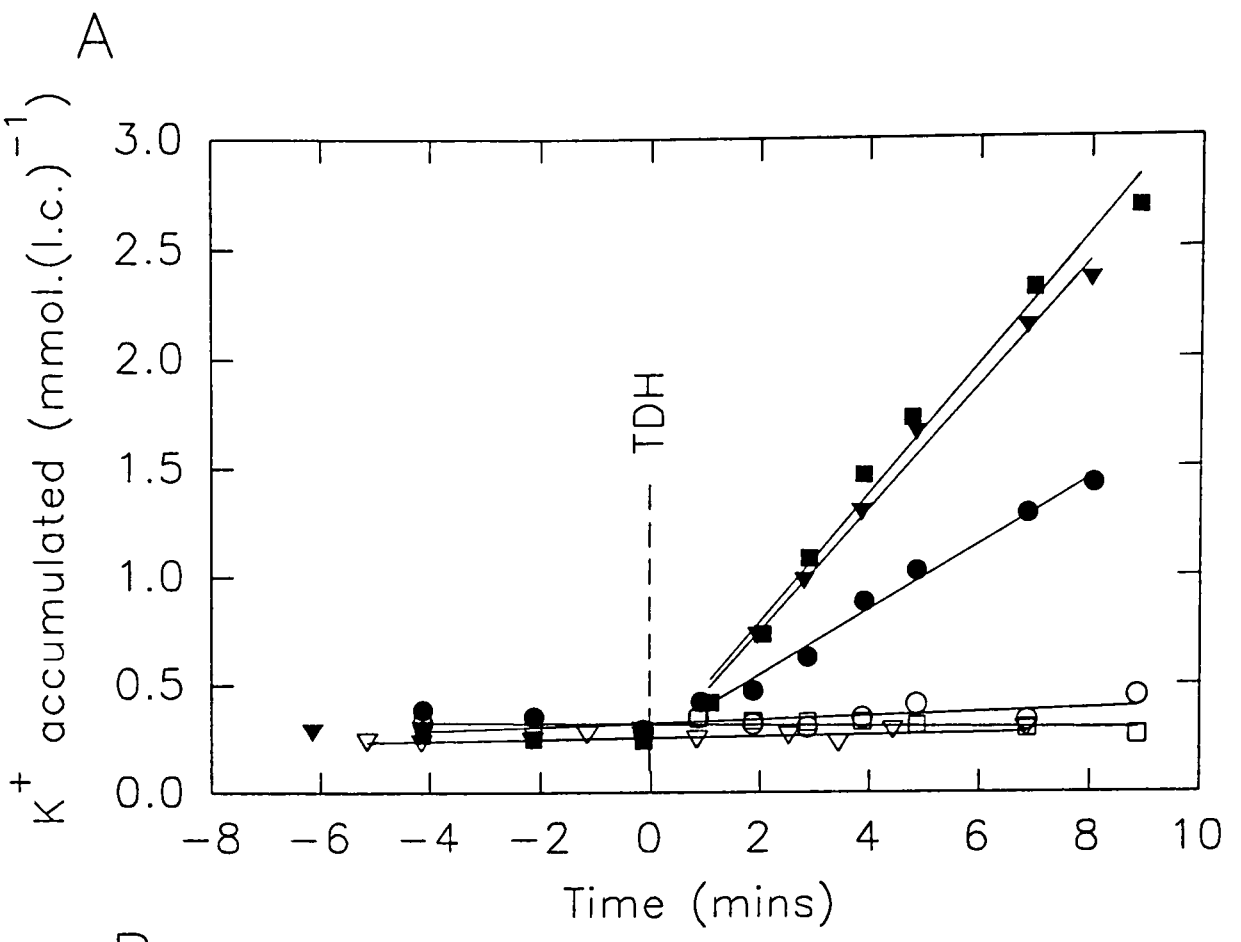
7.2.4 Effect of washout on TDH-induced haemolysis

In this section, the nature of the interaction between haemolytic entities and the HRBC membrane was investigated (Figure 7.6) similarly to the manner previously outlined for KH (section 4.2.5; Figure 4.9). The extracellular solution was washed out 20 seconds or 5 minutes after TDH (0.8625 $\mu g(ml)^{-1}$) addition at 37 °C, by centrifugation (CP3), removal of extracellular solution and resuspension. Haemolysis for the system washed after 5 minutes (% total, assayed after 55 minutes at 37 °C) was significantly (paired *t*-test; $P < 0.05$; $n = 3$) decreased, from 28.8 ± 7.89 (unwashed system) to 7.5 ± 3.30 %.

7.2.5 Further flux studies on the lesion induced by TDH

Table 7.2 examines the effect of Zn^{2+} (100 μM) on TDH-induced influx. For all four donors tested, Zn^{2+} (100 μM) was inhibitory to TDH-induced K^+ influx whilst causing a comparatively small increase in leak in the control cells not treated by TDH. However, there was extensive variation in the degree of inhibition ie the quantitative reproducibility of the effect is poor, reflected in the large S.E.M. for Zn^{2+} inhibition of TDH-induced influx. Concentrations of Zn^{2+} greater than 250 μM caused cell clumping.

The magnitude of K^+ influx also varied with haematocrit, as depicted in Figure 7.7A. The same patterns were seen in all three experiments, although the results were not directly quantitatively reproducible. Increasing the haematocrit (ie the target cell concentration) beyond a certain level (between 0.015 and 0.025, depending on donor) caused the induced flux to fall dramatically, before reaching a plateau phase (at Hct > 0.035). Note that the basal leak, in TDH-treated cells, obtained at haematocrits > 0.035 did not change with



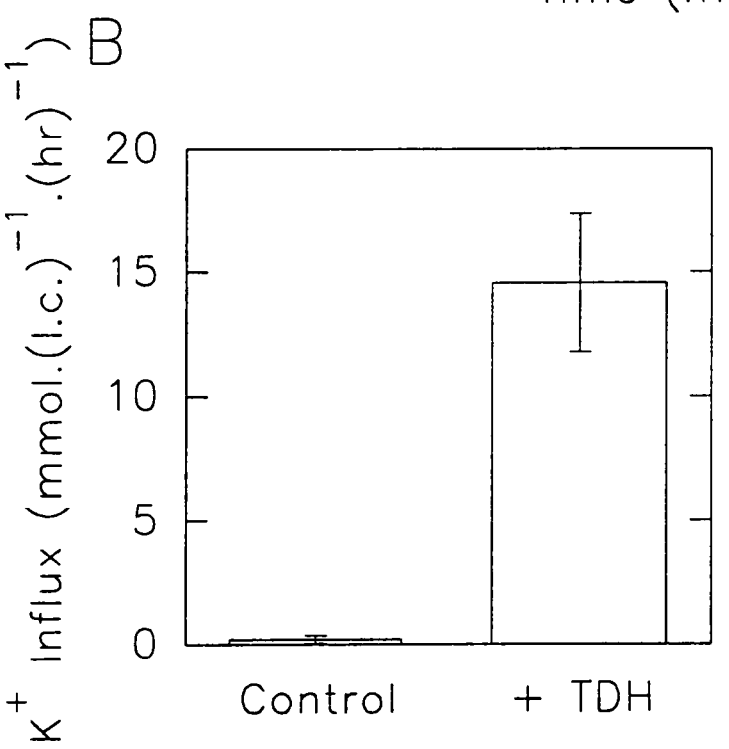


Figure 7.5 Effect of TDH on cation (K^+) influx. Fluxes were initiated by the addition of 0.5 ml K(^{86}Rb)Cl (150 mM) to 4.5 ml of cell suspension (MBS containing sucrose (45 mM) and ouabain, bumetanide and nitrendipine (each at 0.1 mM)), such that $(K^+)_o$ = 15 mM, $(Na^+)_o$ = 135 mM, Hct = 4 %, with 200 µl samples of the flux suspension being taken at 1 - 2 minute intervals, and spun through DBP. After three such samples TDH (3.4 µl *stock* $(ml)^{-1}$ (filled symbols)) or MBS (open symbols) was added to the remaining 4.4 ml of flux suspension. (A) Filled and open symbols represent systems in which TDH was and was not added, respectively. Each symbol shape represents results from a different donor (n = 4). Lines of best fit were obtained by linear regression. (B) K^+ influxes were calculated from the gradients of the lines given in (A).

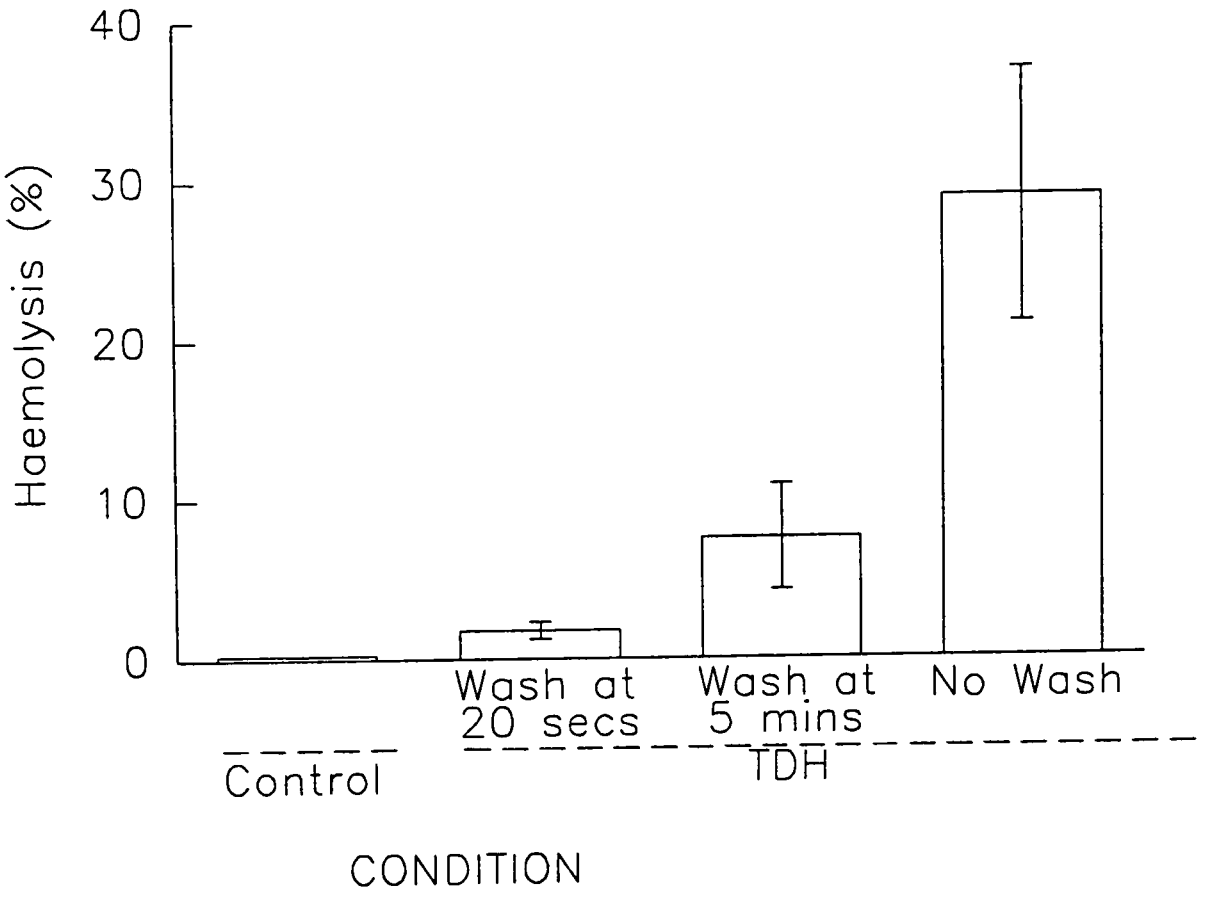
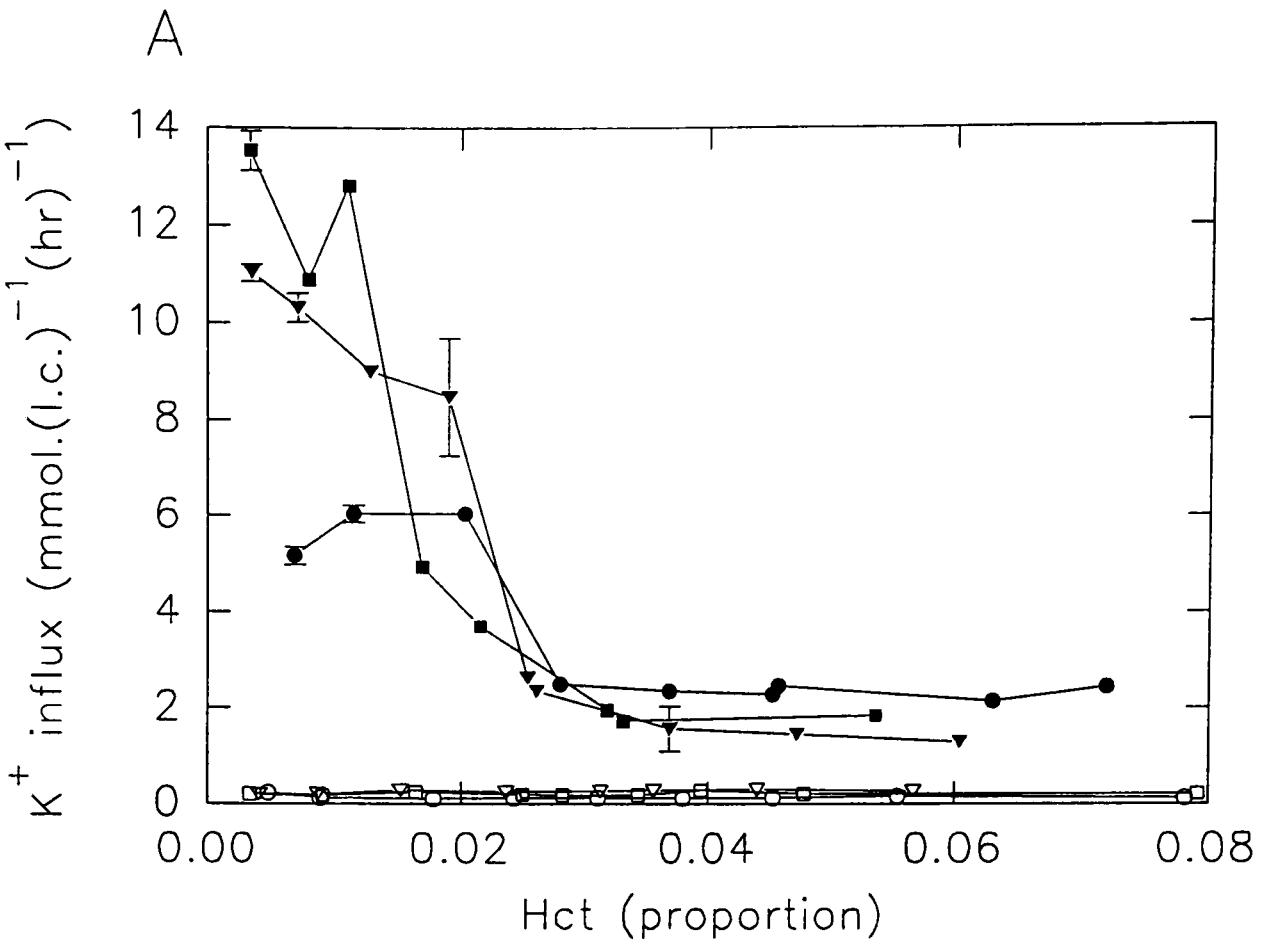


Figure 7.6 The effect of washout on TDH-induced haemolysis. Results are pooled data from three experiments. MBS (0.5 ml) containing ouabain, bumetanide and nitrendipine (each at 0.1 mM) with or without (as indicated) TDH (1.725 μg(ml)$^{-1}$) was added to 0.5 ml of HRBC suspension (Hct = 4 %) in MBS (similarly containing inhibitors). At the time indicated, cells were spun down by CP3; 0.8 ml of the supernatant was taken off, 0.8 ml of MBS (with inhibitors) being added back to the pelleted cells, which were then resuspended. Haemolysis was measured 55 minutes after the initial addition of TDH to HRBC.

	flux (mean ± S.E.M.) in mmol./l.c./hr		
Condition	A (alone)	B (+Zn^{2+} (100μM))	A - B
Control	0.21 ± 0.00675	0.30 ± 0.024	-0.09 ± 0.0258
+ TDH	18.89 ± 1.09	11.31 ± 2.93	+7.58 ± 3.13

Table 7.2 Effect of Zn^{2+} on TDH-induced K^+ influx. Fluxes were initiated by the addition of 100 μl K(^{86}Rb)Cl (150 mM) to 0.9 ml of cell suspension (MBS + ouabain + bumetanide + nitrendipine ± Zn^{2+} ± TDH) as indicated, such that in the flux tube $(K^+)_o$ = 15 mM, $(Na^+)_o$ = 135 mM, HCt = 4%; 10 μl TDH added per ml. Values are mean ± S.E.M. for 4 experiments.



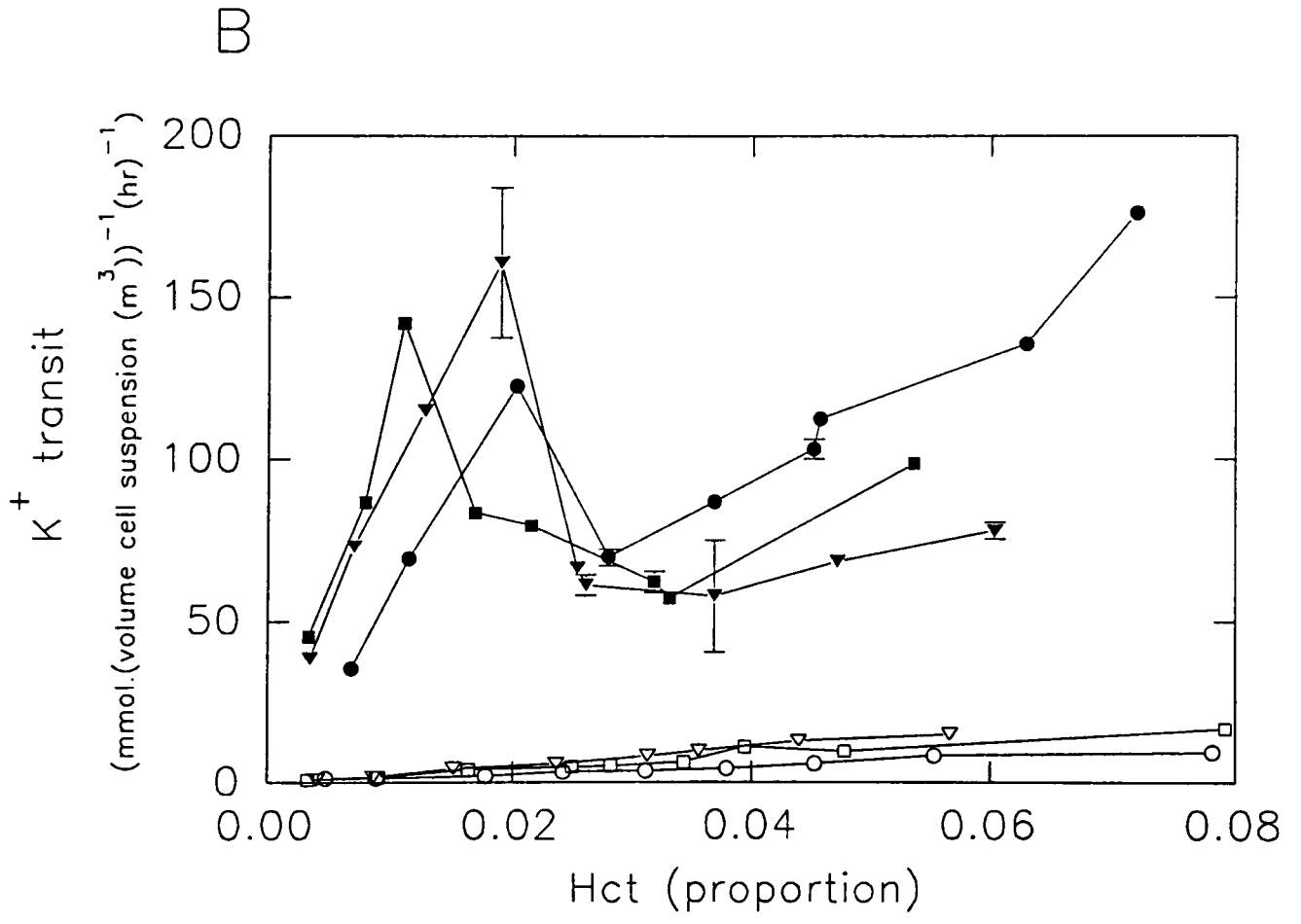


Figure 7.7 Multi-hit analysis of TDH-induced K^+ influx. Fluxes were initiated by the addition of 100 µl K(^{86}Rb)Cl (150 mM) to 0.9 ml of HRBC suspension, in MBS with ouabain, bumetanide and nitrendipine (each at 0.1 mM) ± TDH (1.2 µl *stock* (ml)$^{-1}$)) of varying Hct as indicated, such that in the flux tube $(K^+)_o$ = 15 mM, $(Na^+)_o$ = 135 mM. Each symbol type represents a different experiment and donor (n = 3). TDH was present (filled symbols) or absent (open symbols). For definition of K^+ transit see section 3.6.

increasing haematocrit but was greater than the basal leak (by approximately an order of magnitude) in untreated control cells (the control data show that in untreated cells K^+ influx does not change with increasing haematocrit). Furthermore, as shown in Figure 7.7B), an index of total counts accumulated irrespective of haematocrit (K^+ transit; see section 3.5) was observed to increase with increasing haematocrit over the lowest haematocrit range (up to c. 0.02). In the middle haematocrit range (c. 0.02 to 0.03), K^+ transit actually decreased rather than flattening off at a higher value. This suggests that there was a protective effect of higher target cell concentration, K^+ transit values being lower than those which would have been predicted if TDH acted only by a single hit mechanism. In the higher haematocrit range, K^+ transit increased again; the slope of this relation was less marked than that observed in the initial phase but greater than that for control cells untreated by TDH (reflecting the increased basal leak caused by TDH-treatment). Results concerning multi-hit mode of action and Zn^{2+} inhibition are consistent with those obtained in analagous experiments (Figures 5.7 and 5.8 respectively) for KH.

7.2.6 Selectivity of the TDH-induced lesion

Figure 7.8 shows (A) the time courses of TDH-induced haemolysis in isosmotic solutions of the alkali metal chlorides for a typical experiment, and (B) the Eisenman plot for pooled data from 3 such experiments, permeability indices being calculated using methods described in section 3.4. Assuming that the identity of the extracellular ion does not affect the action of agents that promote haemolysis by a mechanism other than leak induction, then the relative rates of TDH-induced haemolysis can be ascribed to selectivity of the induced leak (Weiner *et al.*, 1985). The selectivity series of the TDH-induced lesion for monovalent cations is $Cs^+ > Li^+ > K^+ > Rb^+ > Na^+$, a sequence that approximates closely to a reversed Eisenman VIII sequence with a small K^+ anomaly, as was also identified for KH (Figure 4.17).

7.2.7 Comparison of the flux-inducing and haemolytic activities of KH and TDH

All data to this point are consistent with TDH being the sole haemolytic agent of KH. It was important to compare the potencies of the induced leaks for pure and crude TDH preparations. Evidently some TDH-induced leak activity would have been lost during the purification protocol. TDH and KH preparations were reconstituted into 4 and 10 ml (hence stock KH solution had an activity of 100 $HU(ml)^{-1}$), respectively.

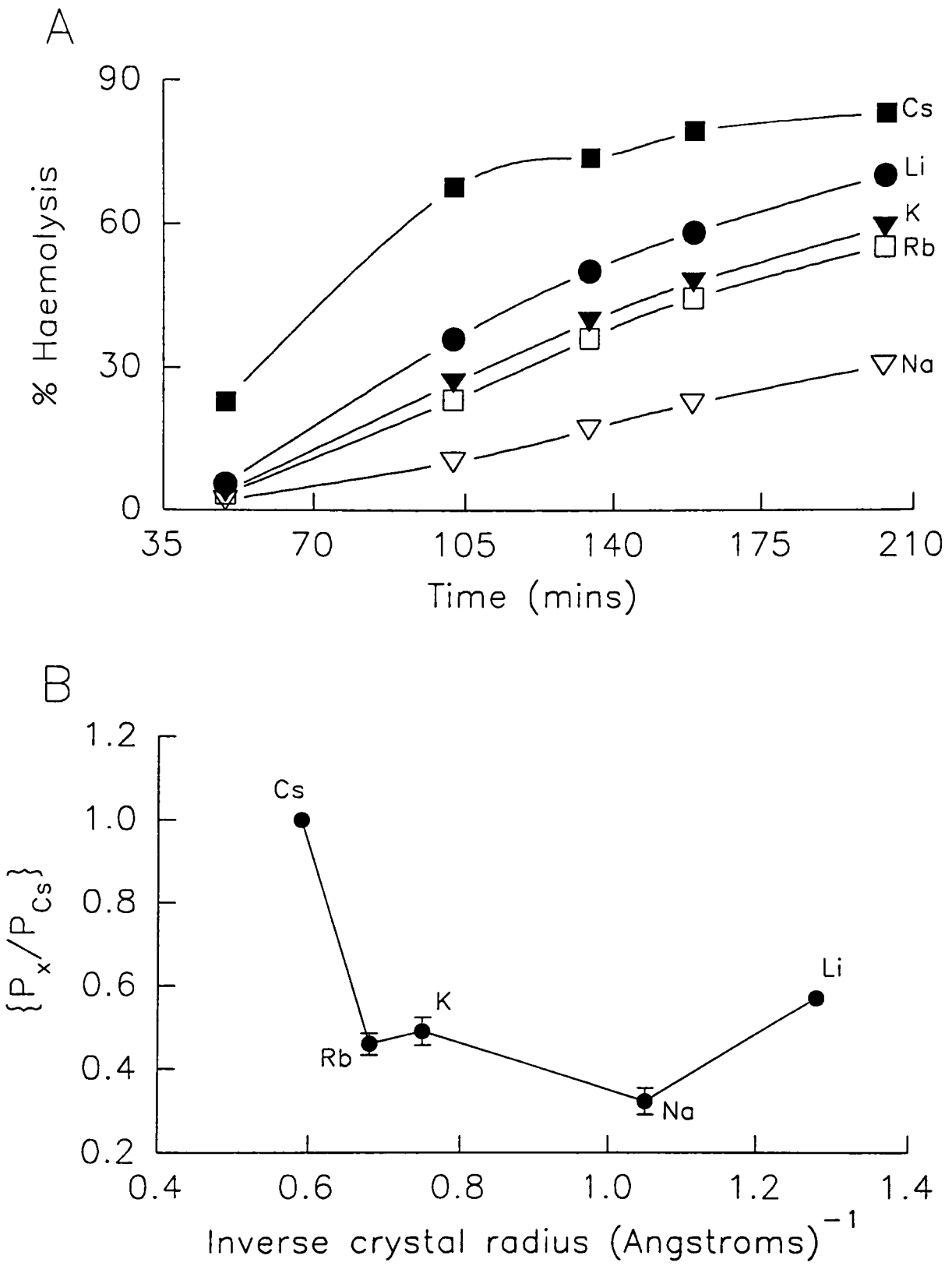


Figure 7.8 Monovalent cation selectivity of TDH-induced haemolysis. Cell suspensions were made up of isosmotic monovalent cation chloride with ouabain, bumetanide and nitrendipine (each at 0.1 mM). TDH (6 µl *stock* $(ml)^{-1}$) was added to each of the solutions before the final addition of 100 µl packed cells (total final volume 5 ml; t = 0). (A) Results of a typical experiment (n = 3). (B) Pooled results of $\{P_x/P_{Cs}\}$ from 3 experiments.

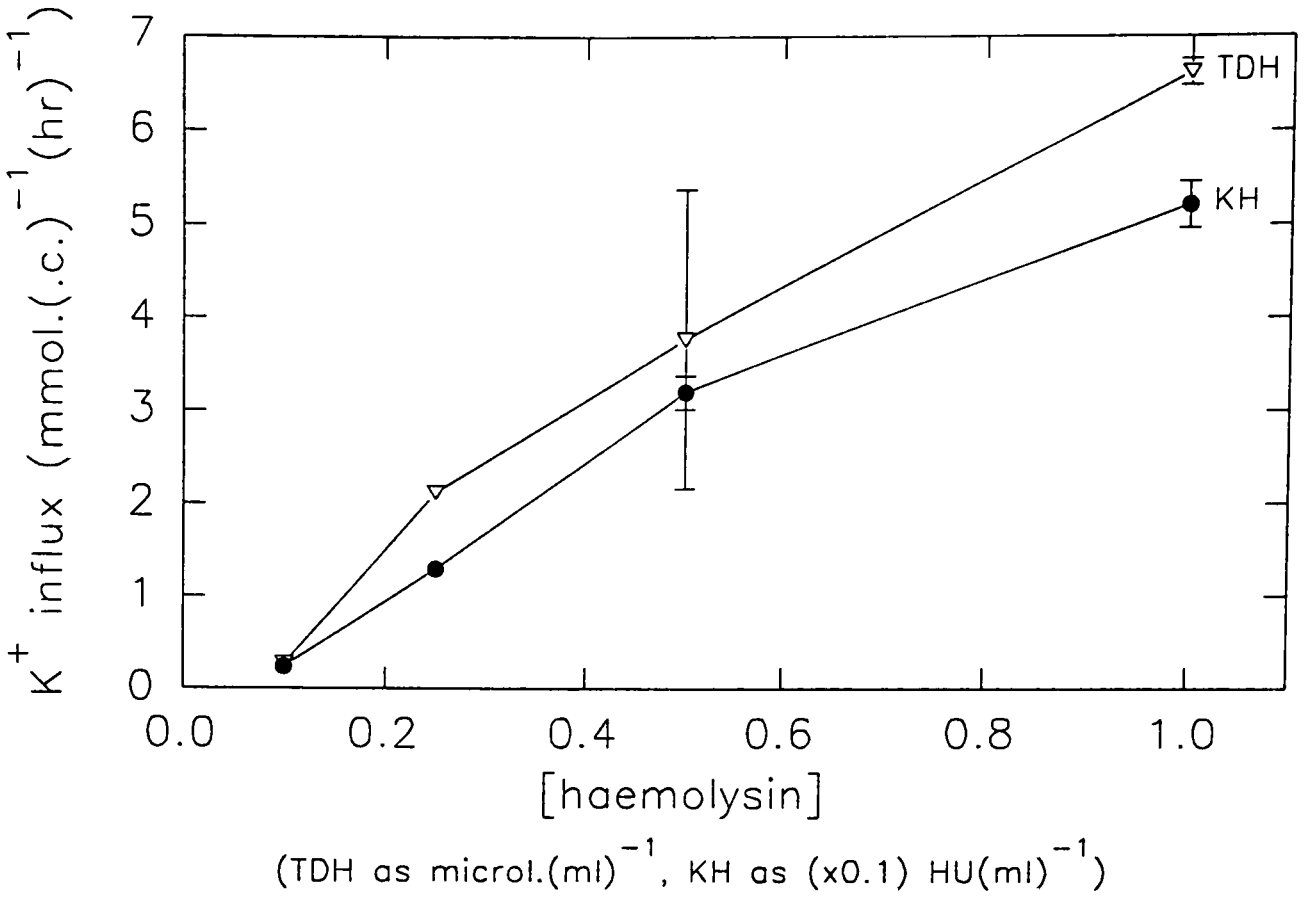


Figure 7.9 Comparative dose-response data of TDH and KH-induced K^+ influx. A TDH preparation was compared with a KH preparation for haemolysin-induced K^+ influx. HRBC were suspended (Hct = 3.4 %) in MBS + sucrose (50 mM) with ouabain, bumetanide and nitrendipine (each at 0.1 mM). Aliquots (0.88 ml) of cell suspension were added to MBS (20 µl) containing TDH or KH (at the indicated dose) and vortexed. Fluxes were initiated immediately by the addition of 100 µl K(^{86}Rb)Cl (150 mM) to 0.9 ml of HRBC suspension (experimental conditions: $(K^+)_o$ = 15 mM, $(Na^+)_o$ = 135 mM, and Hct = 3 %). Results are from one experiment representative of three such experiments. Open inverted triangles and closed circles indicate data for TDH and KH respectively.

Figure 7.9 shows dose-response data indicating that, for a given volume (2 - 20 µl) of these preparations, TDH caused a larger K^+ influx than KH. The shape of this relation is consistent with the lag relation found earlier (section 5.2.1), in that at lower doses of toxin, a small increase in toxin concentration did not have as large an effect as at larger doses of toxin. At the concentrations indicated, purified TDH was found to be a more potent leak-inducing agent than KH. This was confirmed in experiments in which 20 µl of both preparations per ml of cell suspension ($0.020 \leq$ Hct (p) ≤ 0.025, experiments performed on three separate donors over a five minute time course) caused a dramatic increase in K^+ influx, the magnitude of the leak induced by KH being 68.7 ± 5.4 % (significantly decreased; $P < 0.05$ in unpaired *t*-test of normalised data, $n = 3$) of that produced by purified TDH (Figure 7.10A). K^+ influx attributable to KH and TDH was 21.66 ± 3.835 and 32.20 ± 6.389 $mmol.(l.c.)^{-1}(hr)^{-1}$ respectively. At the same relative dose (ie 100 µl of agent added to cell suspension (4.9 ml; Hct = 2 %), KH had a greater haemolytic activity than TDH (Figure 7.10B) in sucrose-free MBS, the H^{50} for purified TDH being increased significantly ($P < 0.01$, $n = 3$) by 96 ± 4.5 % over that given by KH.

7.3 DISCUSSION

These data reemphasise the fact that relatively straightforward osmotic fragility and membrane transport experiments on red cells (Bernheimer, 1988) give useful information about the nature of toxin-induced membrane lesions, in particular for TDH. As noted previously (section 4.2.2), the unsuspected kinetic characteristics of haemolysis (Figures 7.1 - 7.2) are peculiar, and (as far as is known) have not been identified for any other bacterial toxins. Except for section 7.2.7, which documents comparative haemolytic and flux-inducing activities of KH and TDH, all evidence gained in this Chapter is consistent with TDH being the only haemolytic constituent of KH. Taking the evidence of section 7.2.7 into account, it is suggested that in this particular KH preparation another factor may be present that can augment haemolysis, although by a mechanism that does not involve leak induction (eg affecting osmotic fragility). Another explanation for these results may be that the presence/absence of co-factors (eg Ca^{2+}; EDTA is included in the TDH preparation as a protease inhibitor) may alter the dynamics of the induced cation influx (section 5.2.1) and

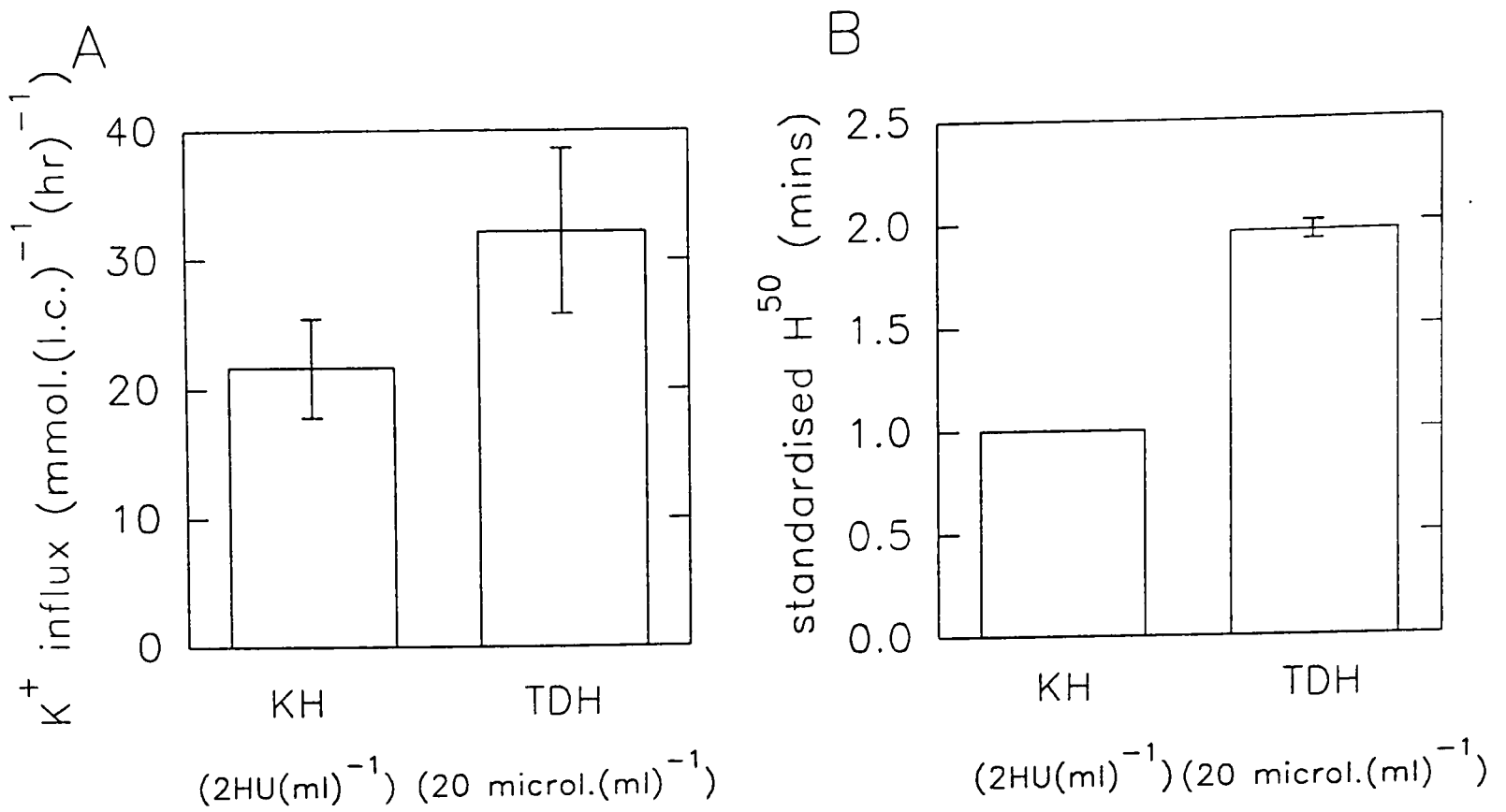


Figure 7.10 Comparative flux-inducing and haemolytic activities of KH and TDH. At relative doses for which TDH had been shown to produce the greater $^{86}Rb^+$ leak (see Figure 7.9), K^+ fluxes and haemolysis time-courses were measured in MBS with ouabain, bumetanide and nitrendipine (each at 0.1 mM). $K^+(^{86}Rb^+)$ fluxes were measured over a 5 minute time course at the haemolytic doses indicated with sucrose (45 mM) as an osmotic protectant ($(K^+)_o$ = 15 mM, $(Na^+)_o$ = 135 mM and Hct = 2 - 2.5 %). For haemolysis experiments, KH (100 µl; 100 HU(ml)$^{-1}$) or TDH (100 µl *stock*) was added to 4.8 ml MBS before the experiment was initiated by the addition of 100 µl packed HRBC.

hence haemolysis.

In addition, since the amount of haemolytic agent (TDH) was quantified by mass rather than by its biological activity for data in Figure 7.2, an estimate was obtained for the ratio of TDH:HRBC. Under the osmotic conditions defined, 0.981 $\mu g(ml)^{-1}$ TDH, approximately in the mid-range of haemolytic TDH concentrations, caused haemolysis such that $H^{50} = 60.0 \pm 17.15$ minutes. At this concentration, the ratio of TDH molecules:HRBC was calculated to be c. 1.1×10^5. Any implication this may have for the mechanism involved must take into account the prolonged binding phase of haemolysin (Chapter 4), the dynamics of induction of the cation leak pathway (section 5.2.1), and the fact that TDH appears to exist in multimeric form in aqueous solution (Honda *et al.*, 1976c).

TDH-induced haemolysis was inhibited by the addition of the osmolyte sorbitol (23.5 mM) to the extracellular solution (Figure 7.4), supporting the hypothesis that haemolysis occurs due to colloid osmosis, secondary to an increase in the cation permeability of the membrane. The effect of TDH on cation permeability was investigated directly (as for KH; Figure 4.6) by measuring K^+ (congener $^{86}Rb^+$) influx into human erythrocytes in which the endogenous cation transporters had been blocked (using ouabain, bumetanide and nitrendipine). TDH increased K^+ influx into these cells; as for KH (section 4.2.2; Figure 4.6), this increase was rapid in onset and constant in magnitude, suggesting a direct action by TDH on the membrane. These flux data do not necessarily imply that TDH is the only leak-inducing agent in the culture supernatants of pathogenic strains. The data presented here can be interpreted as good evidence that the induced leak is likely to be the result of non-enzymic action directly on the cell membrane, rather than either enzymic or intracellular action. If these latter mechanisms were involved, it is likely that the magnitude of influx would increase with time after a more pronounced lag period. These observations on dynamics of induced K^+ influx are in marked contrast to those defining the cation leak induced by other so called pore-forming agents such as melittin (Tosteson *et al.*, 1985), which causes a leak of only transient nature, and palytoxin (Tosteson *et al.*, 1991), which generates a raised cation permeability that increases with time. Protection of haemolysis by (i) addition of extracellular osmolytes, and (ii) washout after 5 minutes by CP3 (Figure 7.4), and the rapidity of onset and constancy of magnitude of the K^+ influx pathway, described here for pure TDH,

have been reported previously for KH (Chapter 4; Figures 4.3, 4.9, and 4.6 respectively).

The kinetics of leak generation due to TDH were examined (Figure 7.7); the relationship between K^+ transit and haematocrit indicate that the TDH-induced *lesion* is multi-hit in nature, in a manner similar to that obtained for KH (Figure 5.7). Having established the independence of influx and time after addition of toxin for the first ten minutes (Figure 7.5), the effect of erythrocyte concentration on influx was examined to clarify the nature of the lesion. The results are interpreted as follows: (1) over the initial haematocrit range (relatively constant high flux and increasing K^+ transit (Figure 7.5B)) the sites available for leak formation are close to saturation, ie the amount of TDH present in solution is greater than that required for generation of a leak pathway of maximum magnitude, and (2) several components of TDH (as it exists in the aqueous phase) are required to form the leak pathway responsible for high K^+ influx. This is because not only flux but also K^+ transit (an index of total rather than fractional uptake of cation) decreases after reaching a maximum, rather than simply levelling out. Thus, leak induction is a multi-hit process, in which TDH components have a proportionately greater effect when they operate in greater rather than lesser unison. Interestingly, K^+ transits at higher haematocrits are significantly higher than those corresponding to the basal leak of control cells. The increased K^+ transit, with increasing haematocrit (in this higher haematocrit range), may indicate that single components of TDH are capable of raising the cation permeability of the membrane, though not as effectively as several components acting in concert. Previous multi-hit studies have used haemolysis (expressed both as percentage and total, whilst varying haematocrit) as their assay of toxin action (Oberley & Duncan, 1971; Johnson, 1972; Inoue *et al.*, 1976; Johnson & Boese-Marrazzo, 1980; Kothary & Kreger, 1985). However, the conclusions that can be drawn from such studies are limited in that they indicate only the number of hits required for *cell death*, rather than for *lesion formation* itself. The experiments of Nishibuchi *et al.* (1992) indicate that TDH is likely to have its enteric effects by a mechanism other than cell lysis. In light of these, and other data indicating osmotic protection by sorbitol and a defined selectivity series for monovalent cations for the TDH-induced lesion (sections 4.2.2 and 5.2.3), it is suggested that the said lesion is capable of a degree of solute discrimination. The fact that the lower haematocrit range indicates a saturation of sites available for leak

formation may bolster previous evidence (Takeda *et al.*, 1975, 1976) suggesting the presence of a neuraminidase-sensitive ganglioside receptor (G_{T1} or G_{D1} rather than G_{M1} or G_{M2}) necessary for TDH action.

Further data described here indicate that: (1) Zn^{2+} significantly inhibits the induced K^+ influx pathway (Table 7.2), and (2) the lesion underlying haemolysis is of a defined monovalent cation selectivity series ($Cs^+ > Li^+ > Rb^+ > K^+ > Na^+$), a reversed Eisenman VIII sequence with a small K^+ anomaly, (Figure 7.8). These two features of the TDH-induced leak have also been ascribed to the leak induced by KH of *V. parahaemolyticus* (Chapters 4 and 5). The evidence here suggests that the increase in cation permeability induced by KH is due to TDH alone because the fluxes induced by cruder (KH) and purer TDH preparations cannot be separated on either the basis of Zn^{2+} inhibition or substrate discrimination (reflected by the unusual profile of monovalent cation selectivity). Although the site(s) of Zn^{2+} action remains obscure (Bashford *et al.*, 1988, 1989; Pasternak *et al.*, 1991), TDH can be added to a lengthening list of Zn^{2+}-sensitive leak-forming agents.

Diarrhea caused by *V. parahaemolyticus* is usually watery (as opposed to dysenteric) (Joseph *et al.*, 1982), which is taken as evidence that TDH (at 'normal' pathophysiological concentrations) perturbs membrane function rather than simply causing lysis. This suggestion is consistent with a study implicating TDH as capable of increasing I_{SC} without causing histological damage (Nishibuchi *et al.*, 1992). In the cases of *V. parahaemolyticus*-associated dysenteric syndrome it is likely that TDH and other factors (such as the enzymes already mentioned (Yanagase *et al.*, 1970; Sarkar *et al.*, 1987)) affecting membrane structure cause cell lysis, thus destroying the functional integrity of the intestinal epithelium. The work presented in this Chapter confirms TDH as the important leak-inducing agent of KH and directly characterises several salient features of TDH action. However, there is an indication that conditions not yet clearly understood influence the haemolytic process or that other factors may be important in *the action of KH* (section 7.2.7). A suggestion has already been made that Kanagawa haemolysin preparations may differ in their potency (sections 5.2.1 and 5.3), so the questioning of its constitution may not be entirely without precedent, especially given the importance of growth conditions to the production of different proteins by *V.*

parahaemolyticus (section 6.2.2). These functional features of the TDH-induced lesion are likely to be important in analysis of the cell physiological events that underly the pathogenesis of *V. parahaemolyticus*-associated diarrhoea.

The next chapter describes analagous work performed on the action of the El Tor haemolysin (ETH), obtained and purified as described in Chapter 6, on human erythrocytes. Having established the importance of TDH as the flux-inducing agent of KH, it is also important to examine the possible role of this toxin on a more pathologically relevant cell-type; Chapter 9 contains data describing KH action on isolated enterocytes, using erythrocytes as a positive control. The overall significance of these data is discussed in Chapter 10.

CHAPTER 8

Studies on the lesion induced in human erythrocyte membranes by the El Tor haemolysin (ETH)

'Be gentle with me and I will yield up my secrets...'
Anon.

8.1 INTRODUCTION

The potential role of the El Tor haemolysin (ETH) in the pathogenesis of disease associated with non-O1, non-O139 *V. cholerae* (Hughes, 1978; Blake, 1980; Morris & Black, 1985) has been considered in section 2.7. In this chapter, the effects of ETH have been investigated on erythrocytes (which constitute an ideal model membrane system as described in sections 4.1 and 2.8.1).

ETH is considerably less well characterised than TDH, although it has also been shown to be haemolytic (Honda & Finkelstein, 1979), cytotoxic (Yamamoto *et al.*, 1984, 1986), lethal (Nath *et al.*, 1978), and enterotoxic (in both rabbit ileal loop and suckling mice studies; Ichinose, 1987). Furthermore, areas of the structural gene encoding the toxin vulnificolysin of *V. vulnificus* greatly resemble the *V. cholerae* ETH structural gene in both sequence and organisation (Yamamoto *et al.*, 1990c). This has been held to suggest that the toxins ETH and vulnificolysin may be derived from a common ancestor and therefore belong to a haemolysin-family distributed within the *Vibrio* genus (Yamamoto *et al.*, 1990c), much in the manner of the TDH-family of toxins (section 2.6.4(iii)).

A seminal characterisation of the haemolytic mechanism of ETH, was performed by Honda and Finkelstein (1979) who they found the purified toxin to have a M_w of c. 20 kd by gel filtration. ETH differed from TDH in its spectrum of lytic activity on erythrocytes from different species and in its thermostability/lability properties. However, it was similarly inhibited by mixed gangliosides and unaffected by the addition of lecithin. Recently, Krasilnikov *et al.* (1992) have examined the mode of action of a related but not identical (by amino acid composition) *V. cholerae* cytolysin in erythrocytes and planar lipid bilayers. The induced pore was anion selective and strongly influenced by pH, and on the basis of osmotic

protectant studies, they suggested that the pores formed had a diameter of 1.8 - 2.0 nm. Haemolytic and pore-forming activities were not affected by addition of cholesterol or dithiothreitol.

Notwithstanding these findings, work on this toxin has been hampered by the uncertainty of its identity, and a variety of molecular weights have been proposed (section 2.7.4). Following the cloning and sequence analysis of the haemolytic determinants (Goldberg & Murphy, 1984, 1985; Manning *et al.*, 1984), Hall and Drasar (1990) clarified the heterogeneity of biochemical data, using an amino-terminal microsequencing technique to show that ETH is processed extracellularly by amino-terminal proteolysis, yielding three forms of the toxin, of calculated molecular weights, 83.2. 68.6, and 65.3 kd.

Work described in Chapter 6 showed how a homogeneous preparation of one of these extracellular proteins can be obtained from the culture supernatants of *V. cholerae* strain 8731. The single extracellular form of ETH was identified by SDS-PAGE and amino-terminal micro-sequencing (Matsudaira, 1993) to be the 68.6 kd form (refer to Hall & Drasar, 1990). Using this preparation, analagous experiments to those described for KH (Chapters 4 and 5) and highly purified TDH (Chapter 7) were performed on human erythrocytes (sections 3.4 and 3.5). However, following initial observations on the instability of the toxin preparation, conditions for maintaining ETH in a viable form had to be established. Results which follow are expressed in terms of percentage haemolysis, time taken to reach 50 % haemolysis (H^{50}), or K^+ accumulated $(mmol.(l.c.)^{-1})$.

8.2 RESULTS

8.2.1 Stability of the ETH preparation

Attempts were initially made to store ETH in a fashion similar to that employed earlier for KH/TDH (section 3.2.1). In these circumstances, however, the activity of the toxin preparation declined markedly within twenty-four hours. To prepare the *stock* preparation, lyophilised sample of ETH was reconstituted in MBS (2 ml; previously bubbled with N_2 to drive off other dissolved gases) and under pure corn oil (PCO). Sample aliquots of the *stock* were then stored overnight at 4 °C under either air, or a layer of PCO. The activities of these

two toxin preparations were then compared (Figure 8.1). That stored under PCO was considerably more potent, although the preparation stored under air retained some activity.

Further experiments were performed (Figure 8.2) to test the combined effects of air exposure and vigorous vortexing (in a whirlimixer). ETH (30 µl) was taken from under PCO and added to a variety of solutions (see legend to Figure 8.2). After these solutions had stood at 37 °C for 30 minutes, an aliquot of erythrocyte suspension was added to each tube and the haemolysis time courses measured at 37 °C. The H^{50} values (Figure 8.2B) for the untreated (ii) system did not increase significantly beyond control ((i): N_2 for 5 minutes + covered by PCO) values. However, the vortexed system (iii) increased H^{50} more markedly than (ii), though statistical testing for three experiments failed to accord this change significance at the 5 % level (unpaired *t*-test; P = 0.063, *n* = 3). However, if vortexing of the system was performed after addition of the toxin (iv), then haemolytic activity was dramatically reduced (Figure 8.2A). Therefore, for subsequent experiments, the ETH *stock* was kept under PCO, until addition to an erythrocyte suspension when complete mixing was performed not by vortexing but by gentle pipette action.

8.2.2 Nature of the haemolytic lesion induced by ETH

Figure 8.3 shows the time courses of haemolysis induced by ETH at 37 °C in MBS (Hct = 1.2 %). Similar to those derived earlier for KH (section 4.2.1), these curves are of a skewed sigmoid shape, exhibiting a lag phase before haemolysis. After a middle phase of relatively rapid haemolysis, the rate diminished but did not stop. The rate of haemolysis was strongly dependent on ETH concentration under the conditions examined.

As noted previously (section 4.2.2), haemolysins can act either by generating large lesions in the cell membrane, through which haemoglobin can leak out, or by instigating an increase in the cation permeability which causes lysis after colloid osmosis. Time courses of ETH-induced haemolysis in the presence or absence of extracellular sucrose (24 mM) are given in Figure 8.4; the H^{50} values were significantly (paired *t*-test; P < 0.005) increased in the presence of sucrose, from (mean ± S.E.M.) 70.7 ± 6.44 to 143.0 ± 16.27 minutes. Normalisation of these data (Figure 8.4B) showed that (sucrose 24 mM) caused the H^{50} value to increase to just over twice (2.02 ± 0.067 x) its initial value. The most likely explanation for this inhibition is that haemolysis occurs secondary to an increase in the cation

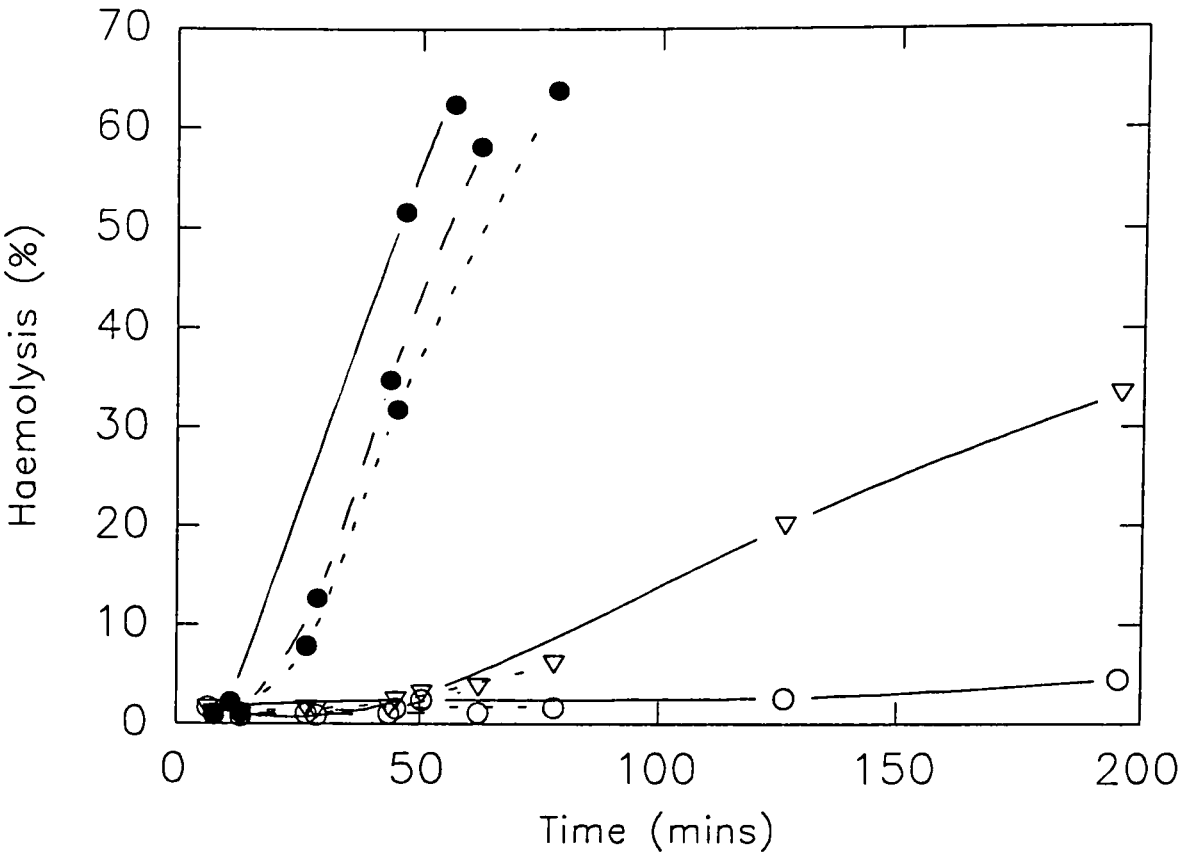
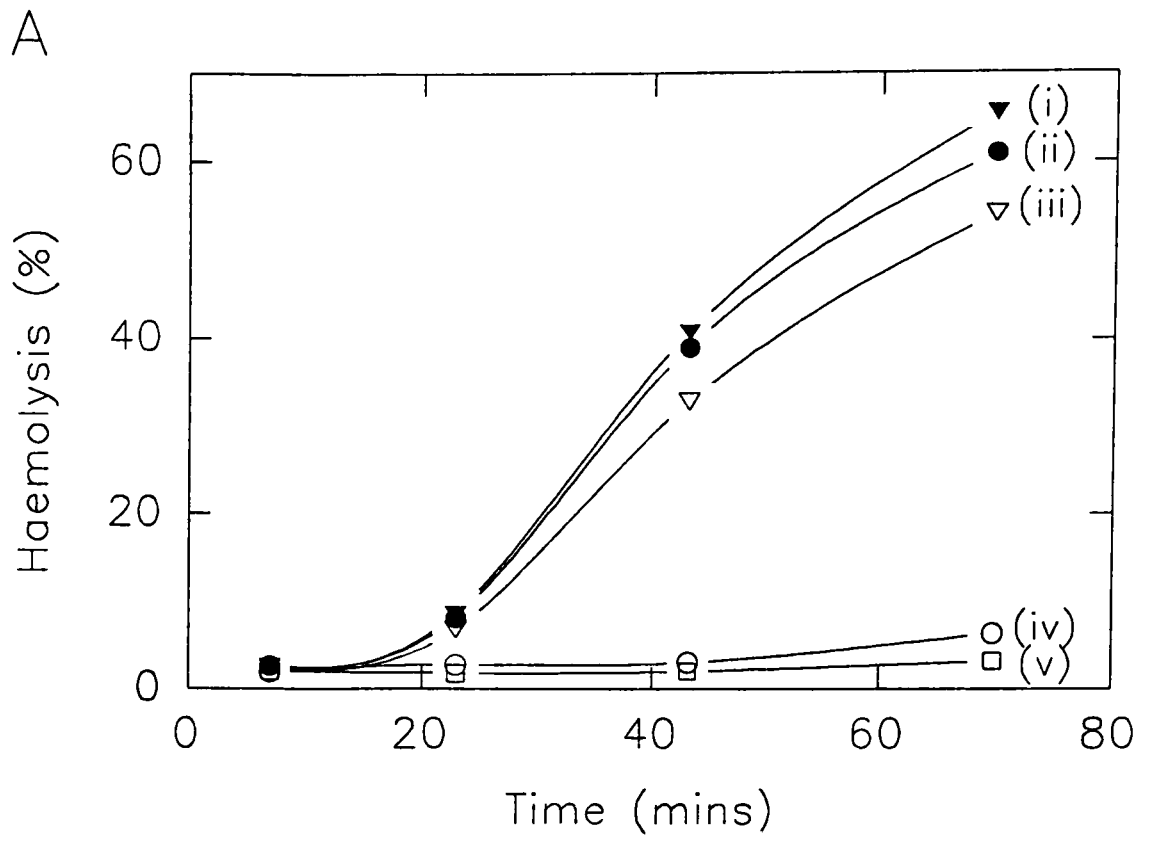


Figure 8.1 Effect of air-exposure on subsequent ETH activity. Aliquots (0.5 ml) of HRBC suspended (Hct = 4 %) in MBS were added to MBS (0.5 ml) ± ETH (30 µl *stock*) that had been stored overnight at 4 °C under either air or pure corn oil. Upon addition, the suspension was gently inverted several times to ensure complete mixing, and incubated at 37 °C. Results are from three donors (indicated by line-type). Open triangles/closed circles = ETH stored under air/oil respectively; open circles describe haemolysis in the absence of toxin.



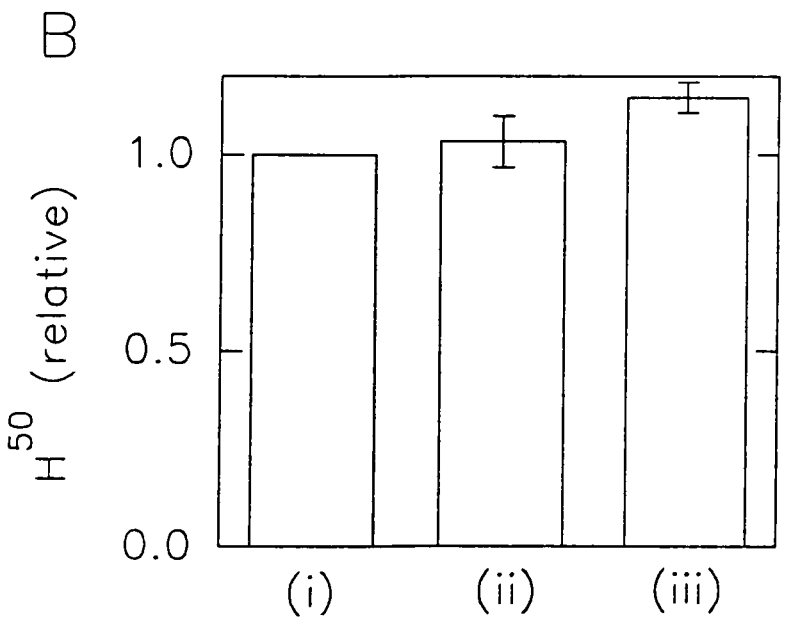


Figure 8.2 Effect of vortex-treatment and air-exposure on ETH activity. Aliquots (0.5 ml) of MBS in Eppendorf tubes were treated as follows: (i) gassed for 5 minutes with N_2 and then 300 µl PCO layered on top, (ii) untreated, (iii) vortexed for 1 minute, (iv) gassed for 5 minutes with N_2, and (v) untreated. An ETH aliquot (30 µl *stock*, stored at 4 °C under PCO) was added to tubes (i), (ii), (iii), and (iv). Tube (iv) was then vortexed for 1 minute. All tubes were then left to stand for 30 minutes at 37 °C. Haemolysis time courses were initiated by the addition of HRBC suspension (experimental Hct = 2 %) aliquots to the aqueous portion of each tube, and incubation at 37 °C. (A) Results from one experiment typical of three such experiments. (B) Pooled and normalised (with respect to values obtained for system (i)) results from three such experiments.

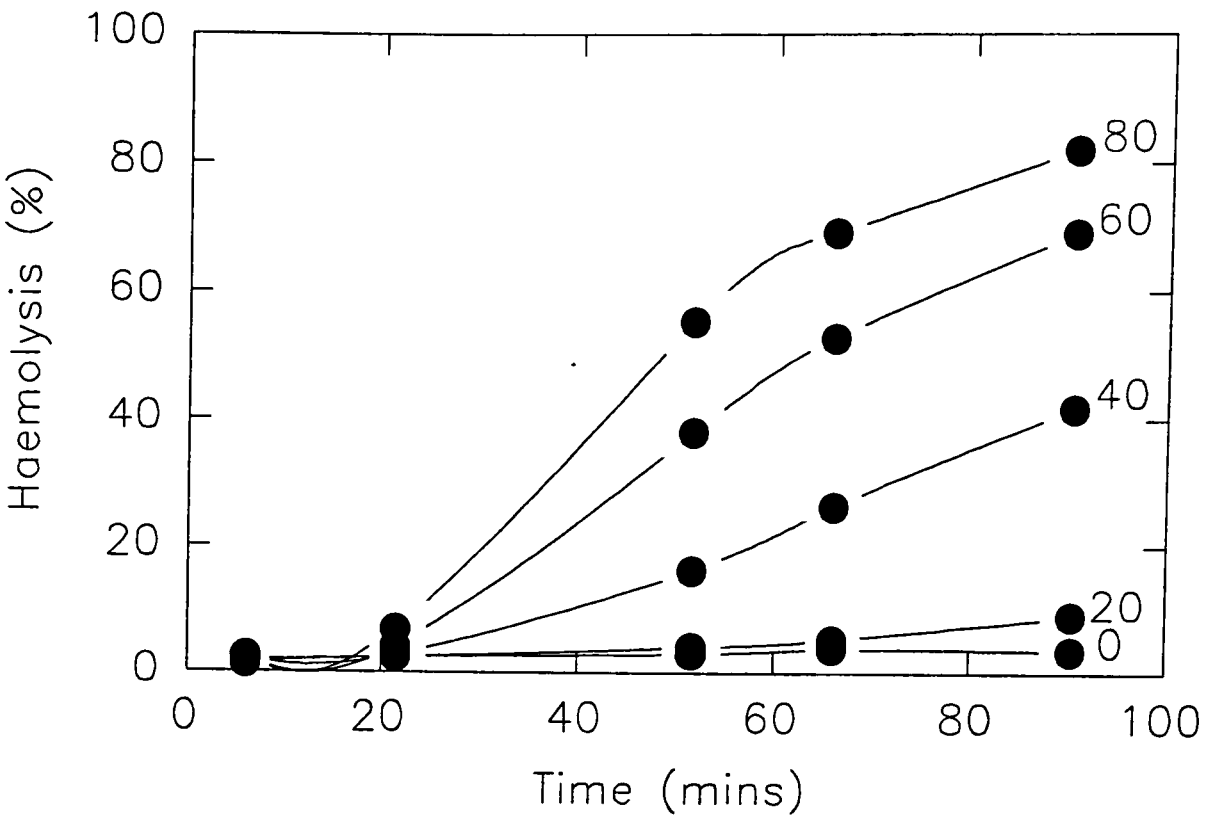
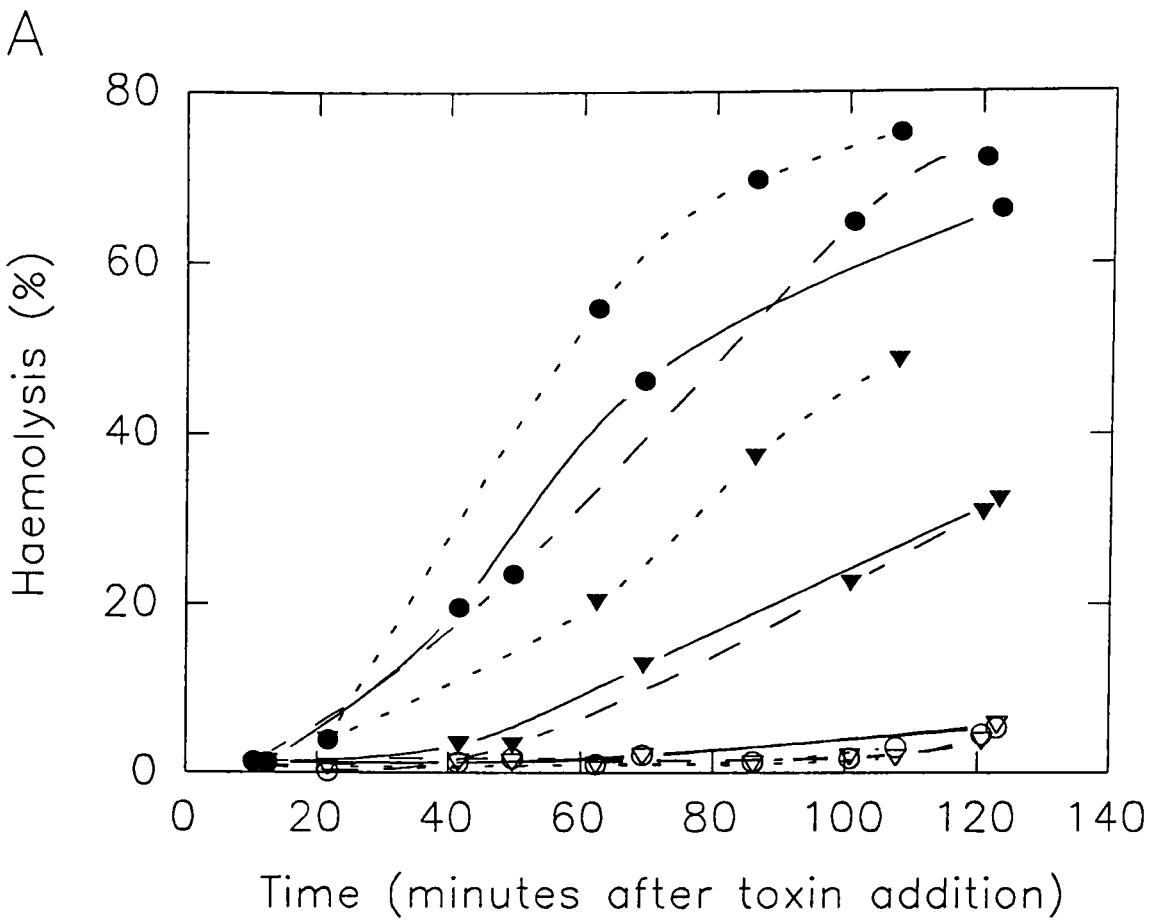


Figure 8.3 Time courses of ETH-induced haemolysis: dose-response data. HRBC were suspended (experimental Hct = 1.2 %) in MBS (2 ml total) containing the indicated amount of ETH (from stock under oil), and incubated at 37 °C.



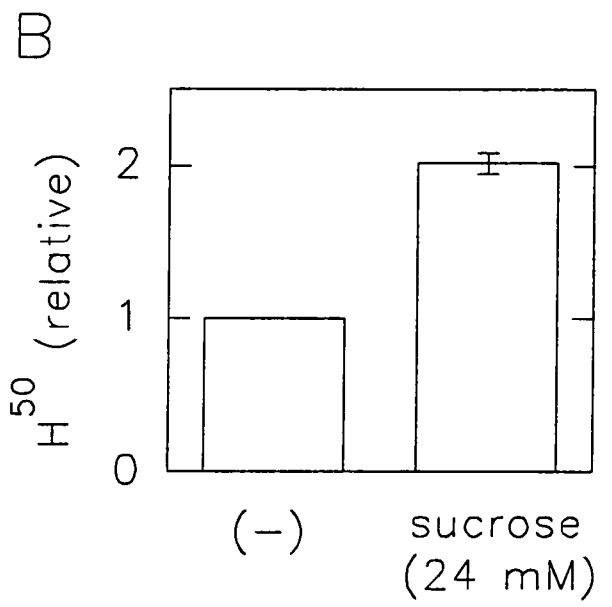


Figure 8.4 Osmotic attenuation of ETH-induced haemolysis. Aliquots (0.5 ml) of HRBC suspension (Hct = 4 %) were added to 0.52 ml MBS ± extracellular sucrose (47.7 mM) ± 20 µl *stock* ETH (from under PCO). Upon addition, the suspension was gently inverted several times to ensure complete mixing, and incubated at 37 °C. (A) Results from three experiments (each donor indicated by line type). Circles/triangles = absence/presence of sucrose; filled/open symbols = with/without ETH. (B) Pooled and normalised H^{50} values.

permeability of the membrane which causes a volume increase, which can be protected against by the addition of extracellular osmotic impermeants.

In light of preliminary evidence that ETH did not affect inhibitor-insensitive K^+ influx in the ten minutes subsequent to ETH addition (data not shown), samples were taken from a preparation treated for a more protracted time period (Figure 8.5). The inhibitor-insensitive K^+ influx in response to ETH addition was indeed found to show a marked lag phase (c. 9 - 15 minutes) prior to leak induction, before increasing to a relatively constant level. The magnitude of the induced leak appeared to vary between donors, as has been previously noted for both ETH (haemolysis; Figure 8.4) and KH (Chapters 4 and 7).

The pattern of the magnitude of ETH-induced cation influx was therefore markedly different from that found for KH (Chapter 5; Figures 5.2, 5.4 and 5.8). In order to confirm these differences and that haemolysis was indeed dependent on the raised cation permeability, the kinetics of TDH and ETH -induced haemolysis were compared (Figure 8.6). Although of a similarly skewed sigmoidal shape (as previously described (Figure 4.1B (KH) and Figure 8.3 (ETH)), the curves are not of the same type. In particular, the rate of ETH-induced haemolysis at a given relative dose of toxin is smaller initially than the rate of TDH-induced haemolysis. However, after the lag period, ETH-induced haemolysis occurs more rapidly than that observed as a result of TDH action.

The selectivity of the induced cation leak was examined using the technique of isosmotic haemolysis as outlined previously (section 3.4). Figure 8.7 shows the time courses of haemolysis from a preliminary experiment performed by a protocol analagous to that used for TDH (section 4.2.6; Figure 4.17). Although haemolysis was not measured over a sufficiently long time period to derive H^{50} values as given for TDH (section 4.2.6; Figure 4.17), the apparent monovalent cation selectivity series for ETH-induced haemolysis was a straight Eisenman IV (contrasted with a reversed Eisenman VIII, for TDH) sequence: $K^+ > Rb^+ > Cs^+ > Na^+ > Li^+$. If the identity of the ion does not affect either leak-induction or the nature of the induced lesion itself, then the relative haemolytic rates can be ascribed to the selectivity of the said lesion.

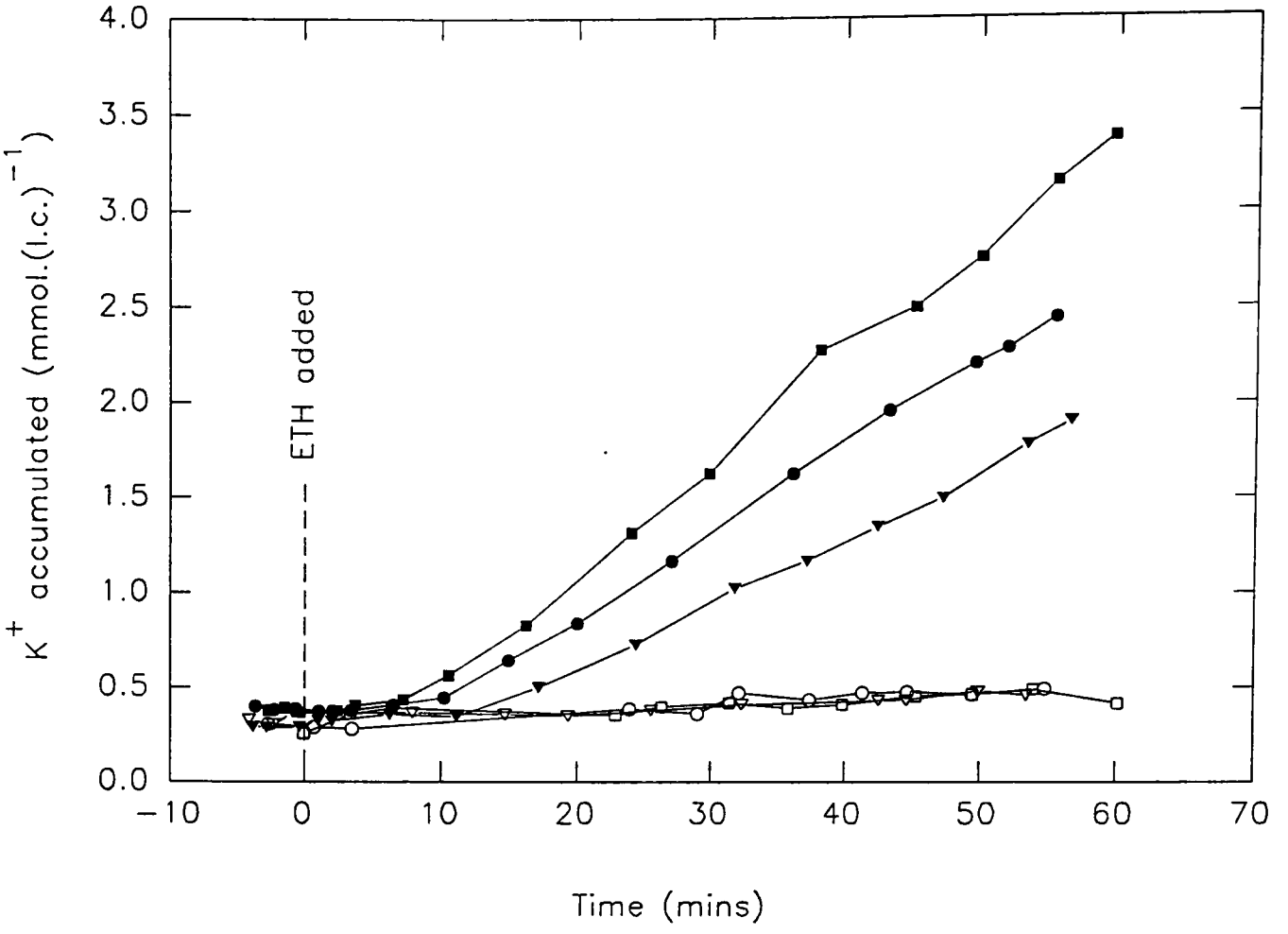


Figure 8.5 Time courses of ETH-induced ($^{86}Rb^+$)K^+ uptake into human erythrocytes. Flux time courses (at 37 °C) were initiated by the addition of 300 µl (^{86}Rb)KCl (150 mM) to 3.7 ml HRBC suspension (MBS + sucrose (50 mM)) in the presence of ouabain, bumetanide and nitrendipine (each at 100 µM), such that the experimental $(K^+)_o$ = 11.25 mM and Hct = 6.5 %. Results are from three separate experiments each on blood from a different donor (indicated by symbol-type). Filled/open symbols = 50 µl *stock* ETH added/not added at time = zero.

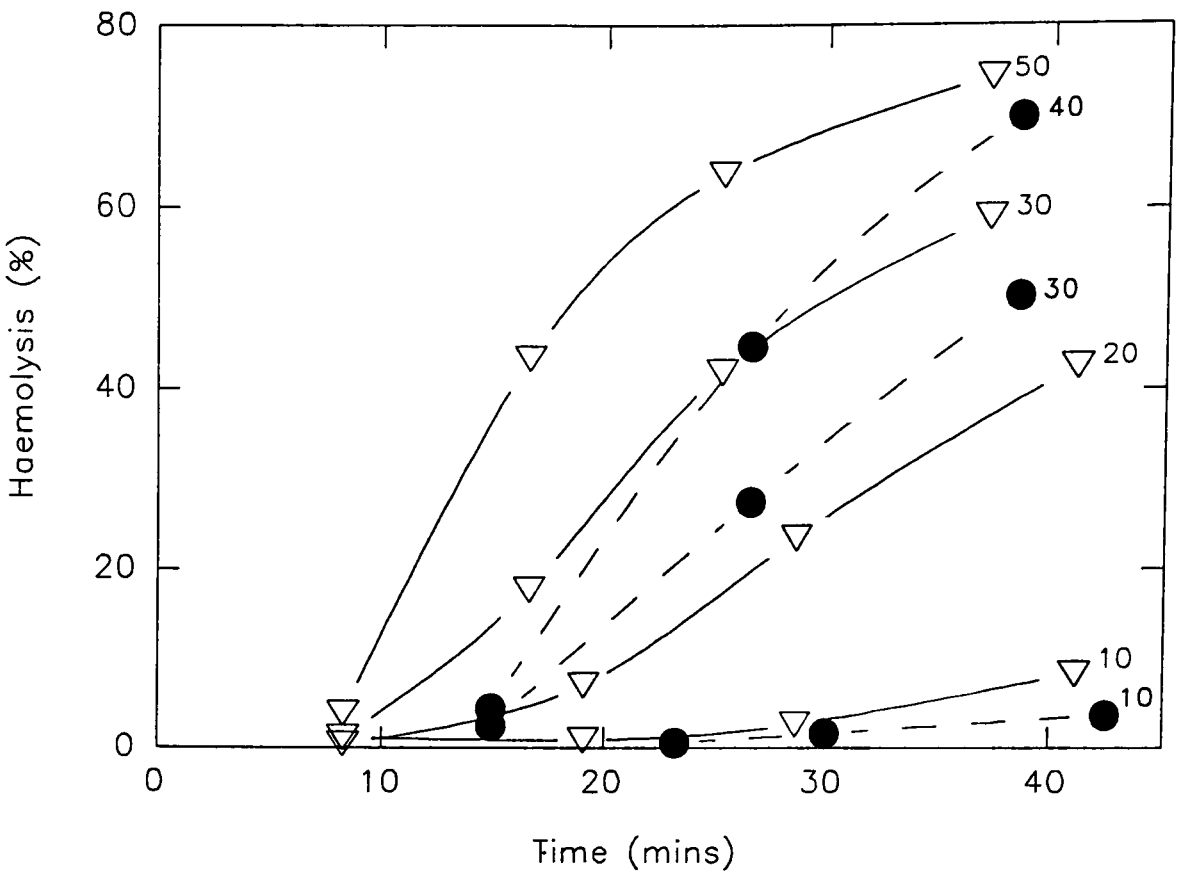


Figure 8.6 Comparative haemolytic time-courses induced by ETH and TDH. Aliquots (0.5 ml) of HRBC suspension (Hct = 4 %) in MBS were added (t = 0; subsequently incubated at 37 °C) to MBS (0.55 ml) containing/not containing TDH (open triangles) or ETH (closed circles) at indicated amounts (µl) of *stock* solutions. The TDH stock solution in this experiment contained 86.25 µg protein(ml)$^{-1}$. Results are from a single experiment representative of three such experiments. Haemolysis of cells untreated by toxin was < 1 % over the same time-course.

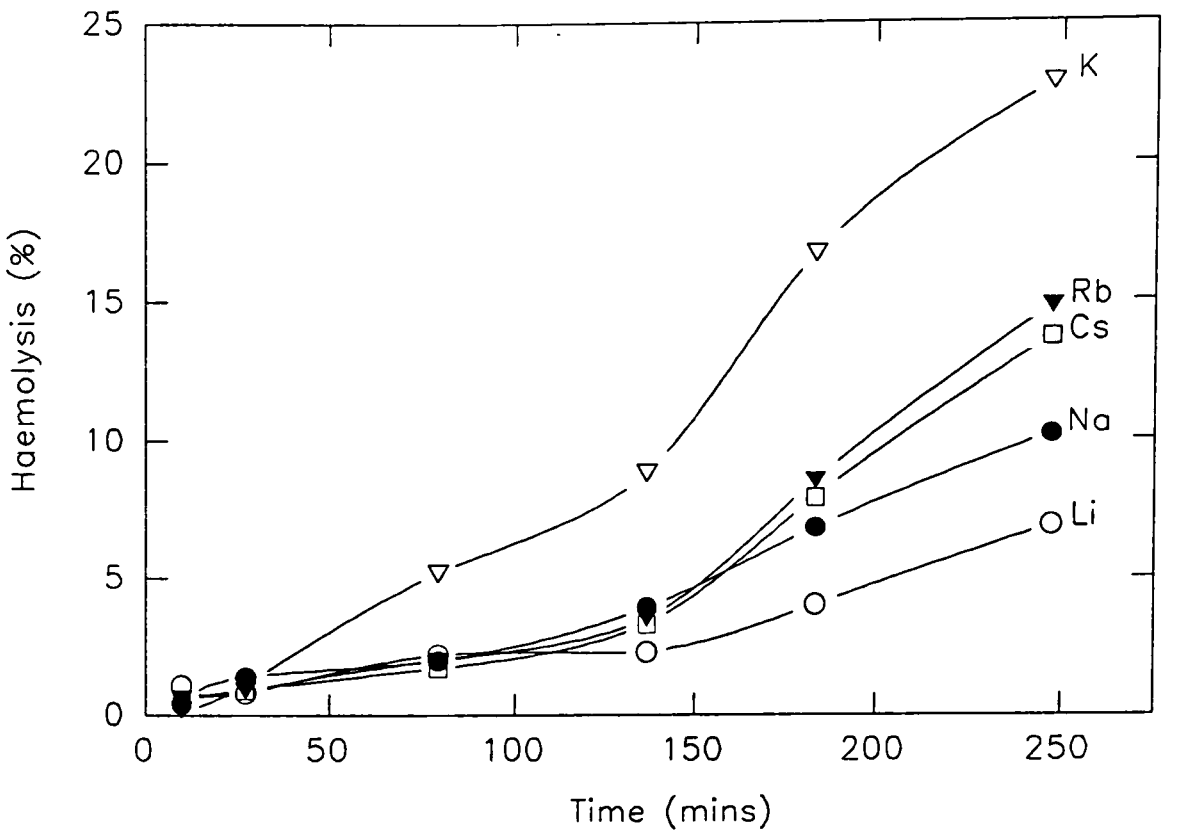


Figure 8.7 Monovalent cation selectivity of the ETH-induced lesion. Haemolysis was measured in monovalent cation chloride (150 mM, corrected to 291 ± 2 mosmoles(kg)$^{-1}$ in HEPES (10 mM) at pH 7.4) solutions. Haemolysis time-courses were initiated by the addition of 100 µl packed HRBC to 4.9 ml containing 25 µl *stock* ETH. This experiment was performed as paired with selectivity experiments for KH (Figure 4.19).

8.3 DISCUSSION

Yet again (see section 4.3), the erythrocyte has proved a useful target cell with which to analyse the mechanism of toxin action. Unfortunately, ETH is not commercially available or easy to obtain in yields equivalent to those for KH/TDH; nor was it as convenient a preparation to investigate as TDH, being unstable to air exposure and excessive mechanical movement (Figures 8.1 and 8.2). Research on ETH has thus been hampered, but appropriate conditions have been identified for stable storage of ETH (solutions bubbled with N_2, and ETH *stock* kept under oil; minimisation of mechanical agitation both before and during experiments) allowing analysis of the mechanism of action. Such instability has not been reported by other investigators (Honda & Finkelstein, 1979; Yamamoto *et al.*, 1984, 1986, 1990b; Hall & Drasar, 1990) and related cytolysins (Krasilnikov *et al.*, 1992).

It is, as yet, unclear whether the basis for mechanical sensitivity is direct or that vibration increases the interactions between toxin molecules and the component(s) of the air with which it reacts. The primary candidate for the gas that inactivates ETH is oxygen, since a number of other haemolysins (the thiol-activated group eg streptolysin O and tetanolysin (Alouf, 1980; Alouf & Geoffroy, 1991)) progressively lose their activity when stored in the presence of oxygen. By analogy, ETH will be investigated for features characteristic of this group, such as reactivation of deactivated toxin by reducing agents and inhibition by nanomolar concentrations of cholesterol.

Interestingly, besides differing from TDH in its stability to air and vibration, ETH appeared to be dissimilar in the more involved details of its action. Honda and Finkelstein (1979) previously documented a difference in the spectrum of haemolytic activity according to the species of erythrocyte employed. In the present study, although both toxins caused an increase in the cation permeability of the erythrocyte membrane (instigating lysis by colloid osmosis), the patterns of induced cation fluxes (and resultant time courses of haemolysis (Figure 8.6)) were very different, being high (initially) to low (after c. 20 minutes) for TDH (sections 5.2.2 and 5.3) and low (initially) to high (after c. 9 - 12 minutes) for ETH (Figure 8.5). Although it is as yet not possible to speculate on the physical basis of these induced leaks, it is likely that these contrasting data indicate a fundamental difference between the mechanisms of action of the two toxins.

Preliminary data on the rates of ETH-induced haemolysis in isosmotic monovalent cation chlorides (Figure 8.7) indicated a very different selectivity series to that obtained for KH/TDH (reversed Eisenman VIII, with a small K^+ anomaly). That obtained for ETH was a straight Eisenman IV sequence: $K^+ > Rb^+ > Cs^+ > Na^+ > Li^+$ (Eisenman & Horn, 1983). It has long been recognised that various effects of the monovalent cations in biology follow neither the simple sequence associated with naked ion size, nor the Hofmeister series of hydrated radii (Diamond & Wright, 1969; Eisenman & Horn, 1983; Armstrong, 1989). If the identity of the ion does not affect either leak-induction or the nature of the induced lesion itself, then the relative haemolytic rates found in the present study can be ascribed to the selectivity of the lesion: this is the assumption on which later reasoning will be based. Observed selectivity series for monovalent cations may be generally viewed as arising from energetic interactions with an anionic site, in which selectivity is generated as a result of the difference in the hydration energy and binding energy (to the anionic site) for particular ions. Hydration and site-binding energies themselves vary with the inverse cation radius such that they can be represented by suitable power series of this variable. Thus when an index of effect (eg permeability of particular ions) is plotted against the inverse naked cation radius, the observed relations exhibit either selectivity maxima (Eisenman sequences, for which binding energies are such that lower power terms dominate for the binding-site compared to hydration) or selectivity minima (polarisability sequences (reversed Eisenman sequences), for which binding energies are such that higher power terms dominate for the site compared with hydration; Eisenman & Horn, 1983). The observation that TDH and ETH yield a polarisability sequence (reversed Eisenman VIII, albeit with a small K^+ anomaly) and a straight Eisenman IV sequence, respectively, indicates a fundamental difference in the underlying energetics of the ion-transducing events mediated by the lesions induced by these toxins. These selectivity sequences also distinguish the toxin-induced leaks from that induced by diamide cross-linking of spectrin, which yields an Eisenman sequence I or II ($Cs^+ > Rb^+ \geq K^+ > Na^+ \geq Li^+$), following the sequence of free solution mobilities (Deuticke *et al.*, 1984).

Sequencing of the structural gene for vulnificolysin of *V. vulnificus* showed that regions of the gene are homologous to that encoding ETH (section 8.1; Yamamoto *et al.*, 1990c; Kreger, 1991). Therefore there are grounds to suspect similarities in the actions of these two toxins,

although results of cross-neutralisation studies with homologous and heterologous antisera (Kreger, 1984) suggest that vulnificolysin is antigenically distinct from ETH, as well as other *Vibrio* toxins (TDH of *V. parahaemolyticus*, and damselysin of *V. damsela*). Characterisation of vulnificolysin preparations (Gray & Kreger, 1985; Miyoshi *et al.*, 1985; Shinoda *et al.*, 1985) suggest that it is heat-labile, inactivated by cholesterol, but unnaffected by oxygen or sulphydryl-blocking agents. Miyoshi *et al.* (1985) therefore proposed this haemolysin to be a unique cholesterol-binding but non-thiol-activated toxin. Gray and Kreger (1985) found that haemolysis of mouse erythrocytes exhibited multi-hit characteristics, thus differing from the mode of action observed for KH (section 4.2.1). Krasilnikov *et al.* (1992) also characterised the manner of attack of a *V. cholerae* cytolysin (designated CC) related, but not identical, to ETH, which they found to be unaffected by cholesterol and dithiothreitol. In light of these reports subsequent research should be directed at the questions of ETH susceptibility to inhibition by cholesterol and other reagents, in an attempt to clarify the degree of relatedness of ETH to other *Vibrio* toxins.

Now that conditions for storage of this toxin have been established, in addition to the definition of culture growth conditions and purification procedures (Chapter 6), the way is paved for future research much along the lines of that performed on KH. The next chapter returns to the examination of KH action, but on isolated enterocytes rather than erythrocytes because having established the likely importance of TDH as a flux-inducing agent, it was important to examine the putative role of this toxin on a more pathophysiologically relevant cell-type.

CHAPTER 9

Effects of Kanagawa haemolysin on rabbit intestinal cells

'"It's no use going after him. He's just had the potion; he's practically invincible!" (Getafix the druid)'
Goscinny, *in* Asterix the Gaul

9.1 INTRODUCTION

The possible role of the thermostable direct haemolysin (TDH) in the pathogenesis of disease associated with *Vibrio parahaemolyticus* and other *Vibrio* species has been discussed in section 2.6. In this study, the mechanism of membrane attack by this toxin has been broadly characterised on erythrocytes (Chapters 4, 5 and 7). Furthermore a possible role for the Ca^{2+}-activated K^+ channel in toxin-induced pathophysiology has been highlighted (section 5.2.4). Although useful, studies on erythrocytes are somewhat removed from the pathophysiological situation with respect to *Vibrio*-instigated gastroenteritis. It was therefore desirable to test the effects of this toxin on an intestinal membrane preparation.

The effect of TDH on intestinal function has been investigated by other workers (section 2.6.4). In particular: (i) alimentary injection of TDH was shown to cause fluid accumulation in ligated rabbit ileal loops (Zen-Yoji *et al.*, 1974), and (ii) Nishibuchi *et al.* (1992) provided good evidence for TDH being an active enterotoxin of *V. parahaemolyticus*, since anti-TDH serum greatly reduced the ability of supernatants to cause an increase in I_{SC} across rabbit ileum.

As previously noted (section 2.4.2), studies of the transport functions (and perturbations thereof) of the small intestine are hindered by the anatomical complexity of this tissue. Results obtained with the whole tissue are often difficult to interpret at the cellular level (Brown & Sepulveda, 1985a). Rabbit jejunocytes were selected as an appropriate membrane preparation for study, because: (a) methodology for isolating viable cells is well established (section 3.3.2; Kimmich, 1975; Brown & Sepulveda, 1985a,b; Ilundain, 1991), (b) such cells constitute the smallest functional transport unit of the tissue though considerably less complex than intact tissue preparations (Ilundain, 1991), and (c) rabbit jejunum is sensitive to TDH^+

but not TDH$^-$ bacteria (ligated loop assay; Nishibuchi *et al.*, (1992)). In addition, because all cell boundaries are equally exposed to the suspending medium, this preparation is ideally suited to the study of all transport events including the putative role of the basolaterally sited (in the intact tissue) Ca^{2+}-activated K^+ channel (Brown & Sepulveda, 1985a,b).

All techniques pertaining to jejunocyte isolation and quantification have been described in section 3.3.2. Ouabain and bumetanide have been used as described earlier (sections 3.6.1 and 4.1). Ba^{2+} (5 mM) (as opposed to nitrendipine (see section)) has been used to inhibit the Ca^{2+}-activated K^+ channel of these cells, as validated by Brown and Sepulveda (1985a,b). These workers also (i) performed a microscopic evaluation of the hyaluronidase-treated jejunum, finding that tissue damage was almost exclusively confined to the epithelial layer of the villus, sheets of epithelial enterocytes being stripped from the underlying *lamina propria*, and (ii) showed that $^{86}Rb^+$ is a suitable tracer for K^+ movement in these cells; efflux was characterised as following a single exponential time course, suggesting that the isolated enterocyte may be treated as a one compartment system. Human erythrocytes have been used as a positive control, testing the viability of the toxin preparation, the cell isolation procedure and drugs used. Results are expressed in terms of either percentage cells surviving, K^+ accumulated (either mmol.$(10^{12}$ cells$)^{-1}$ or mmol.(l.c.)$^{-1}$), efflux parameters (see section 3.5.2) or percentage haemolysis (for control experiments on red cells).

9.2 RESULTS

Although observations of cellular morphology confirmed homogeneity of isolated cells (section 3.3.3), comparisons of quantifications of protein (bicinchoninic acid assay; section 3.3.2) and the number of jejunocytes revealed that the average amount of protein per cell varied markedly between isolations, there being (mean ± S.E.M.) 0.174 ± 0.0809 mg protein per 10^6 cells (n = 3). The reason for this variation is, as yet, unclear. This quantity also differed from that obtained by Kimmich (1975) for chicken enterocytes (c. 1 mg protein per 10^6 cells). Subsequently, therefore, parameters of ion movement were quantified according to the number (rather than the volume) of cells (see section 3.3.2, equation *7*). Experiments

on a concentrated cell suspension showed that all cells (ie both alive and dead) were centrifuged through the DBP:DNP mixture, there being no visible debris above the oil layer after CP4.

Demonstration of endogenous components of K^+ influx was performed (Figure 9.1) by measuring isotope uptake in the presence of transport inhibitors, ouabain (0.1 mM) for the Na^+/K^+ ATPase, bumetanide (0.1 mM) for the $Na^+/K^+/2Cl^-$ cotransporter and Ba^{2+} (5 mM) for the Ca^{2+}-activated K^+ channel - concentrations of inhibitors previously used by Brown and Sepulveda (1985a), and Ilundain (1991). As already shown by Brown and Sepulveda (1985a), ($^{86}Rb^+$)K^+ influx was predominantly mediated by the ouabain-sensitive route. It appeared that large amounts of isotope were trapped with the cell pellet (which included a proportion of dead cells (c. 32 %)) on spinning through the oil mixture. Hence the baseline for the parameter of accumulated K^+ was markedly raised, even at values given by extrapolation to time = zero.

Enterocyte survival was followed, in the presence and absence of KH ($2HU(ml)^{-1}$), by using Trypan Blue exclusion (section 3.3.2) as a measure of cell viability. After the isolation procedure 68.47 ± 2.24 % (n = 3) of cells were capable of excluding the vital dye. Over a 30 minute time course, KH had no significant effect on the % cells surviving, this percentage being 65.9 ± 2.92 and 65.1 ± 3.65 (n = 3) in the absence and presence of toxin (Figure 9.2).

Preliminary experiments at doses (0.1 - 0.2 $HU(ml)^{-1}$) comparable to those used earlier for erythrocytes (section 5.2.1) gave no indication of raised influx in response to KH. Accordingly, a larger dose (0.95 $HU(ml)^{-1}$) was used (Figure 9.3). No noticeable response was elicited in three experiments at this dose. Once again, extracellular isotope associated with the cell pellet made precise determination of the basal (inhibitor-insensitive) flux difficult; it is conceivable that the 'noise' inherent in this determination was obscuring an altered influx. In an attempt to circumvent this problem, efflux protocols were used on cells which had been washed after loading with isotope (sections 3.5.2 and 3.5.3), with quantification of extracellular isotope after centrifugation (CP4) of cells through oil.

A preliminary experiment (Figure 9.4) did not indicate a raised efflux constant for a preparation treated with KH (0.95 $HU(ml)^{-1}$); the efflux rate contant (min^{-1}) was 0.040 ± 0.0011 (control) and 0.039 ± 0.0040 (after KH addition). A trivial explanation for this

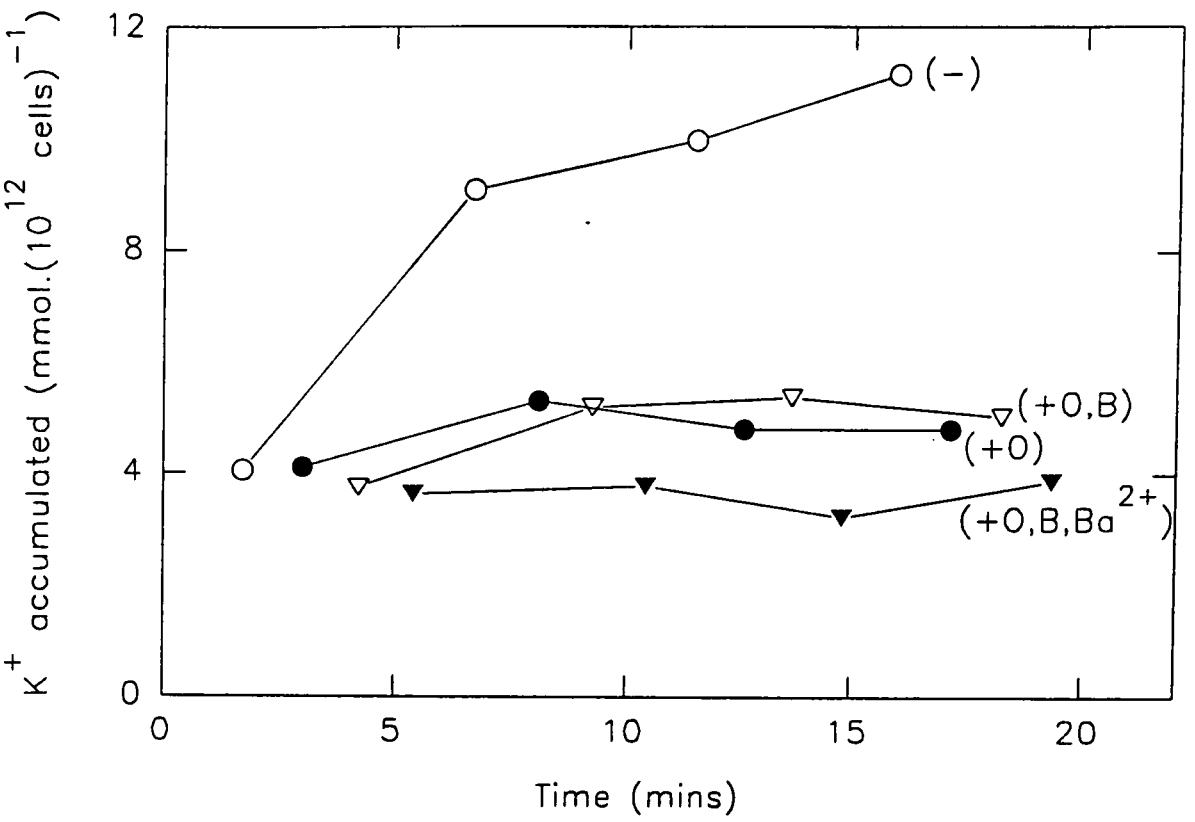


Figure 9.1 Time course of K^+ influx mediated by endogenous components into isolated jejunocytes. Isolated cells were resuspended in GCS (1.8 ml) with inhibitors as indicated. At time = zero, (^{86}Rb)KCl (150 mM; 0.2 ml) was added to these suspensions such that experimental parameters were: cell density varied between experiments but was in the range 1.7 - 6.6 x 10^7 cells(ml)$^{-1}$, $(K^+)_o$ = 21 mM, (ouabain (O)) (if appropriate) = 0.1 mM, (bumetanide (B)) (if appropriate) = 0.1 mM, $(Ba^{2+})_o$ (if appropriate) = 5 mM, and the temperature was 37 °C. At time-points, 200 µl samples of suspension and spun through a DBP:DNP oil mixture, as described in section 3.5.1 Results given are representative of three such experiments.

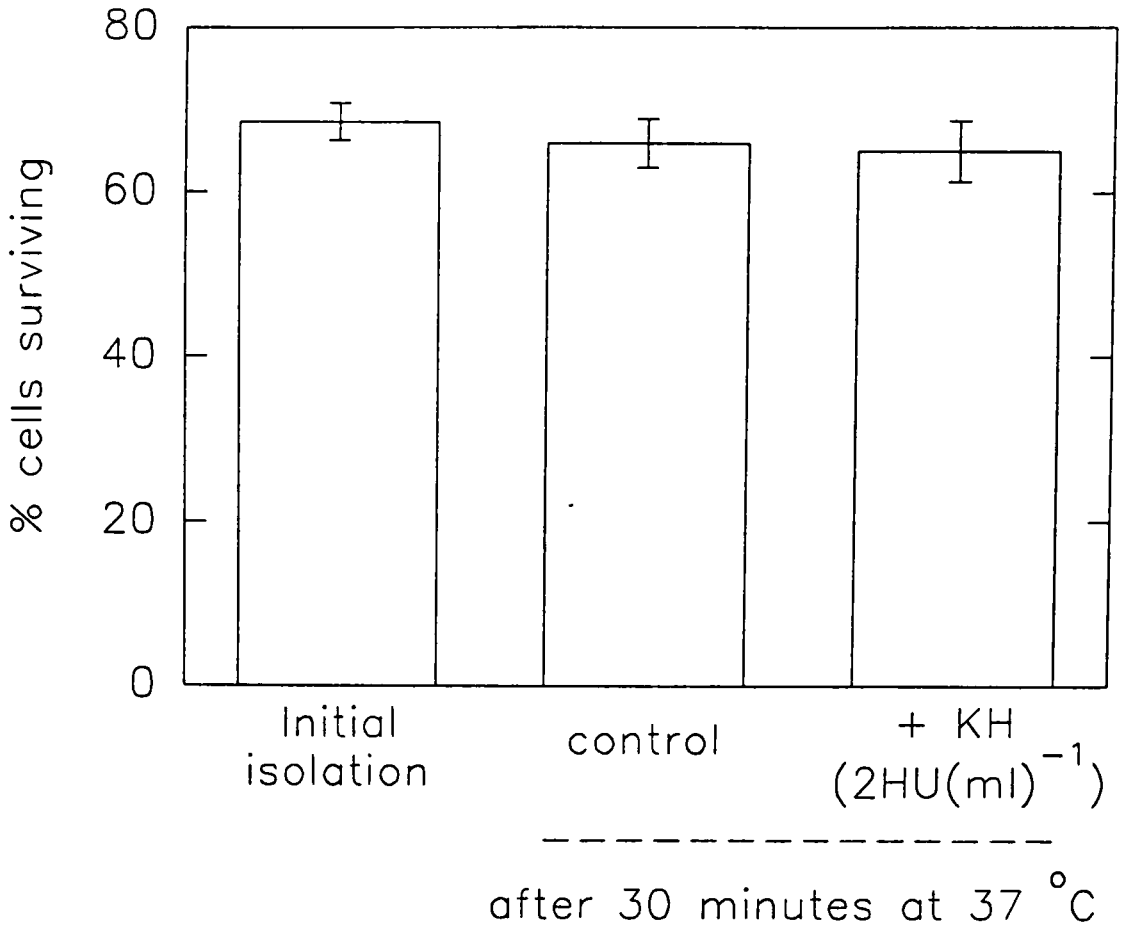


Figure 9.2 Lack of effect of KH (2 HU(ml)$^{-1}$) on survival of isolated rabbit jejunocytes. Isolated enterocytes were resuspended to c. 7 x 10^6 cells (ml)$^{-1}$, treated as appropriate with KH (2HU(ml)$^{-1}$) or an equal volume of MBS (control), and incubated at 37 °C for 30 minutes.

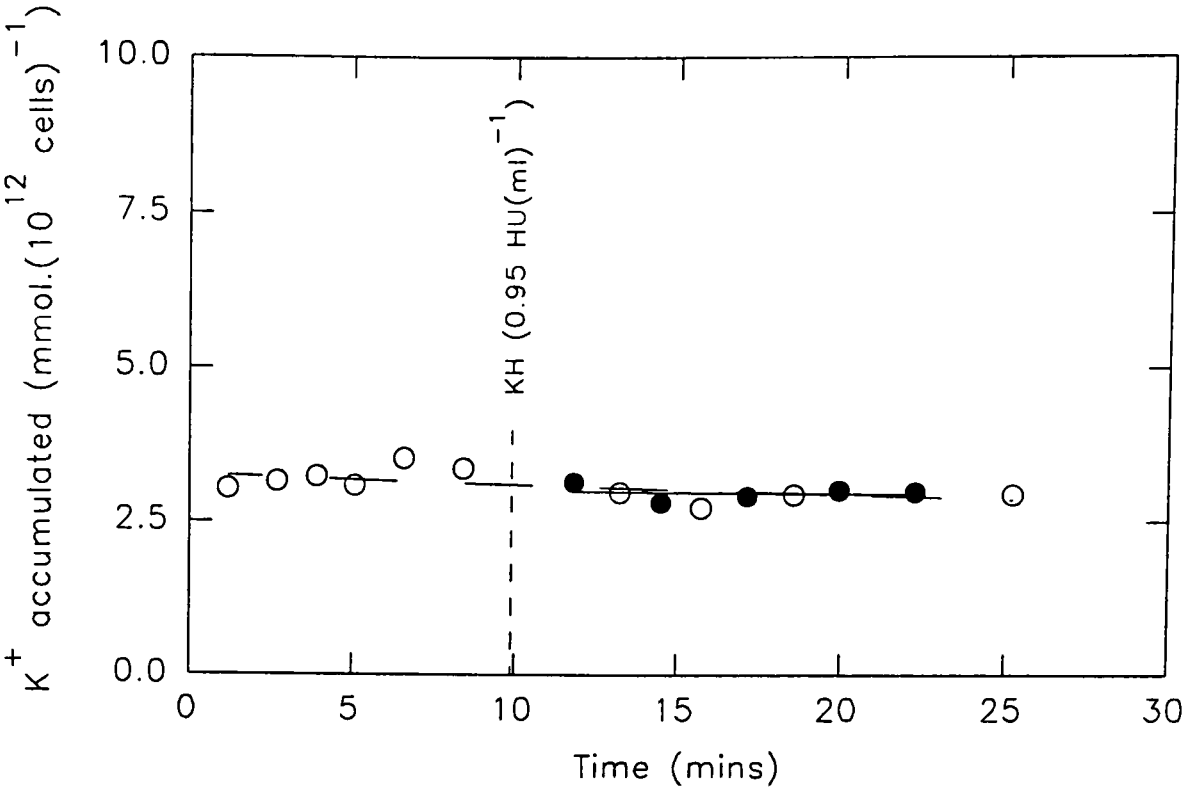


Figure 9.3 Lack of effect of KH on K^+ movement into isolated jejunocytes in the presence of ouabain, bumetanide and Ba^{2+}. Isolated cells were resuspended in GCS (5.4 ml) with inhibitors. At time = zero, (^{86}Rb)KCl (150 mM; 0.6 ml) was added to these suspensions such that experimental parameters were: cell density varied but was in the range 1.46 - 3.18 x 10^7 cells(ml)$^{-1}$, $(K^+)_o$ = 21 mM, ouabain (0.1 mM), bumetanide (0.1 mM), Ba^{2+} (5 mM), and temperature = 37 °C. At time-points, samples (300 µl) were spun through DBP:DNP (section 3.5.1). Typical results are given that are representative of three such experiments. Open/closed circles indicate untreated and KH (0.95 HU(ml)$^{-1}$)-treated systems respectively; lines of best fit were calculated by least squares regression for these two sets of data (dashed/solid lines). There was no significant difference in the gradients of these two relations.

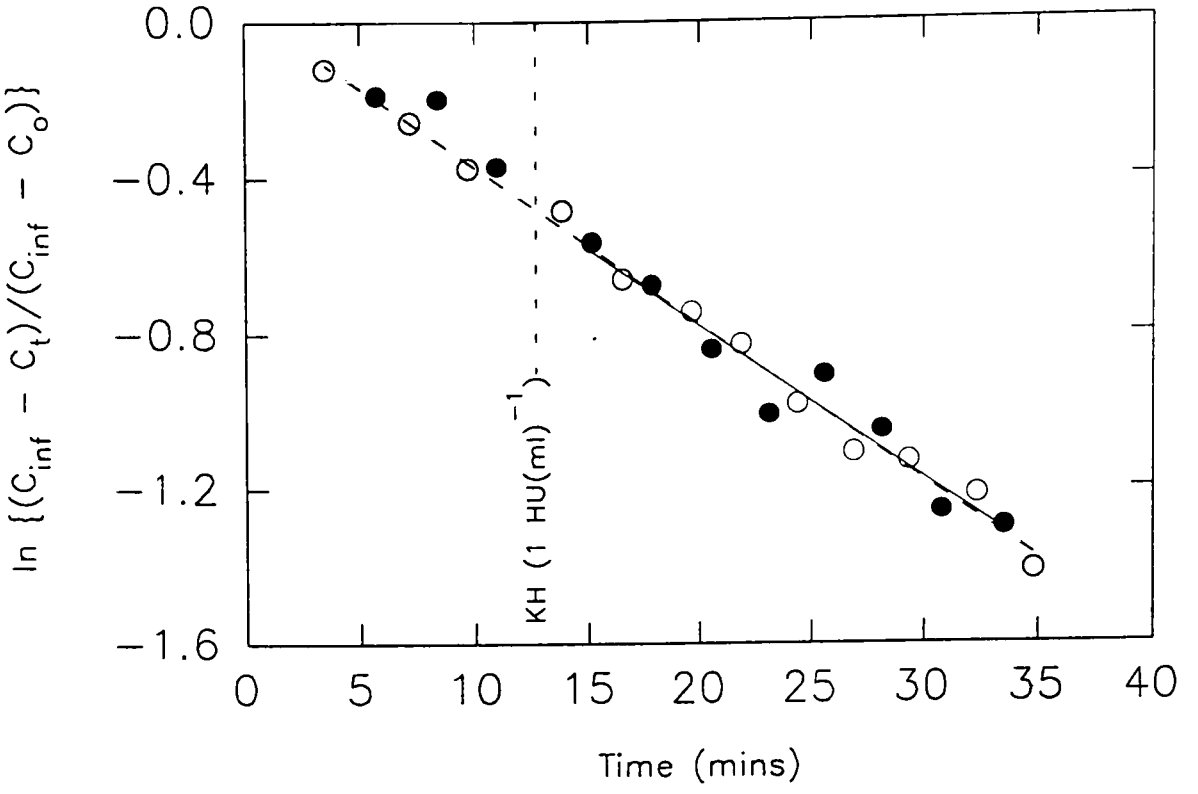


Figure 9.4 Preliminary experiment describing the time course of K^+ efflux from isolated rabbit jejunocytes and the lack of effect of KH (0.95 HU(ml)$^{-1}$). Isolated jejunocytes were resuspended to a total volume of which c. 80 % was equal to that previously occupied by the cell pellet after centrifugation (CP2). The suspension was incubated (15 minutes at 37°C) with ^{86}RbCl at 5 μCi(ml)$^{-1}$, prior to washing (2 x) by addition of a large extracellular volume (extracellular:cellular = c. 50:1) of cold (from ice) GCS, centrifugation (CP2 at 8 °C), and aspiration of supernatant. Packed cells (250 μl) were resuspended in pre-warmed (37 °C) GCS (15 ml total) containing Ba^{2+} (5 mM). After 12.67 minutes, KH (0.95 HU(ml)$^{-1}$), was added to the system given by closed circles (control system is given by open symbols). Samples (1 ml) were spun through DBP/DNP and the supernatant removed for counting. The parameter $\ln\{(C_{inf} - C_t)/(C_{inf} - C_o)\}$ is plotted against time (mins); the negative of the slope of this relation yields the efflux rate constant (min^{-1}). Lines of best fit were fitted by least squares regression for the whole time course (control cells; dashed line) and after toxin addition (KH-treated preparation; solid line). The gradients did not differ significantly.

apparent insensitivity was that it was an artefact of either the preparative or experimental conditions, ie use of GCS (as opposed to MBS), hyaluronidase treatment of cells, or use of Ba^{2+} (5 mM). The possibility that these factors might render cells insusceptible to toxin action was assessed by treating human erythrocytes similarly (Figure 9.5). As already found (sections 4.22 and 5.21), K^+ influx ($[K^+]_o$ = 15 mM) into human erythrocytes suspended in MBS with sucrose (45 mM) treated with maximally inhibitory concentrations of ouabain, bumetanide and nitrendipine (section 3.6.1) was significantly increased ($P < 0.05$, $n = 4$) from - 0.08 ± 0.043 to 20.15 ± 4.524 mmol.(l.c.)$^{-1}$(hr)$^{-1}$. GCS medium has a K^+ concentration of 6 mM (see Table 3.1) and GCS-based systems were 'spiked' in a similar manner to that for MBS-based systems (ie addition of 400 µl (^{86}Rb)KCl (150 mM) to 3.6 ml cell suspension). To obtain comparable measures of K^+ K^+ influx (possible because KH-induced K^+ influx is not saturatable with respect to $[K^+]_o$ in this range - see section 5.2.1, Figure 5.5), this value must be corrected for $[K^+]_o$ (see *y*-axis, in Figure 9.5).

Interestingly, the parameter of scaled KH-induced K^+ influx (mmol.(l.c.)$^{-1}$(hr)$^{-1}$) was significantly (paired *t*-test; $P = 0.01$, $n = 4$) higher in GCS (22.02 ± 4.623) than MBS (20.15 ± 4.524). However, this increase apeared to be accounted for by the parallel and significant ($P = 0.005$, $n = 3$) inequality in basal leak for systems untreated by KH, this being -0.08 ± 0.043 and 0.90 ± 0.188 mmol.(l.c.)$^{-1}$(hr)$^{-1}$ for cells suspended in MBS and GCS, respectively. These fluxes were calculated from the gradient of the line fitted to data with respect to time by least squares regression. Hyaluronidase (1 mg(ml)$^{-1}$ in GCS; Hct = 10 %; 30 minutes at 37 °C) treatment of red cells did not cause a significantly increased leak in either the control or KH-treated condition. The presence of Ba^{2+} (5 mM) in GCS caused an increase ($P < 0.1$, $n = 3$) in scaled KH-induced K^+ influx into HRBC from 23.74 ± 5.115 to 29.60 ± 6.102 mmol.(l.c.)$^{-1}$(hr)$^{-1}$; this was not attributable to raised basal leak.

Having ascertained that neither the cell isolation procedure nor treatment by Ba^{2+} (5 mM) protected against toxin action, a larger dose (2 HU(ml)$^{-1}$) was used in conjunction with the efflux protocol (Brown & Sepulveda, 1985a,b) - see Figure 9.6A). After loading with isotope (cells incubated for 15 minutes at 37 °C, in GCS with ^{86}Rb at 5 µCi(ml)$^{-1}$), cells were washed twice (addition of ice-cold GCS, centrifugation (CP2) and asiration of supernatant) in an

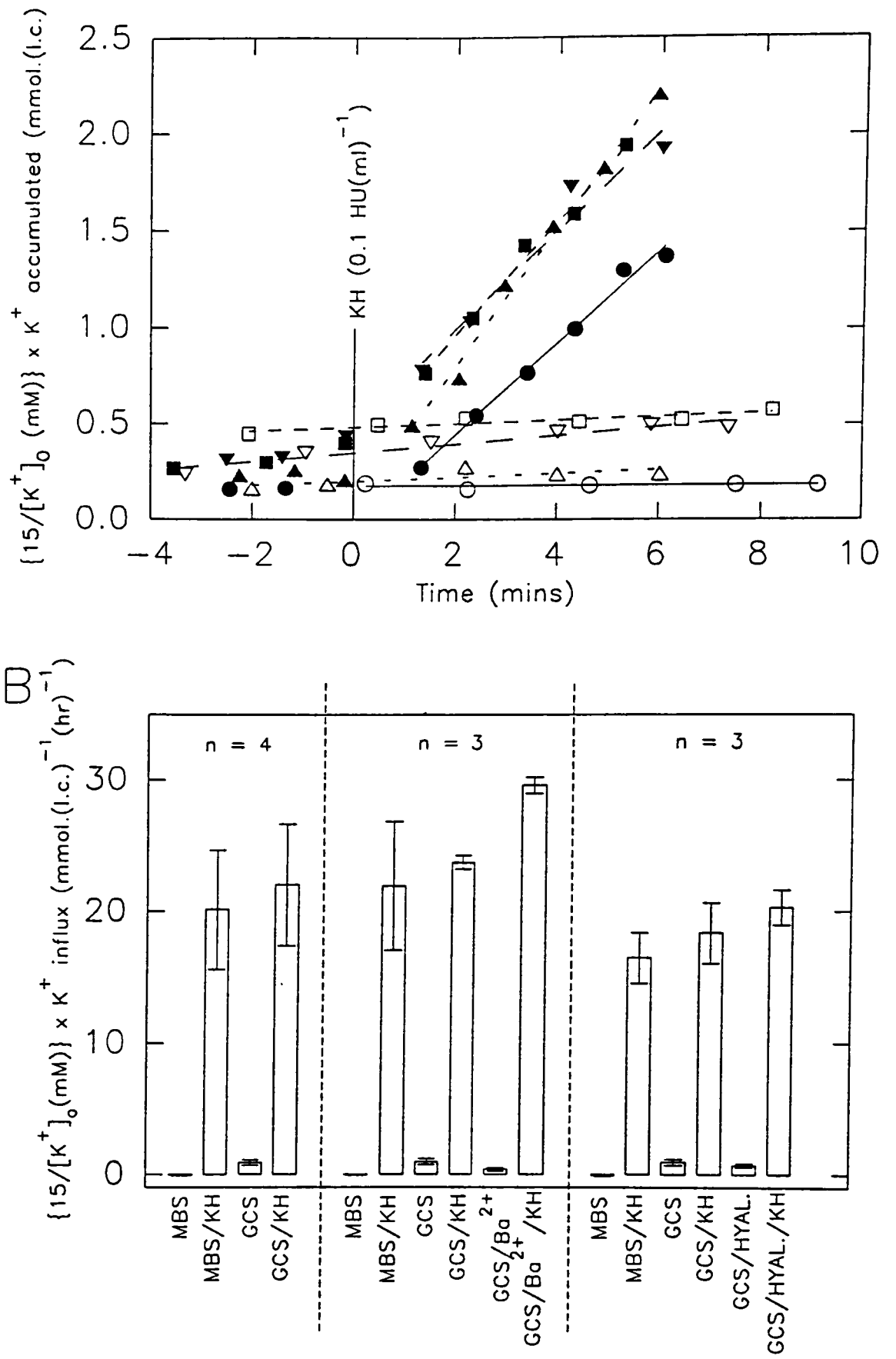


Figure 9.5 Effects of GCS, GCS + hyaluronidase, and GCS + Ba^{2+} (5 mM) on KH-induced K^+ influx in human erythrocytes. HRBC suspended (Hct = 10 %) in either MBS (+ sucrose (50 mM)), GCS, and GCS + hyaluronidase (1 mg(ml)$^{-1}$) were incubated at 37 °C for 30 minutes. Cells were then washed twice in MBS or GCS (hyaluronidase-free - irrespective of starting condition), as appropriate and resuspended in the same (280 µl packed cells to 3.32 ml solution also containing ouabain, bumetanide and nitrendipine (final concentration = 100 µM for each drug). Cells initially incubated in GCS alone were ultimately resuspended in both GCS and GCS + Ba^{2+} (experimental concentration = 5 mM). Aliquots (0.4 ml) of (^{86}Rb)KCl (150 mM) were added to suspensions, initiating time courses. KH (0.1 HU(ml)$^{-1}$ was added as appropriate at time = zero; samples (200 µl) were taken over the next 6.5 minutes (5 < experimental Hct (%) < 6) for centrifugation (CP3) through DBP. Experimental $(K^+)_o$ = 15 mM for the MBS-based system, and 21 mM for others. (A) Time courses of K^+ accumulated for different conditions. Open/filled symbols represent systems in which KH was not/was added. Circles (solid line), inverted triangles (medium dashed line), squares (small dashed line) and flat-based triangles (dotted line) represent the solution/treatment in (i) MBS, (ii) GCS, (iii) GCS + hyaluronidase (1 mg(ml)$^{-1}$), and (iv) GCS + Ba^{2+} (5 mM), respectively. Results are from a typical experiment representative of a number of experiments (*n* as indicated in (B)). (B) K^+ influxes calculated by least square regression for both control and KH-treated systems, pooled for the indicated number of experiments. The significance of differences between conditions is described in text (section 9.2).

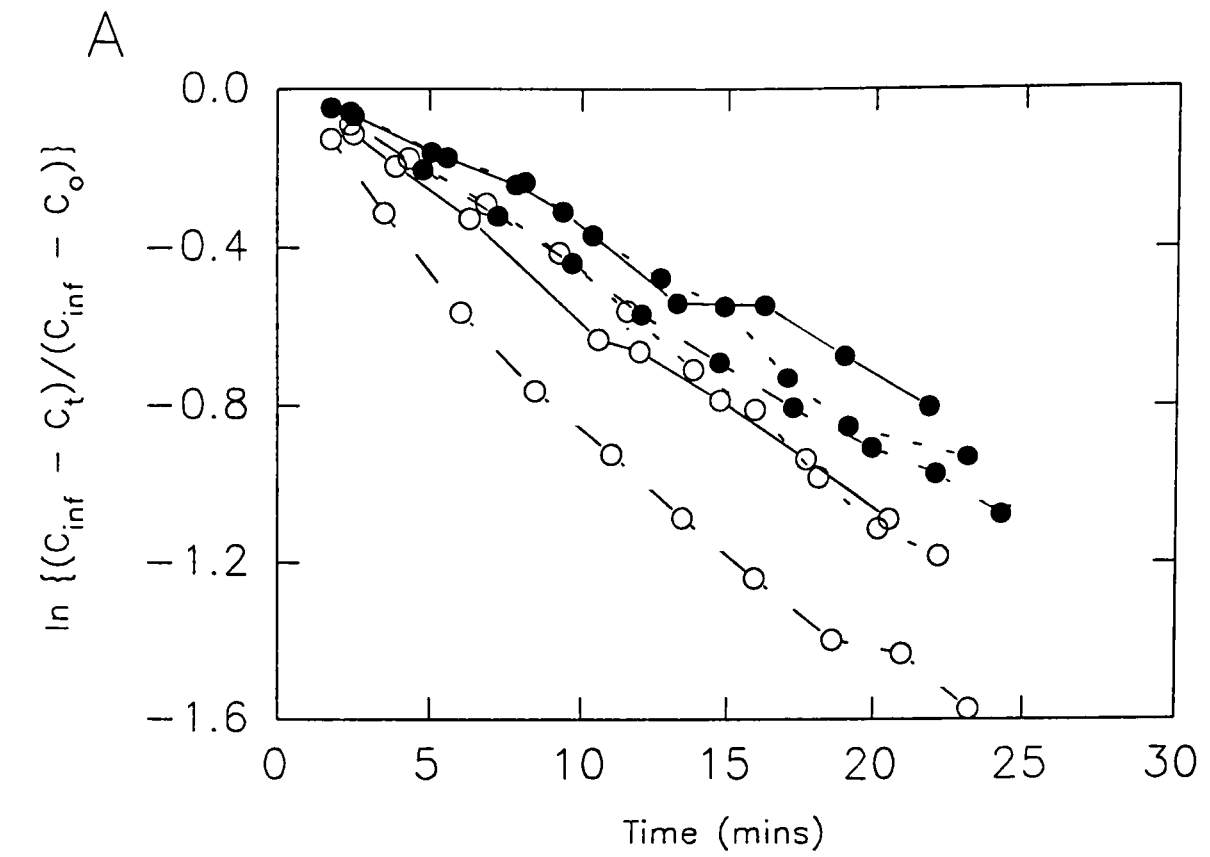


Figure 9.6 Time course of K^+ efflux from isolated jejunocytes in the presence of Ba^{2+} (5 mM) and the presence/absence of KH. Isolated jejunocytes were resuspended to a total volume of which c. 80 % was equal to that previously occupied by the cell pellet after centrifugation (CP2). The suspension was incubated (15 minutes at 37°C) with $^{86}RbCl$ at 5 $\mu Ci(ml)^{-1}$, prior to washing (2 x) by addition of a large extracellular volume (extracellular:cellular = c. 50:1) of cold (from ice) GCS, centrifugation (CP2 at 8 °C), and aspiration of supernatant. Packed cells (250 µl) were resuspended in pre-warmed (37 °C) GCS (15 ml total) containing Ba^{2+} (5 mM) and KH (2 $HU(ml)^{-1}$), as appropriate. Samples (1 ml) were spun through DBP/DNP and the supernatant removed for counting. Positive controls for toxin activity were performed on HRBC suspended (Hct = 5 %, at 37 °C) in MBS with/without KH (1 $HU(ml)^{-1}$). Toxin-induced haemolysis assayed after 25 minutes was 30.5 ± 5.52 % (n = 3) compared with < 1 % for the untreated condition. (A) The parameter $\ln\{(C_{inf} - C_t)/(C_{inf} - C_o)\}$ is plotted against time (mins); the negative of the slope of this relation yields the efflux rate constant (min^{-1}). KH-treated/untreated cells are given by empty/filled circles respectively; each cell isolation is assigned an individual line-type. (B) Efflux rate constants calculated by linear regression over the time period 0 - 10.5 minutes (n = 3) after initiation of the efflux time-course.

excess of extracellular volume (ratio of extracellular to intracellular volumes = c. 50:1) to minimise initial (time = zero) extracellular counts. Human erythrocytes (HRBC) were used as a positive control to confirm activity of the toxin preparation; KH (1 $HU(ml)^{-1}$) caused 30.5 ± 5.2 % haemolysis (n = 3; assayed after 25 minutes at 37 °C) of an HRBC suspension (initial Hct = 5 %) in MBS, whereas the control suspension (untreated by KH) had < 1 % haemolysis. There appeared to be a degree of variation in induced efflux from enterocytes according to particular isolation. Efflux rate constants were calculated over the first four time-points (ie 0 - 10.5 minutes of toxin exposure) by calculating the gradient of the line of best fit, given by least squares regression through the origin. The basal rate constant of efflux in Ba^{2+}-treated (not in the presence of ouabain or bumetanide) enterocytes was 0.036 ± 0.0040 min^{-1}. The rate constant was increased (by 71.5 ± 21.01 %) by KH-treatment (Figure 9.6B) to 0.063 ± 0.014 min^{-1} (n = 3); however, this increase was not significant at the 5 % confidence level (n = 3).

9.3 DISCUSSION

Enterocytes were isolated in a viable form for experimentation, in particular: (i) over two-thirds of the cells excluded Trypan Blue, (ii) the cell suspension accumulated $^{86}Rb^{+}$ (Figure 9.1) predominantly by the ouabain-inhibitable route (presumably the Na^{+}/K^{+} ATPase; Brown & Sepulveda, 1985a), and (iii) the efflux rate constant for $^{86}Rb^{+}$ was in good agreeement with values obtained by other workers (Brown & Sepulveda, 1985b). Preparative procedures did not affect the susceptibility of HRBC to KH action, and KH (albeit at a high dose) was capable of increasing the efflux rate constant.

Data indicated that KH (2 $HU(ml)^{-1}$) did not affect enterocyte survival over a 30 minute incubation period (Figure 9.2). This was not surprising given certain properties ascribed to enterocytes, namely a large reserve volume and consequent capacity to swell (Knutton *et al.*, 1976), and the ability to regulate their volume (section 2.4.1(*iv*)). Furthermore, Nishibuchi *et al.* (1992) have documented TDH-induced increases in I_{SC} across rabbit ileum, for which there were no accompanying histological changes. Therefore, isotope flux methodology was applied to search for altered ion movement in the presence of toxin, in a manner analagous

to that previously performed on erythrocytes (Chapter 5).

Subjecting human erythrocytes to analagous treatments to those performed on isolated jejunocytes, by exposure to hyaluronidase or Ba^{2+}, had no protective effect on KH-induced K^+ influx (Figure 9.5). Suspension of erythrocytes in GCS as opposed to MBS caused a slight stimulation of KH-induced influx but this increase was attributable to raised ion movement by a pathway other than that instigated by the toxin. However, the addition of Ba^{2+} (5 mM) to cells suspended in GCS appeared to enhance KH-induced influx independently of the basal leak. These results are interesting in light of other findings (section 5.2.2), indicating that the divalent cations Cd^{2+} (1 mM) and Zn^{2+} (250 μM) inhibited toxin-induced cation influx. Future studies on the effects of divalent cations on the TDH-induced lesion, possibly using the lipid bilayer preparation, should therefore compare the effects of different divalent cations (including Ba^{2+}) and at different concentrations (Pasternak, 1991).

For $^{86}Rb^+$ movement across the jejunocyte membrane, the efflux rate constant obtained under basal conditions in this study (0.036 ± 0.004) is in good agreement with the value (0.035 ± 0.002 min^{-1}) obtained by Brown and Sepulveda (1985b) in their study on this cell preparation. KH (2 $HU(ml)^{-1}$) increased this parameter (Figure 9.6) indicating sensitivity of the tissue to toxin, confirming the possibility that this protein may be an important enterotoxin of *V. parahaemolyticus*. However, the concentration of KH required to elicit this effect was extremely high; positive controls for toxin activity in these experiments showed that after 25 minutes incubation (in MBS with [KH] = 1 $HU(ml)^{-1}$), human erythrocyte suspensions exhibited substantial haemolysis.

Previous studies have also shown that large amounts of TDH (500 μg; 100 μg was insufficient, per loop) were required to cause fluid accumulation in ligated rabbit ileal loops (Zen-Yoji *et al.*, 1974). This has been elaborated on by other authors (Sakazaki *et al.*, 1974; Honda & Finkelstein, 1979; Sarkar *et al.*, 1987) who have been sceptical of the role of TDH in gastrointestinal illness. Sakazaki *et al.* (1974) found that concentrated culture filtrates from both KP^+ and KP^- strains were similarly reactogenic in rabbit ileal loops. Furthermore, the results of tests of live cultures (10^8 cells) on the same intestinal preparations produced dilatation and inflammation predominantly for KP^+ strains; the factor(s) responsible for

changes instigated by culture filtrates, irrespective of the Kanagawa phenomenon, was thermolabile. Therefore, these workers suggested that the ability of *V. parahaemolyticus* to multiply in the intestine (which they found to be linked to $KP^{+/-}$) might be the critical variable governing enteropathogenicity and that a factor other than TDH might be important in enteropathogenesis. Sarkar *et al.* (1987) found that repeated subculturing of KP^{-} *V. parahaemolyticus* strains on Wagatsuma agar induced the production of exoprotein(s) other than TDH. The exoprotein mixture derived from the culture supernatants of these induced bacteria exhibited haemolytic, lethal and enterotoxigenic (rabbit ileal loop) activities, supporting the contention that factors other than TDH may be important in *V. parahaemolyticus*-associated disease.

However, only 2.0 - 12.5 µg TDH was needed to cause diarrhoea (and death in some animals) in the suckling mouse (Obara *et al.*, 1974; Miyamoto *et al.*, 1980), and 9 µg was sufficient to cause fluid accumulation in ligated mouse ileum (Douet *et al.*, 1992). One possible explanation for the relative insensitivity of rabbit tissues to TDH is that TDH-receptor expression is species-dependent, and that rabbit small intestine has a relatively low number of receptors. Whether or not these observations do indicate a species variation in sensitivity to TDH, it is clearly important to evaluate the likelihood of a role for TDH in tissues from other species, in particular *Homo sapiens*. In addition to cell physiology methodology, an appropriate investigative line may be an attempt to identify the intestinal receptor by autoradiographic studies, using radiolabelled toxin (eg radio-iodinated toxin; Sugii & Tsuji, 1989). Alternatively, identification of an intestinal receptor might be possible using an approach analagous to that employed by Griffiths and Critchley (1991). These workers separated brushborder proteins by SDS-PAGE, blotted them to nitrocellulose and labelled the filters with [^{125}I]-labelled (Lazarovici & Yavin, 1985a; Sugii & Tsuji, 1989) heat-labile toxin (LT-1) of *E. coli*, which bound to a series of brushborder galactoproteins, ranging in size from 130 - 140 kd. Such overlay assays can also be used on thin layer chromatogram preparations of glycolipid preparations (Bock *et al.*, 1987).

In summary, although an increased K^{+} efflux has been described for isolated jejunocytes treated by KH (Figure 9.6), the short-lived nature of this preparation and the large amount

of toxin required to elicit an effect therein are major limitations to its use in examination of cell physiological changes induced by toxin action. In this respect, the future use of small intestinal tissue (Nishibuchi *et al.*, 1992) or cultured cell-lines (Dharmsathaphorn & Madara, 1990; Sepulveda, 1991; Zweibaum *et al.*, 1991; Evans *et al.*, 1994) in Ussing Chamber or isotope flux studies is advocated (see Chapter 10; McEwan *et al.*, 1993, 1994). The following chapter presents a summary of all results in this study, and suggests future lines of investigation.

CHAPTER 10

Concluding summary

'"Oh, I see," said Pooh.'
Benjamin Hoff *in* The Tao of Pooh

This investigation has centred on the mechanisms of action of bacterial toxins, in particular those of the *Vibrio* genus. The nature of membrane attack and the sequelae to such action has been examined, using erythrocytes, Chinese hamster ovary (CHO) cells, and isolated intestinal cells as target membranes. This chapter summarises the findings of the previous six chapters and considers the implications of these data with regard to the plausible *in vivo* pathophysiological action of these toxins. An emphasis for continuing research in this area is suggested.

V. parahaemolyticus and *V. cholerae* non-O1 are important contributors to the spectrum of diarrhoeal disease (Sakazaki & Balows, 1981; Doyle, 1990; Spriggs & Sack, 1990; Hughes, 1978; Blake, 1980; Morris *et al.*, 1981; Morris & Black, 1985) and the importance of exotoxins in the pathogenesis of these diseases has been discussed (section 2.5.1). Both *V. parahaemolyticus* and *V. cholerae* produce major exoproteins that can lyse erythrocytes - these being the thermostable direct haemolysin (TDH) and the El Tor haemolysin (ETH), respectively. Both have been partially characterised as haemolysins (Sakurai *et al.*, 1973, 1975; Takeda *et al.*, 1975, 1976; Zen-Yoji *et al.*, 1976; Takeda, 1983; Honda *et al.*, 1992; Honda & Iida, 1993; Honda & Finkelstein, 1979; Krasilnikov *et al.*, 1993), and are cytolytic/cytotonic for cells other than erythrocytes (Zen-Yoji *et al.*, 1974; Obara *et al.*, 1974; Goshima *et al*, 1976, 1977; Takeda, 1983; Nishibuchi *et al.*, 1992; Nath *et al.*, 1978; Ichinose *et al.*,1987; Hall & Drasar, 1990). In the case of TDH, a high degree of correlation was noted between haemolytic ability in culture (the Kanagawa phenomenon) and the pathogenicity of the strain (Kato *et al.*, 1965, 1966; Miyamoto *et al.*, 1969).

Toxicological research is obviously dependent on the use of both *toxin* and *target* preparations. The toxin preparation must be prepared in large enough amounts and to high purity (Chapter 6). The choice of target system is dependent on the area under investigation. For example, if testing whether a toxin is likely to be a pathogenic determinant, a system

close to that encountered *in vivo* should be used. However, the complexity of systems approximating to the pathogenic condition means that they are perhaps not optimal for detailed analysis of toxin mechanism. This is especially the case if large quantities of toxin are required in order to elicit an effect - as appears to be the case for TDH on distal rabbit ileum (Nishibuchi *et al.*, 1992) and isolated rabbit jejunocytes (Figure 9.6). Erythrocytes, well evaluated in terms of membrane physiology and established as a cell-type sensitive to toxin action (horse and hagfish erythrocytes being the exceptions), were deemed to be an ideal initial system.

The experimental findings of this project are considered under four headings:
(i) bacterial culture and purification of toxic exoproteins that are candidate virulence factors, (ii) biophysical analysis of the nature of the lesion formed in erythrocyte membranes, (iii) identification of toxin-induced derangements of endogenous membrane transporter function, and (iv) preliminary evaluation of toxin action on a more relevant putative target tissue.

(i) Bacterial culture and purification of toxic exoproteins that are candidate virulence factors.

Chapter 6 describes experiments designed to optimise culture conditions for the production of TDH by *V. parahaemolyticus* AQ 3185. Growth conditions were found to be of paramount importance, not only in determining the amount of TDH produced but also the relative amounts of other extracellular proteins. The conditions used by Nishibuchi *et al.* (1992) to culture *V. parahaemolyticus* were found to be the poorest with respect to TDH production, though the best for bacterial growth. Because of a report questioning the purity of KH (Sigma) as a TDH preparation (Douet *et al.*, 1992), TDH was purified to homogeneity (single band on SDS-PAGE) from both KH and culture supernatants of *V. parahaemolyticus*. This chapter was also concerned with (a) the production of ETH, and (b) the evaluation of culture supernatants of CT^- *V. cholerae* strains for other toxic factors. The haemolytic characteristics of ETH were evaluated by analagous experiments after suitable conditions for storing this unstable toxin preparation had been established (Chapter 8; section 8.2.1). Several of the *V. cholerae* strains appeared to secrete factors capable of altering CHO cell morphology (Table 6.1), indicating that these bacteria may possess as yet unidentified factors capable of perturbing parameters of cell physiology. Further characterisation of these factors

is in progress (Hall, 1994).

(ii) Biophysical analysis of the nature of the lesion formed in erythrocyte membranes. In a manner similar to some bacterial toxins (eg α-toxin of *Staphylococcus aureus* (Fussle *et al.*, 1991) and aerolysin of *Aeromonas hydrophila* (Chakraborty *et al.*, 1990)) and different to others (eg streptolysin O (Duncan, 1974)), both TDH and ETH caused haemolysis by colloid osmosis, causing an increase in cation permeability that resulted in net cation influx and (unless osmosis was inhibited by extracellular osmolytes) cell swelling until lysis (Figures 4.3 - 4.6, 7.4, 7.5, 8.4). However the dynamics of induced fluxes were different for these two toxins. TDH caused a rapid onset increase in K^+ influx, with no demonstrable lag phase, that subsequently decreased in magnitude to a relatively constant level (Figures 5.2, 5.8, 5.22 and 7.5). In contrast, the increase in cation influx instigated by ETH had a pronounced lag phase before increasing to a relatively constant level (Figure 8.5). The patterns of magnitude of induced cation influx correlate to the observed difference in the kinetics of haemolysis. Although haemolysis induced by both toxins occurred after a time delay, that instigated by ETH exhibited a more pronounced lag phase but thereafter proceeded more rapidly (Figure 8.6).

The binding of TDH (as the preparation KH) was investigated in more detail than has previously been the case (Sakurai *et al.*, 1975; Honda *et al.*, 1992). In particular, although the onset of induced K^+ influx was very rapid, binding of TDH to HRBC membranes was shown to occur over a rather longer period than the 1 - 2 minutes suggested by Honda *et al.* (1992). Furthermore, once induced, the lesion appeared not to be susceptible to washout even by high centrifugal forces (10000g: CP3) for both isotopic flux (Figure 5.8) and haemolysis (Figures 4.8 - 4.14, 5.9 and 7.6) experiments.

The induced lesion had characteristics compatible with those of a pore, since KH-induced K^+ influx was unsaturated with respect to $[K^+]_o$ over the range 0 - 30 mM (Figure 5.5). In addition, Cl^--dependent processes did not appear to contribute significantly to the induced influx in the presence of ouabain, bumetanide and nitrendipine (Figure 5.10). The rate of KH (1 $HU(ml)^{-1}$) -induced haemolysis was dependent on donor and KH concentration alone (Figures 4.1, 4.2, and 7.1 - 7.3). However, for lower doses of toxin, a non-monotonic dose-response relation (Figure 5.6) and target cell inhibition (Figure 5.7) of KH-induced K^+ influx

and transit indicated the likelihood of a multi-hit relation. The reason for the discrepancy between haemolysis and flux data is not clear but is likely to be related to the different doses used and the dynamics of induced flux. Whilst a highly purified TDH preparation had similar characteristics of induced flux (Figures 7.5, 7.7 and 7.8) and haemolysis (Figures 7.1 - 7.4, and 7.6) it was demonstrated to have different relative haemolysis-inducing:flux-inducing potential (Figures 7.9 and 7.10). It is not clear what underlies this effect; it is, however, possible that KH preparations vary with respect to their homogeneity for TDH (Douet *et al.*, 1992), and/or that the isolation of toxin in concentrated preparations produces variations in their buffer content (eg EDTA/Ca^{2+}) that may alter the dynamics of induced influx.

Many membrane-active agents had no demonstrable effect on KH-induced haemolysis (Figure 4.16) or induced cation flux (Figures 5.12 and 5.13). Such evidence serves to demonstrate the distinction between the TDH-induced cation leak and leaks induced by oxidative damage (Deuticke *et al.*, 1991), low ionic strength (Bernhardt *et al.*, 1991), and *Plasmodium falciparum* infection (Ginsburg *et al.*, 1983, 1985; Kirk *et al.*, 1994).

Aggregation of endogenous membrane protein(s) is a possible mechanism for pore-formation by toxins and other agents such as polylysine and Zn^{2+} (section 5.3; Bjerrum *et al.*, 1980; Benz *et al.*, 1984; Clague & Cherry, 1989). In the present study, DIDS had no effect on KH-induced K^+ influx (Figure 5.13). Hagfish (*Eptatretus stouti*) erythrocytes, unusual in that they have very low numbers of Band 3, were insensitive to toxin action (Figure 5.11) - however, the absence of an effect of TDH on this cell type does not support the hypothesis of Band 3 aggregation, since horse erythrocytes have a similar insensitivity to KH (Figures 4.7 and 4.16), yet possess Band 3 in abundance. Thus no evidence was obtained in support of a hypothesis postulating toxin-induced Band 3 aggregation.

Neuraminidase treatment of human erythrocytes enhanced subsequent KH-induced haemolysis as a result of increased KH-induced cation permeability (Figures 5.15 - 5.19). It is uncertain how this relates to previous evidence pertaining to putative receptor(s) for this toxin (Takeda *et al.*, 1975, 1976). These latter studies implicated the neuraminidase-sensitive gangliosides G_{D1a} and G_{T1} as receptors for TDH on the basis of their inhibitory effects. The effect of neuraminidase is unlikely to be of direct relevance to a pathogenic mechanism as

no pathogenic *V. parahaemolyticus* strains tested have been neuraminidase positive (Kabir *et al.*, 1984). However, this treatment indicates a class of receptor that can be unmasked by enzyme action, and that an increase in the number of such receptors augments KH action at a given dose.

In common with other pore-forming agents (eg staphylococcal α-toxin (Pasternak *et al.*, 1992) and *C. perfringens* θ-toxin (Menestrina *et al.*, 1990)), the action of TDH could be inhibited by Zn^{2+} (100 μM) (Figure 5.12 and Table 7.2). The interactions of divalent cations with pathways mediating ion leakage across biological membranes has been considered in section 5.3. Pasternak *et al.* (1992) have reviewed the common mechanisms of induction and prevention of membrane damage by pore-forming toxins. Although by no means comprehensive, this paper nevertheless highlights some key features of pore formation by bacterial toxins. Some of these are not found for TDH. In particular, pore size may not increase either with the amount of agent added (Honda *et al.*, 1992), or with time - as evinced by the strange dynamics of magnitude of induced cation influx by TDH. Several useful areas of further research into the biophysics of pore-forming toxins are identified by these authors, in particular, analysis of the effects of pH, lowered ionic strength and high osmotic pressure. The influence of these variables could once more be assessed using the erythrocyte as the target membrane.

The selectivity series for both KH (Figures 4.17 - 4.19) and highly purified TDH (Figure 7.8) is a reversed Eisenman VIII sequence, with a small K^+ anomaly: $Cs^+ > Li^+ > K^+ > Rb^+ > Na^+$ (Eisenman & Horn, 1983). This series was found for a variety of KH doses, and was unchanged by the addition of the endogenous cation transport system inhibitors ouabain, bumetanide, and nitrendipine. Preliminary evidence derived from analagous monovalent cation selectivity experiments for ETH depicted a straight Eisenman IV sequence: $K^+ > Rb^+ > Cs^+ > Na^+ > Li^+$ (Eisenman & Horn, 1983). Although this result has yet to be obtained for different doses of ETH, it is a further demonstration of functional differences in mechanism of action between TDH and ETH. These two toxins have also been distinguished on the basis of their thermostability (TDH)/thermolability (ETH), and species spectrum of activity on erythrocytes (Honda & Finkelstein, 1979).

Increasing TDH concentration in the haemolytic range did not have a major effect on the *nature* of the lesion, rather it was the *number* of lesions which was affected (Figure 4.19). Sizing experiments performed by Honda *et al.* (1992) suggest that the TDH-induced pore is of a small functional diameter (1 - 2 nm) and also that pore size is not increased by addition of more toxin. In this respect, TDH appears to behave similarly to staphylococcal α-toxin (Fussle *et al.*, 1981; Bhakdi *et al.*, 1985a) and complement (Koski *et al.*, 1983) in producing small lesions of a fixed diameter. This is rather different to the thiol-activated toxins typified by streptolysin O (Buckingham & Duncan, 1983; Alouf & Geoffroy, 1991) which produce progressively larger lesions by a barrel-stave type mechanism.

(iii) Identification of toxin-induced derangements of endogenous membrane transporter function. Data presented in Chapter 5 describe the effects of TDH on cation transport, in the first ten minutes after toxin addition, and over a longer time course for ion movement by the Na^+/K^+ ATPase. A stimulation of nitrendipine-sensitive K^+ influx was noted, even in nominally Ca^{2+}-free MBS (Figure 5.1). This stimulation was enhanced when measured in MBS containing raised $[Ca^{2+}]_o$ in the range 0 - 1 mM (Figure 5.19), but a similar effect was not observed in the presence of Mg^{2+} up to 6 mM (Figure 5.20), or EGTA at 0.15 mM (Table 5.1). In the presence of $[Mg^{2+}]$ = 1.5 mM, there was a significant inhibition of the $Na^+/K^+/2Cl^-$ cotransporter, indicating that this divalent cation may also have a cellular pathophysiological role as a result of TDH action. Ca^{2+} has been implicated as playing an important role in many toxin-induced processes, including cell death (Schanne *et al.*, 1976; Nicotera *et al.*, 1992), derangement of cellular function (Bhakdi *et al.*, 1985a; Pasternak, 1986), and more specifically cardiac myocyte degeneration by TDH (Goshima *et al.*, 1978). Results presented here show that at physiological values of $[Ca^{2+}]_o$, TDH is capable of perturbing certain membrane transport processes in a manner manifestedly dependent on Ca^{2+}.

Subtle effects on cellular physiology have been documented for bacterial toxins at lower doses than those required for lysis. For example: (a) there can be effects on the cells of the immune system, associated with changes in adhesiveness, or responsiveness to chemotactic or mitogenic agents (Alouf, 1985), (b) *Escherichia coli* haemolysin and *S. aureus* α-toxin potently induce release of nitric oxide in porcine endothelial cells (Suttorp *et al.*, 1993), and

(c) staphylococcal α-toxin causes a stimulation of the arachidonic cascade in isolated rabbit lung preparations, raised $[Ca^{2+}]_i$ being proposed as the instigating factor (Bhakdi *et al.*, 1985a). These latter workers deemed effects on cellular physiology to be particularly relevant to the pathogenesis of bacteria secreting staphylococcal α-toxin, because of the toxin's low binding efficacy and small pore size (c. 3 nm; Fussle *et al.*, 1981). Such arguments similarly apply to TDH of *V. parahaemolyticus* (protracted binding period (Chapters 4 and 5) and 1 - 2 nm functional diameter (see above; Honda *et al.*, 1992)), although TDH and α-toxin bind to different receptors, and the bacteria involved (*V. parahaemolyticus* and *S. aureus*) colonise different sites.

(iv) Preliminary evaluation of toxin action on a more relevant putative target tissue. Extrapolating from the erythrocyte model to the epithelial/pathophysiological situation (section 2.4) may be tenuous, especially with regard to the nature of the toxin receptor. Experiments described in Chapter 9 addressed the response of a more pathogenically relevant target, isolated rabbit enterocytes (Kimmich, 1975; Brown & Sepulveda, 1985; Ilundain, 1991). Although these cells were found to be sensitive to KH in terms of $^{86}Rb^+$ efflux, high concentrations of KH (viability checked by positive control experiments on HRBC) were needed to elicit this effect. Furthermore, the enzymic (hyaluronidase) isolation procedure for jejunocytes, had no protective effect on the induced flux into human erythrocytes. The short-term viability of isolated cells was also an important limiting factor (section 3.4.2; Evans *et al.*, 1994) in further examination of toxin-induced perturbations of cellular physiology. Possible explanations for the low sensitivity of rabbit jejunocytes to TDH include species-dependent or alimentary location-dependent paucity of receptors. Large amounts of TDH (500 μg; 100 μg was insufficient) were required to cause fluid accumulation in ligated rabbit ileal loops (Zen-Yoji *et al.*, 1974). However, only 2.0 - 12.5 μg TDH was needed to cause diarrhoea (and death in some animals) in the suckling mouse (Obara *et al.*, 1974; Miyamoto *et al.*, 1980). Recently, 9 μg TDH was found to be sufficient to cause massive fluid accumulation in mouse ileal loops (Douet *et al.*, 1992). The sensitivity of different preparations is an area warranting further investigation, and is further considered below.

On the basis of results outlined in section 5.2.4 and (iii) above, McEwan *et al.* (1994) investigated the effects of KH on the human intestinal T_{84} cell-line. The T_{84} cell-line has been well-validated as a model Cl^--secreting epithelium (section 2.4.1, Figure 2.15), exhibiting a basolateral Cl^- accumulating (sodium pump and $Na^+/K^+/2Cl^-$ cotransport) mechanism, a K^+ recycling mechanism and an apical Cl^- conductance (Dharmsathaporn & Madara, 1990; Sepulveda, 1991; Zweibaum *et al.*, 1991). However, although polarised, T_{84} monolayers do not display an apical brush border (Madara & Dharmsathaphorn, 1985). McEwan *et al.* (1994) cultured cells on filter supports and measurements of short-circuit current (I_{SC}), transepithelial conductance (G_T) and intracellular Ca^{2+} (using fura-2) were made as previously described (McEwan *et al.*, 1993). At low levels of I_{SC}, KH (10 $HU(ml)^{-1}$) applied to the apical surface had no effect on either I_{SC} or G_T. However, when applied basolaterally at this concentration there was a transient increase in inward I_{SC}. This stimulation was accompanied by a sustained increase in G_T which continued to rise despite I_{SC} values returning to prestimulation values. This was held to suggest that KH may act to increase paracellular conductance. If monolayers were prestimulated by forskolin (0.1 mM), basolateral KH application augmented the secretory response, suggesting that KH was activating mechanisms distinct from the adenylate cyclase/cAMP pathway. Consistent with these results, basolateral application of KH caused an increase in $[Ca^{2+}]_i$, the onset of which was similar in time course to that observed for the rise in I_{SC}, suggesting that it may indeed be a rise in intracellular Ca^{2+} that underlies the diarrhoea induced by *V. parahaemolyticus* (see Chapter 5). Finally, these interesting results were interpreted to suggest that *V. parahaemolyticus* must penetrate across the intestinal epithelium to exert its enteric effects (sections 2.6.2 and 2.6.3).

However, these workers failed to address the findings of other studies which conflict with their ultimate conclusion. In particular, in the absence of *V. parahaemolyticus* itself, (i) injection of purified TDH causes fluid accumulation in ligated rabbit ileal loops, (Zen-Yoji *et al.*, 1974), (ii) oral challenge of suckling mice by TDH causes diarrhoea, and death in some animals (Obara *et al.*, 1974), and (iii) the culture filtrate of TDH^+ (as opposed to TDH^-) strains causes an increase in I_{SC} that is blocked by anti-TDH antiserum (Nishibuchi *et al.*, 1992). Furthermore, the receptors to TDH are thought to be glycolipids (specifically the

neuraminidase-sensitive gangliosides G_{D1a} and G_{T1} or ganglioside-associated proteins (Takeda *et al.*, 1975, 1976)) and the enterocyte apical membrane is rich in such substances (Umesaki *et al.*, 1989).

Model epithelia are known to have altered properties, even with regard to apical/basolateral siting of membrane proteins. For example, the MDCK cell-line expresses a hemicholinium-3 inhibitable Na^+-independent choline transporter the apical/basolateral disribution of which appears to depend on the growth conditions of the monolayers (Zlatkine *et al.*, 1993). For cells grown on plastic dishes, specific binding of [^{3}H]-hemicholinium-3 was detected on the apical membranes, whereas it was confined to the basolateral domain of cells grown on a permeant support. Furthermore for cells grown on the permeant support, choline uptake at the basolateral surface was c. 10 x that at the apical.

Therefore it is conceivable that T_{84} cell-lines may have a switched membrane asymmetry (from that encountered *in vivo*) for the TDH-receptor. Caution in drawing conclusions from model epithelia is important and reservations have been emphasised by Sepulveda (1991) who states 'Although it is clear that studies with permanent lines of cultured cells have and will be useful in understanding epithelial function and development, they do not replace studies with the naturally occurring epithelia. In that sense they remain models, though very good ones. But regardless of the source, cell lines must be abnormal in some aspects of growth regulation and probably also in other respects. Thus cells grown in culture might express functions not expressed *in vivo* and lack some which are present in the tissue of origin'.

Future areas of research, based on the present study and that outlined above, have been indicated in the discussion sections of Chapters 4 - 9. However for clarity, they are summarised below:

(i) The red cell is a practicable system for further assessment of the putative involvement of endogenous membrane proteins and gycolipids as receptor molecules (Takeda *et al.*, 1975, 1976) in TDH and ETH action. This important area of investigation may be broached by attempting to confer sensitivity on horse erythrocytes which are normally insusceptible to TDH action. Indeed, the erythrocyte facilitates such studies, having the ability to accumulate

gangliosides from an experimental medium (the methodology for such accumulation being simple and well established for human erythrocytes; Lazarovici & Yavin, 1985a). The studies of Lazarovici and Yavin (1985a,b) used radio-iodinated tetanus toxin as a measure of incorporated gangliosides G_{T1b} and G_{D1b}; induced K^+ influx would be a simpler method to measure conferred sensitivity to TDH. Other studies have evaluated the role of glycolipids as toxin receptors by inserting the glycolipid into an otherwise insusceptible cell type, for example insertion of ganglioside G_{M1} into rat glioma C6 cells (Spiegel, 1988), and insertion by liposomal fusion of the neutral glycolipid G_{b3} into Daudi human lymphoma cells (Waddell *et al.*, 1990). The effects of other enzyme treatments (eg trypsin, chymotrypsin; Carraway, 1982) on erythrocyte susceptibility to TDH action is another sensible approach with which to assess the importance of endogenous membrane proteins in pore-formation.

(ii) A more reductionist approach is that involving artificial phospholipid membranes (lipid bilayers and/or liposomes). This strategy has been used extensively in recent years to analyse the membrane actions (pore-forming actvities) of various toxins, including diphtheria toxin (Alder *et al.*, 1990), *Aeromonas hydrophila* aerolysin (Wilmsen *et al.*, 1990), staphylococcal α-toxin (Menestrina, 1986; Belmonte *et al.*, 1987; Bashford *et al.*, 1988a; Menestrina *et al.*, 1990), *E. coli* haemolysin (Menestrina *et al.*, 1990), *Clostridium perfringens* θ-toxin (Menestrina *et al.*, 1990) and the sea anemone cytolysin equinatoxin II (Belmonte *et al.*, 1993). Use of such a preparation might also prove valuable in discerning interesting biophysical aspects of toxin action which may be pertinent to the pathophysiological action of the toxin (eg cation/anion selectivity). An assessment of the effects of divalent cations on channel conductances arising from many pore-forming toxins is difficult because of the heterogeneity of channel sizes in the case of progressive, barrel-stave pore-forming toxins - however, this should not be the case for TDH, for which pore size appears to be a fixed parameter with respect to TDH concentration (Honda *et al.*, 1992). Therefore, the use of a lipid bilayer model (as advocated above) would be an appropriate approach for this analysis.

(iii) Whilst ganglioside incorporation and artificial lipid membrane experiments may yield information about the TDH-binding event, evaluation of the receptor interaction in the pathophysiological tissue is a critical aim. Identification of an intestinal receptor for TDH

and ETH might be possible using an approach analagous to that employed by Griffiths and Critchley (1991). These workers separated brushborder proteins by SDS-PAGE, blotted them to nitrocellulose and labelled the filters with [^{125}I]-labelled (Lazarovici & Yavin, 1985a; Sugii & Tsuji, 1989) heat-labile toxin (LT-1) of *E. coli*. This toxin bound to a series of brushborder galactoproteins, ranging in size from 130 - 140 kd. Similarly, Cohen *et al.* (1993) identified a receptor for the *E. coli* heat-stable enterotoxin. Such overlay assays may also be used on thin layer chromatogram preparations of glycolipid preparations (Bock *et al.*, 1987).

(iv) Whether or not observations of Chapter 9 are actually accounted for by a species difference in susceptibility to toxin action, it is clearly important to evaluate the likelihood of a role for TDH in human tissue, and in particular small intestine. Given the likely cadaveric nature of human tissue specimens, the most appropriate investigative line may be autoradiographic studies using radiolabelled toxin (eg radio-iodinated toxin; Sugii & Tsuji, 1989).

Beyond the immediate wish to understand bacterial toxinology as it pertains to the disease state, the specificity of many toxin interactions indicates a possible future therapeutic role (Hakomori, 1986; Read & Stein, 1993). For instance, in the processes underlying malignant change, the patterns of glycosphingolipid expression are dramatically altered and interactions of varying degrees of specificity for bacterial toxins with such glycosphingolipids are well-documented (Table 2.4). Much information is becoming available on the structure of a wide variety of toxins as a result of advances in molecular biology and biophysics (Read & Stein, 1993). Amino acid sequences can be obtained either directly (Tsunasawa *et al.*, 1987; Matsudaira *et al.*, 1993) or derived from nucleotide sequences, facilitating the identification of regions of homology with other factors and allowing structural predictions (Chou & Fasman, 1974; Rees *et al.*, 1989; Ojcius & Young, 1991). Notwithstanding this, no likely amphipathic transmembrane α-helical regions could be identified for TDH by α-helical wheel analysis (section 2.5.2; Figure 2.20; method described by Ojcius & Young, 1991). Another aim of subsequent research for TDH and ETH must involve the prediction of tertiary structure

and how this relates to biological activity, especially in terms of: (i) specificity of receptor-binding, (ii) hydrophilic-hydrophobic transition, and (iii) pore structure.

With reference to the devastating and worldwide effects of diarrhoeal illness, socioenvironmental intervention, in particular hygiene and sanitation, has dramatically decreased the incidence of diarrhoea. Various opiates, anti-inflammatory drugs, calmodulin inhibitors, neurotransmitters and proteases have had some success in clinical trials for gastrointestinal illness (Field *et al.*, 1989). However, diarrhoeal disease remains responsible for human suffering on an immense scale. Research into the effects of toxins on membrane physiology, such as that outlined in this dissertation and elsewhere (Mynott *et al.*, 1993) may allow identification of viable pharmacological sites, and thus yield effective therapies. These, in conjunction with improvements in sanitation and nutrition, will further reduce the mortality and morbidity of infectious diarrhoea.

BIBLIOGRAPHY

Abbott, S. L., C. Powers, C. A. Kaysner, Y. Takeda, M. Ishibashi, S. W. Joseph, & J. M. Janda. 1989. Emergence of a restricted bioserovar of *Vibrio parahaemolyticus* as the predominant cause of *Vibrio*-associated gastro-enteritis on the west coast of the United States and Mexico. J. Clin. Microbiol. **27**:2891-2893.

Ackers, G. K. 1967. A new calibration procedure for gel filtration columns. J. Biol. Chem. **242**:3237-3238.

Ada, G. L., E. L. French, & P. E. Lind. 1961. Purification and properties of neuraminidase from *Vibrio cholerae*. J. Gen. Microbiol. **24**:409-425.

Adkins, H. J., J. Escamilla, L. T. Santiago, C. Ranoa, P. Echeverria, & J. H. Cross. 1987. Two year survey of aetiological agents of diarrhoeal disease at San Lazaro Hospital, Manila, Republic of the Philippines. J. Clin. Microbiol. **25**:1143-1147.

Agar, N. S., & P. G. Board. 1983. Red blood cells of domestic mammals. Elsevier, Amsterdam.

Ahnert-Hilger, G., M.-E. Dauzenroth, E. Habermann, A. Henschen, K. Krieglstein, F. Mauler, & U. Weller. 1990. Chains and fragments of tetanus toxin and their contribution of toxicity. J. Physiol. **84**:229-236.

Ahsan, N., R. L. Conter, & P. C. Appelbaum. 1988. Post-operative wound infection associated with *Vibrio parahaemolyticus* in a patient without exposure to sea-water. J. Clin. Microbiology. **26**:1214-1215.

Albert, M. J., A. K. Siddique, M. S. Islam, A. S. G. Faruque, Ansaruzzamanm, S. M. Faruque, & R. B. Sack. 1993. Large outbreak of clinical cholera due to *Vibrio cholerae* non-O1 in Bangladesh. Lancet **341**:704.

Alberts, B., D. Bray, J. Lewis, M. Raff, K. Roberts, & J. D. Watson. 1989. Molecular biology of the cell. 2nd edition. Garland, New York.

Alder, G. M., C. L. Bashford, & C. A. Pasternak. 1990. Action of diphtheria toxin does not depend on the induction of large, stable pores across biological membranes. J. Memb. Biol. **113**:67-74.

Allewell, N. M. 1993. Untangling the energetics of protein folding. Trends Biochem. Sci. **18**:1-2.

Alouf, J. E. 1980. Streptococcal toxins (streptolysin O, streptolysin S, erythrogenic toxin). Pharmac. Ther. **11**:661-717.

Alouf, J. E. 1985. Interactions of bacterial protein toxins with host defense mechanisms. pp.121-130, *in* Bacterial protein toxins (Falmagne, P., J. E. Alouf, F. J. Fehrenbach, M. Thelestam, & J. Jeljsaszewicz (ed.s)). Zbl. Bakt. Suppl. 5. Gustav Fischer, Stuttgart.

Alouf, J. E., & J. H. Freer. 1991. Sourcebook of bacterial protein toxins. Academic Press, London.

Alouf, J. E., & C. Jolivet-Reynaud. 1981. Purification and characterisation of *Clostridium perfringens* delta-toxin. Infect. Immun. **31**:536-546.

Andersen, O. S. 1984. Gramicidin channels. Ann. Rev. Physiol. **46**:531-548.

Andersen, O. S., A. Finkelstein, I. Katz, & A. Cass. 1976. Effect of phloretin on the permeability of thin lipid membranes. J. Gen. Physiol. **67**:749-771.

Anderson, P. C., & R. E. Lovrien. 1977. Human red cell hemolysis rates in the subsecond to seconds range. Biophys. J. **20**:181-191.

Anthone, G. J., M. S. Orandle, Z. V. Mavrophilipos, B. H. Wang, & C. J. Yeo. 1991a. Neuropeptide Y is a

proabsorptive agent in the small intestine. J. Surg. Res. **50**:589-594.

Anthone, G. J., M. S. Orandle, B. H. Wang, & C. J. Yeo. 1991b. Neuropeptide Y-induced intestinal absorption: mediation by alpha-2-adrenergic receptors. Surgery **110**:1132-1138.

Aoyagi, T., T. Takeuchi, A. Matsuzaki, K. Kawamura, S. Kendo, M. Hamada, K. Maeda, & H. Umezawa. 1969. Leupeptins, new protease inhibitors from actinomycetes. J. Antibiot. **22**:283-286.

Araki, K., & J. M. Rifkind. 1981. The rate of osmotic haemolysis. A relationship with membrane bilayer fluidity. Biochim. Biophys. Acta **645**:81-90.

Arco, A., M. Faure, Y. Piemont, & J. Thivolet. 1991. Experimental models for studying the effects induced by staphylococcal toxins A and B on human keratinocytes in culture. Arch. Ital. Anat. Embriol. **96**:185-199.

Argos, P., & J. K. MohanaRao. 1986. Prediction of protein structure. Met. Enz. **130**:185-269.

Armstrong, C. L. 1989. Reflections on selectivity. pp.261-274, *in* Membrane transport. People and ideas. American Physiological Society, Bethesda.

Armstrong, W. M., D. L. Musselman, & H. C. Reitzug. 1970. Sodium, potassium and water content of isolated bullfrog small intestinal epithelia. Am. J. Physiol. **219**:1023-1026.

Aronson, J. K. 1990. Methods for expressing the characteristics of transmembrane ion transport systems. Clin. Sci. **78**:247-254.

Avigad, G. 1976. Microbial phospholipases. pp.99-167, *in* Mechanisms in bacterial toxinology (Bernheimer, A. W. (ed.)). Wiley, New York.

Axelrod, D. 1983. Lateral motion of membrane proteins and biological function. J. Memb. Biol. **75**:1-10.

Baba, K., H. Shirai, A. Terai, K. Kumagai, Y. Takeda, & M. Nischibuchi. 1991. Similarity of the *tdh* gene-bearing plasmids of *Vibrio cholerae* non-O1 and *Vibrio parahaemolyticus*. Microb. Path. **10**:61-70.

Baba, K., S. Yamasaki, M. Nischibuchi, & Y. Takeda. 1992. Examination by site-directed mutagenesis of the amino acid residues of the thermostable direct haemolysin of *Vibrio parahaemolyticus* required for its haemolytic activity. Microb. Path. **12**:279-287.

Badin, J., & M.-A. Denne. 1978. A cholesterol fraction for streptolysin O binding on cell membranes and lipoproteins - I. Optimal conditions for the determination. Cell Molec. Biol. **22**:133-143.

Barker, W. H. Jr. 1974. *Vibrio parahaemolyticus* outbreaks in the United States. Lancet **1**:551-554.

Barinaga, M. 1993. New technique offers a window on bacteria's secret weapons. Science **259**:595.

Barnes, S. J., & P. M. Conn. 1993. Cholera toxin and dibutyryl cyclic adenosine 3',5'-monophosphate sensitize gonadotropin-releasing hormone-stimulated inositol phosphate production to inhibition in protein kinase-C (PKC)-depleted cells: evidence for cross-talk between a cholera toxin-sensitive G-protein and PKC. Endocrin. **133**:2756-2760.

Barros, F., P. Dominguez, G. Velasco, & P. S. Lazo. 1986. Na^+/H^+ exchange is present in the basolateral membranes from rabbit small intestine. Biochem. Biophys. Res. Comm. **134**:827-834.

Barua, D., & W. Burrows. 1974. Cholera. W. B. Saunders, Philadelphia.

Bashford, C. L., G. M. Alder, J. M. Graham, G. Menestrina, & C. A. Pasternak. 1988a. Ion modulation of membrane permeability: effect of cations on intact cells and on cells and phospholipid bilayers treated with pore-forming agents. J. Memb. Biol. **103**:79-94.

Bashford, C. L., G. M. Alder, G. Menestrina, K. J. Micklen, J. J. Murphy, & C. A. Pasternak. 1986. Membrane damage by hemolytic viruses, toxins, complement and other cytotoxic agents: a common mechanism blocked by divalent cations. J. Biol. Chem. **261**:9300-9308.

Bashford, C. L., G. Menestrina, P. A. Henkart, & C. A. Pasternak. 1988b. Cell damage by cytolysin, spontaneous recovery and reversible inhibition by divalent cations. J. Immunol. **141**:3965-3974.

Bashford, C. L., L. Rodrigues, & C. A. Pasternak. 1989. Protection of cells against membrane damage by haemolytic agents: divalent cations and protons act at the extracellular side of the plasma membrane. Biochim. Biophys. Acta **983**:56-64.

Baskerville, A., J. W. Conlan, L. A. E. Ashworth, & A. B. Dowsett. 1986. Pulmonary damage caused by a protease from *Legionella pneumophilia*. Brit. J. Exp. Pathol. **67**:527-536.

Baudry, B., A. Fasano, J. Ketley, & J. B. Kaper. 1992. Cloning of a gene (*zot*) encoding a new toxin produced by *Vibrio cholerae*. Infect. Immun. **60**:428-434.

Behrens, M. I., A. Oberhauser, F. Bezanilla, & R. Latorre. 1989. Batrachotoxin-modified sodium channels from squid optic nerve in planar bilayers. J. Gen. Physiol. **93**:23-41.

Beliveau, R., & M. Potier. 1989. The radiation inactivation of membrane transport Systems. News Physiol. Sci. **4**:134-138.

Bell, S. M., J. D. Priddle, & P. L. Stern. 1984. Heterogeneity of rat TCGF defined by Mono P isoelectric focusing. Molec. Immunol. **21**:271-276.

Belmonte, G., L. Cesscatti, B. Ferrari, T. Nicolussi, M. Ropele, & G. Menestrina. 1987. Pore formation by *Staphylococcus aureus* alpha-toxin in lipid bilayers. Eur. J. Biophys. **14**:349-358.

Belmonte, G., C. Pederzolli, P. Macek, & G. Menestrina. 1993. Pore formation by the sea anemone cytolysin equinatoxin II in red blood cells and model lipid membranes. J. Memb. Biol. **131**:11-22.

Benchetrit, L. C., C. C. Avelino, L. Barracund, A. M. S. Figueiredo, & C. M. Oliveira. 1984. Hyaluronidase production by groups A, B, C, and G streptococci: a statistical analysis. Zentralbl. Bakteriol. Mikrobiol. Hyg. A **257**:27-37.

Benchetrit, L. C., C. C. Avelino, & C. M. Oliveira. 1987. Hyaluronidase activity of group B streptococci. Braz. J. Med. Biol. Res. **20**:411-414.

Benedetti, H., M. Frenette, D. Baty, M. Knibiehler, F. Pattus, & C. Lazdunski. 1991. Individual domains of colicins confer specificity in colicin uptake, in pore properties and in immunity requirement. J. Mol. Biol. **217**:429-439.

Benz, R., M. T. Tosteson, & D. Schubert. 1984. Formation and properties of tetramers of Band III Protein from human erythrocyte membranes in planar lipid bilayers. Biochim. Biophys. Acta. **775**:347-355.

Berka, R. M., & M. L. Vasil. 1982. Phospholipase C (heat-labile hemolysin) of *Pseudomonas aeruginosa*: purification and preliminary characterization. J. Bacteriol. **152**:239-245.

Bernhardt, I., A. C. Hall, & J. C. Ellory. 1991. Effects of low ionic strength media on passive human red cell monovalent cation transport. J. Physiol. **434**:489-506.

Bernheimer, A. W. 1947. Comparative kinetics of haemolysis induced by bacterial and other haemolysins. J. Gen. Physiol. **30**:337-353.

Bernheimer, A. W. 1974. Interactions between membranes and cytolytic bacterial toxins. Biochim. Biophys. Acta **344**:27-50.

Bernheimer, A. W. 1988. Assay of hemolytic toxins. Met. Enz. **165**:213-217.

Bernheimer, A. W., B. J. Campbell, & L. J. Forrester. 1985. Comparative toxinology of *Loxosceles reclusa* and

Corynebacterium pseudotuberculosis. Science **228**:590-591.

Bernheimer, A. W., R. Linder, & L. S. Avigad. 1979. Nature and mechanism of action of the CAMP protein of group B streptococci. Infect. Immun. **23**:838-844.

Bernheimer, A. W., & B. Rudy. 1986. Interactions between membranes and cytolytic peptides. Biochim. Biophys. Acta **864**:123-141.

Bhakdi, S., N. Mackman, J.-M. Nicaud, & I. B. Holland. 1986. *Escherichia coli* hemolysin may damage target cell membranes by generating transmembrane pores. Infect. Immun. **52**:63-69.

Bhakdi, S., W. Seeger, N. Suttorp, & J. Tranum-Jensen. 1985a. Damage to the host by channel-forming bacterial toxins. pp.268-279, *in* The pathogenesis of bacterial infections (Jackson, G. G., & H. Thomas (ed.s)). Bayer-Symposium VIII. Springer-Verlag, Berlin.

Bhakdi, S., & J. Tranum-Jensen. 1985. Complement activation and attack on autologous cell membranes induced by streptolysin O. Infect. Immun. **48**:713-719.

Bhakdi, S., & J. Tranum-Jensen. 1986. Membrane damage by pore-forming bacterial cytolysins. Microb. Path. **1**:5-14.

Bhakdi, S., & J. Tranum-Jensen. 1988. Damage to cell membranes by pore-forming bacterial cytolysins. Prog. Allergy **40**:1-43.

Bhakdi, S., & J. Tranum-Jensen. 1991. Alpha-toxin of *Staphylococcus aureus*. Microbiol. Rev. **55**:733-751.

Bhakdi, S., J. Tranum-Jensen, & A. Sziegoleit. 1985b. Mechanism of membrane damage by streptolysin-O. Infect. Immun. **47**:52-60.

Bhakdi, S., U. Weller, I. Walev, E. Martin, D. Jonas, & M. Palmer. 1993. A guide to the use of pore-forming toxins for controlled permeabilisation of cell membranes. Med. Microbiol. Immunol. Berl. **182**:167-175.

Bhattacharya, S. K., G. Balakrish-Nair, P. Datta, D. Datta, S. P. De, & S. C. Pal. 1984. Electrocardiographic changes in *Vibrio parahaemolyticus* infection. J. Assoc. Physic. India **32**:607-608.

Biber, T. U. L. 1971. Effect of changes in transepithelial transport on the uptake of sodium across the outer surface of the frog skin. J. Gen. Physiol. **58**:131-144.

Binder, H. J., G. F. Lemp, & J. D. Gardner. 1980. Receptors for vasoactive intestinal peptide and secretion on small intestinal epithelial cells. Am. J. Physiol. **238**:G190-G197.

Binder, H. J., A. White, D. Whiting, & J. P. Hayslett. 1986. Demonstration of specific high affinity receptors for aldosterone in cytosol of rat colon. Endocrin. **118**:628-631.

Bjerrum, P. J., J. Tranum-Jensen, & K. Mollgard. 1980. Morphology of erythrocyte membranes and their transport function following aggregation of membrane proteins. pp.51-67, *in* Membrane transport in erythrocytes, Alfred Benzon Symposium 14 (U. V. Lassen, H. H. Ussing, & J. O. Wieth (ed.s)). Munksgaard, Copenhagen.

Blake, P. A., R. E. Weaver, & D. G. Hollis. 1980. Diseases of humans (other than cholera) caused by *Vibrio*s. Ann. Rev. Microbiol. **34**:341-367.

Blake, P. A., M. H. Merson, R. E. Weaver, D. G. Hollis, & P. C. Heublein. 1979. Disease caused by a marine *Vibrio*: clinical characteristics and epidemiology. New Engl. J. Med. **300**:1-5.

Blaustein, M. P. 1974. The interrelationship between sodium and calcium fluxes across cell membranes. Rev. Physiol. Biochem. Pharmacol. **70**:33-82.

Bloom, B. R., & C. J. L. Murray. 1992. Tuberculosis: commentary on a reemergent killer. Science **257**:1055-1064.

Bock, K., K.-A. Karlsson, N. Stromberg, & S. Teneberg. 1988. Interaction of viruses, bacteria and bacterial toxins with host cell surface glycolipids. Aspects on receptor identification and dissection of binding epitopes. Adv. Exp. Biol. **228**:153-186.

Bode, R. B., R. R. Brayton, R. R. Colwell, F. M. Russo, & W. E. Bullock. 1986. A new *Vibrio* species, *Vibrio cincinnatiensis*, causing meningitis: successful treatment in an adult. Ann. Intern. Med. **104**:55-56.

Bonting, S. L., & J. H. M. de Pont. 1981. Membrane transport. New comprehensive biochemistry **2**. Elsevier/North-Holland Biochemical Press, Amsterdam.

Bonventre, P. F. 1970. p.29, *in* Microbial toxins **1** (Ajl, S. J., S. Kadis, & T. C. Montie (ed.s)). Academic Press, New York.

Bookchin, R. M., V. L. Lew, R. L. Nagel, & C. Raventos. 1980. Increase in potassium and calcium transport in human red cells infected with *Plasmodium falciparum*. J. Physiol. **312**:65P.

Boudon, A., C. Richard, C. Le Corre, & P. Colombo. 1983. Premier cas autochtone de syndrome diarrhéique à *Vibrio parahaemolyticus* en France. Données bactériologiques, cliniques et épidémiologiques. Méd. Mal. Infect. **13**:443-447.

Boulnois, G. J., T. J. Mitchell, & P. W. T. I. Andrew. 1991. Structure and function of pneumolysin, the multifunctional, thiol-activated toxin of *Streptococcus pneumoniae*. Mol. Microbiol. **5**:2611-2616.

Bourdineaud, J. P., P. Boulanger, C. Lazdunski, & L. Letellier. 1990. *In vivo* properties of colicin A: channel activity is voltage dependent but translocation may be voltage independent. Proc. Nat. Acad. Sci. USA **87**:1037-1041.

Bowdler, A. J., & T. K. Chan. 1969. The time course of red cell lysis in hypotonic electrolyte solutions. J. Physiol. **201**:437-452.

Boyd, C. A. R., & G. J. Walley. 1984. Evidence for Ca^{2+}-activated K^{+}-channels in the basolateral membrane of the vascularly perfused frog small intestine. J. Physiol. **358**:77P.

Bradford, M. M. 1976. A rapid and sensistive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248-254.

Bragg, T. S., & D. L. Robertson. 1989. Nucleotide sequence and analysis of the lethal factor gene (*lef*) from *Bacillus anthracis*. Gene **81**:45-54.

Brahm, J. 1977. Temperature-dependent changes of chloride transport kinetics in human red cells. J. Gen. Physiol. **70**:283-306.

Brahm, J. 1982. Diffusional water permeability of human erythrocytes and their ghosts. J. Gen. Physiol. **79**:791-819.

Brahm, J. 1983. Permeability of human red cells to a homologous series of aliphatic alcohols. J. Gen. Physiol. **81**:283-304.

Braun, V., & T. Focareta. 1991. Pore-forming bacterial protein hemolysins (cytolysins). CRC Crit. Rev. Microbiol. **18**:115-158.

Brayton, P. R., R. B. Bode, R. R. Colwell, M. T. MacDonell, H. L. Hall, D. J. Grimes, P. A. West, & T. N. Bryant. 1986. *Vibrio cincinnatiensis* sp. nov., a human pathogen. J. Clin. Microbiol. **23**:104-108.

Bremer, E. G., & S. Hakomori. 1984. Gangliosides as receptor modulators. Adv. Exp. Med. Biol. **174**:381-394.

Brenner, D. J., F. W. Hickman-Brenner, J. V. Lee, A. G. Steigerwalt, G. R. Fanning, D. G. Hollis, J. J. III Farmer, R. E. Weaver, S. W. Joseph & R. J. Seidler. 1983. *Vibrio furnissii* (formerly aerogenic biogroup of *Vibrio fluvialis*), a new species isolated from human feces and the environment. J. Clin. Microbiol. **18**:816-824.

Bretscher, M. S. 1973. Membrane structure: some general principles. Science **181**:622-629.

Brown, D. R., S. L. Boster, M. F. Overend, A. M. Parsons, & B. G. Treder. 1990. Actions of neuropeptide Y on basal, cyclic AMP-induced and neurally evoked ion transport in porcine distal jejunum. Regul. Pept. **29**:31-47.

Brown, P. D., & F. V. Sepulveda. 1985a. A rabbit jejunal isolated enterocyte preparation suitable for transport studies. J. Physiol. **363**:257-270.

Brown, P. D., & F. V. Sepulveda. 1985b. Potassium movements associated with amino acid and sugar transport in enterocytes isolated from rabbit jejunum. J. Physiol. **363**:271-285.

Brown, D. F., P. L. Spaulding, & R. M. Twedt. 1977. Enteropathogenicity of *Vibrio parahaemolyticus* in the ligated rabbit ileum. Appl. Env. Microbiol. **33**:10-14.

Brugnara, C., M. Canessa, D. Cusi, & D. C. Tosteson. 1986. Furosemide-sensitive Na and K fluxes in human red cells. Net uphill Na extrusion and equilibrium properties. J. Gen. Physiol. **87**:91-112.

Bruins, H. R. 1929. Coefficients of diffusion in liquids. pp.63-76, *in* International critical tables **5** (Washburn, E. W. (ed.)). McGraw-Hill, New York.

Buckingham, L., & J. L. Duncan. 1983. Approximate dimensions of membrane lesions produced by streptolysin S and streptolysin O. Biochim. Biophys. Acta **729**:115-122.

Buckley, J. T. 1991. Secretion and mechanism of action of the hole-forming toxin aerolysin. Experentia **47**:418-419.

Burnet, F. M. 1949. Haemolysis by Newcastle disease virus. Nature **114**:1008.

Burnet, F. M., & J. D. Stone. 1947. The receptor destroying enzyme of *Vibrio cholerae*. Aust. J. Exp. Biol. Med. Sci. **25**:227-233.

Burton, R. M. 1963. The action of neuraminidase from *Clostridium perfringens* on gangliosides. J. Neurochem. **10**:503-512.

Calappi, E., M. Masserini, G. Schiavo, C. Montecucco, & G. Tettamanti. 1992. Lipid interaction of tetanus neurotoxin. FEBS Lett. **309**:107-110.

Canham, P. B., & D. R. Parkinson. 1970. The area and volume of single human erythrocytes during gradual osmotic swelling to hemolysis. Can. J. Physiol. Pharmacol. **48**:369-376.

Caprioli, A., I. Luzzi, F. Rosmini, P. Pasquini, R. Cirrincione, A. Gianviti, M. C. Matteucci, G. Rizzoni, and the HUS Italian Study Group. 1992. Hemolytic-uremic syndrome and vero cytotoxin-producing *Escherichia coli* infection in Italy. J. Infect. Dis. **166**:154-158.

Carafoli, E. 1987. Intracellular calcium homeostasis. Ann. Rev. Physiol. **56**:395-433.

Carraway, K. L. 1982. Proteolytic enzyme treatment. pp.239-250, *in* Red cell membranes - a methodological approach (Ellory, J. C., & J. D. Young (ed.s)). Academic Press, London.

Carruthers, A., & D. L. Melchior. 1988. Effects of lipid environment on membrane transport: the human erythrocyte sugar transport protein/lipid bilayer system. Ann. Rev. Physiol. **50**:257-71.

Cassano, G., B. Stieger, & H. Murer. 1984. Na/H and Cl/HCO_3 exchange in rat jejunal and rat proximal tubular brush border membrane vesicles. Studies with acridine orange. Pflug. Archiv. **400**:309-317.

Cassidy, J. T., G. W. Jourdian, & S. Roseman. 1965. The sialic acids. VI. Purification and properties of sialidase from *Clostridium perfringens*. J. Biol. Chem. **240**:3501-3506.

Cassuto, J., M. Jodal, & O. Lundgren. 1982. The effect of nicotinic and muscarinic blockade on cholera toxin induced intestinal secretion in cats and rats. Acta Physiol. Scand. **114**:573-577.

Cassuto, J., M. Jodal, A. Siewert, & O. Lundgren. 1983. The involvement of intramural nerves in cholera toxin induced intestinal secretion. Acta Physiol. Scand. **117**:195-202.

Castro, G. A. 1985. Fluid and electrolyte absorption. pp.129-139, *in* Gastrointestinal physiology (L. R. Johnson (ed.)). C. V. Mosby Co., St. Louis.

Castro, G. A. 1989. Gut immunophysiology: regulatory pathways within a common mucosal system. News Physiol. Sci. **4**:59-64.

Catto-Smith, A. G., J. A. Hardin, M. K. Patrick, E. V. O'Loughlin, & D. G. Gall. 1991. The effect of atrial natriuretic peptide on intestinal electrolyte transport. Regul. Pept. **36**:29-44.

Cavaillon, J.-M., C. Jolivet-Reynaud, C. Fitting, B. David, & J. E. Alouf. 1986. Ganglioside identification on human monocyte membrane with *Clostridium perfringens* delta-toxin. J. Leuk. Biol. **40**:65-72.

Cescatti, L., C. Pederzolli, & G. Menestrina. 1991. Modification of lysine residues of *Staphylococcus aureus* α-toxin: effects on its channel-forming properties. J. Memb. Biol. **119**:53-64.

Chakraborty, T., A. Schmid, S. Notermans, & R. Benz. 1990. Aerolysin of *Aeromonas sobria*: evidence for formation of ion-permeable channels and comparison with alpha-toxin of *Staphylococcus aureus*. Infect. Immun. **58**:2127-2132.

Chan, K. Y., M. L. Woo, K. W. Lo, & G. L. French. 1986. Occurence and distribution of halophilic *Vibrios* in subtropical coastal waters of Hong Kong. Appl. Env. Microbiol. **52**:1407-1411.

Chang, E. B., D. R. Brown, N. S. Wang, & M. Field. 1986. Secretagogue-induced changes in membrane calcium permeability in chicken and chinchilla ileal mucosa. J. Clin. Invest. **78**:281-297.

Chang, E. B., M. Field, & R. J. Miller. 1982. Alpha-2-adrenergic receptor regulation of ion transport in rabbit ileum. Am. J. Physiol. **242**:G237-G242.

Chang, E. B., M. Field, & R. J. Miller. 1983. Enterocyte alpha-2-adrenergic receptors: yohimbine and *p*-aminoclonidine binding relative to ion transport. Am. J. Physiol. **244**:G76-G82.

Chang, E. B., A. J. Gill, N. S. Wang, & M. Field. 1984. Alpha-2-adrenergic inhibition of Ca-dependent intestinal secretion. Gastroenterol. **86**:1044A.

Chang, E. B., & M. W. Musch. 1990. Calcium-mediated neurohumoral inhibition of chicken enterocyte Na influx: role of phosphatidylinositol metabolites. Life Sci. **46**:1913-1921.

Chang, E. B., & M. C. Rao. 1991. Intracellular mediators of intestinal electrolyte transport. pp.49-72, *in* Current topics in gastroenterology. Elsevier, New York.

Chase, H. S., & Q. Al-Awqati. 1983. Calcium reduces the sodium permeability of luminal membrane vesicles from toad bladder. Studies using a fast reaction apparatus. J. Gen. Physiol. **81**:643-666.

Chaudhuri, A. G., P. C. Sen, & U. Ganguly. 1993. Evidence for protein kinase C stimulation in rat enterocytes pretreated with heat stable enterotoxin of *Escherichia coli*. FEMS Microbiol. Lett. **110**:185-190.

Chaudry, G. J., P. L. Holmans, R. C. Clowes, & R. K. Draper. 1992. *Pseudomonas aeruginosa* exotoxin A. Target. Diagn. Ther. **7**:405-423.

Cherwonogrodzky, J. W., & A. G. Clark. 1981. Effect of pH on the production of the Kanagawa hemolysin by *Vibrio parahaemolyticus*. Infect. Immun. **34**:115-119.

Cherwonogrodzky, J. W., & A. G. Clark. 1982a. Production of the Kanagawa hemolysin by *Vibrio parahaemolyticus* in a synthetic medium. Infect. Immun. **37**:60-63.

Cherwonogrodzky, J. W., & A. G. Clark. 1982b. The purification of Kanagawa hemolysin from *Vibrio parahaemolyticus*. FEMS Microbiol. Lett. **15**:175-179.

Chloe, S., M. J. Bennet, G. Fujii, P. M. G. Curmi, K. A. Kantarajieff, R. J. Collier, & D. Eisenberg. 1992. The crystal structure of diphtheria toxin. Nature **357**:216-222.

Chou, P. Y., & G. D. Fasman. 1974. Prediction of protein conformation. Biochem. **13**:222-244.

Christensen, H. N. 1975. Biological transport. 2nd edition. Benjamin, Massachusetts.

Christensen, O. 1987. Mediation of cell volume regulation by Ca influx through stretch activated channels. Nature **330**:66-68.

Christie, R., N. E. Atkins, & E. Munch-Petersen. 1944. A note on a lytic phenomenon shown by group B streptococci. Aust. J. Exp. Biol. Med. Sci. **22**:197-200.

Chun, D., J. K. Chung, & R. Tak. 1974. Some observations on Kanagawa type hemolysis of *Vibrio parahaemolyticus*. pp.199-204, *in* International symposium on *Vibrio parahaemolyticus* (Fujino, T., G. Sakaguchi, R. Sakazaki, & Y. Takeda (ed.s)). Saikon Publishing, Tokyo.

Chun, D., J. K. Chung, R. Tak, & S. Y. Seol. 1975. Nature of the Kanagawa phenomenon of *Vibrio parahaemolyticus*. Infect. Immun. **12**:81-87.

Clague, M. J., & R. J. Cherry. 1989. A study of band 3 aggregation in erythrocyte membranes by melittin and other cationic agents. Biochim. Biophys. Acta **980**:93-99.

Claret, M., R. Garay, & F. Giraud. 1978. The effect of membrane cholesterol on the sodium pump in red blood cells. J. Physiol. **274**:247-263.

Clark, A. G., & J. Cherwonogrodzky. 1984. Haemolysis production and Kanagawa variability of *Vibrio parahaemolyticus*. pp.369-371, *in* Bacterial protein toxins (Alouf, J. E., F. J. Fehrenbach, J. H. Freer & J. Jeljaszewicz (ed.s)). Academic Press, London.

Cohen, B. E. 1974a. The permeability of liposomes to non-electrolytes. I. Activation energies for permeation. J. Memb. Biol. **20**:205-234.

Cohen, B. E. 1974b. The permeability of liposomes to non-electrolytes. II. The effect of nystatin and gramicidin A. J. Memb. Biol. **20**:235-268.

Cohen, P., C. F. B. Holmes, & Y. Tsukitani. 1990. Okadaic acid: a new probe for the study of cellular function. Trends Biochem. Sci. **15**:98-102.

Cohen, M. B., N. J. Jensen, J. A. Hawkins, E. A. Mann, M. R. Thompson, M. J. Lentze, & R. A. Giannella. 1993. Receptors for *Escherichia coli* heat stable enterotoxin in human intestine and in a human intestinal cell line (Caco-2). J. Cell. Physiol. **156**:138-144.

Collins, S., E. O'Loughlin, J. O. Rourke, Z. Li, A. Lee, & M. Howden. 1992. A cytotonic, cholera toxin-like protein produced by *Campylobacter jejuni*. Comp. Biochem. Physiol. **103B**:299-303.

Colwell, R. R., T. Kaneko, T. Staley, M. Sochard, J. Pickar, & L. Wan. 1974. *Vibrio parahaemolyticus* - taxonomy, ecology, and pathogenicity. pp.169-176, *in* International symposium on *Vibrio parahaemolyticus* (Fujino, T., G. Sakaguchi, R. Sakazaki, & Y. Takeda (ed.s)). Saikon Publishing, Tokyo.

Conlan, J. W., A. Baskerville, & L. E. A. Ashworth. 1986. Separation of *Legionella pneumophilia* proteases and purification of a protease which produces lesions like those of Legionnaires' disease in guinea-pig lung. J. Gen. Microbiol. **132**:1565-1574.

Contreras, R. G., L. Gonzalez-Mariscal, M. S. Balda, M. R. Garcia-Villegas, & M. Cereijido. 1992. The role of calcium in the making of a transporting epithelium. News Physiol. Sci. **7**:105-108.

Cooper, R. A., E. C. Arner, J. S. Wiley, & S. J. Shatti. 1975. Modification of red cell membrane structure by cholesterol-rich lipid dispersions. A model for the primary spur cell defect. J. Clin. Invest. **55**:115-126.

Cowell, J. L., K. Kim, & A. W. Bernheimer. 1978. Alteration by cereolysin of the structure of cholesterol-containing membranes. Biochim. Biophys. Acta **507**:230-241.

Craigie, E. H. 1966. A laboratory guide to the anatomy of the rabbit. 2nd edition. University of Toronto Press, Toronto.

Csaky, T. Z., & G. Esposito. 1969. Osmotic swelling of intestinal epithelial cells during active sugar transport. Am. J. Physiol. **217**:753-755.

Curran, P. F., F. C. Herrera, & W. J. Flanigan. 1963. The effect of calcium and antidiuretic hormone on Na transport across frog skin. II. Sites and mechanisms of action. J. Gen. Physiol. **46**:1011-1027.

Cutter, D. L., & A. S. Kreger. 1990. Cloning and expression of the damselysin gene from *Vibrio damsela*. Infect. Immun. **58**:266-268.

Dacie, J. V., & S. M. Lewis. 1975. Practical haematology. 5th edition. Churchill Livingstone, Edinburgh.

Dai, J.-H., Y.-S. Lee, & H.-C. Wong. 1992. Effects of iron limitation on production of a siderophore, outer membrane proteins, and hemolysin and on hydrophobicity, cell adherence, and lethality for mice of *Vibrio parahaemolyticus*. Infect. Immun. **60**:2952-2956.

Daniel, L. W., L. King, & M. Kennedy. 1988. Phospolipase activity of bacterial toxins. Met. Enz. **165**:298-301.

Datta, A. R., B. A. Wentz, & J. Russell. 1990. Cloning of the listeriolysin O gene and development of specific gene probes for *Listeria monocytogenes*. Appl. Env. Microbiol. **56**:3874-3877.

Davis, B. J. 1964. Disc electrophoresis-II: method and application to human serum proteins. Ann. N. Y. Acad. Sci. **121**:404-427.

Davis, B. R., G. R. Fanning, J. M. Madden, A. G. Steigerwalt, H. B. Jr. Bradford, H. L. Jr. Smith, & D. J. Brenner. 1981. Characterization of biochemically atypical *Vibrio cholerae* strains, and the designation of a new pathogenic species, *Vibrio mimicus*. J. Clin. Microbiol. **14**:631-639.

Davis, C. W., & A. L. Finn. 1982. Sodium transport inhibition by amiloride reduces basolateral membrane K conductance in tight epithelia. Science **216**:525-527.

Davis, C. W., & A. L. Finn. 1985. Cell volume regulation in frog urinary bladder. Fed. Proc. **44**:2520-2525.

Davis, G., C. Santa-Ana, S. Morawski, & J. Fordtran. 1982. Permeability characteristics of human jejunum, ileum, proximal colon and distal colon: results of potential difference measurements and unidirectional fluxes. Gastroenterol. **83**:844-850.

Dawson, D. C. 1991. Principles of membrane transport. pp.1-44, *in* Handbook of physiology. The gastrointestinal system. Section 6. **4** (Schultz, S. G. (ed.)). American Physiological Society, Bethesda.

De, S. N., & D. N. Chatterje. 1953. An experimental study of the mechanism of action of *Vibrio cholerae* on the intestinal mucous membrane. J. Path. Bact. **66**:559-562.

De, S. N. 1959. Enterotoxicity of bacteria-free culture filtrate of *Vibrio cholerae*. Nature **183**:1533-1534.

Dempster, J. A., A. N. Van Hoek, & C. H. Van Os. 1992. The quest for water channels. News Physiol. Sci. 7:172-176.

Derevitskaya, V. A., N. P. Arbatsky, & N. K. Kochetkov. 1978. The structure of carbohydrate chains of blood-group substance. Eur. J. Biochem. **86**:423-437.

Deuticke, B. 1977. Properties and structural basis of simple diffusion pathways in the erythrocyte membrane. Rev. Physiol. Biochem. Pharmacol. **78**:1-97.

Deuticke, B., & C. W. M. Haest. 1987. Lipid modulation of transport proteins in vertebrate cell membranes. Ann. Rev. Physiol. **49**:221-235.

Deuticke, B., P. Lutkemeier, & B. Poser. 1991. Influence of phloretin and alcohols on barrier defects in the erythrocyte membrane caused by oxidative injury and electroporation. Biochim. Biophys. Acta **1067**:111-122.

Deuticke, B., P. Lutkemeier, & M. Sistemich. 1984. Ion selectivity of aqeous leaks induced in the erythrocyte membrane by crosslinking of membrane proteins. Biochim. Biophys. Acta **775**:150-160.

Deuticke, B., B. Poser, P. Lutkemeier, & C. W. M. Haest. 1983. Formation of aqeous pores in the human erythrocyte membrane after oxidative cross-linking of spectrin by diamide. Biochim. Biophys. Acta **731**:196-210.

Dharmsathaphorn, K., H. J. Binder, & J. W. Dobbins. 1980. Somatostatin stimulates sodium and chloride absorption in the rabbit ileum. Gastroenterol. **78**:1559-1565.

Dharmsathaphorn, K., & J. L. Madara. 1990. Established intestinal cell lines as model systems for electrolyte studies. Met. Enz. **192**:354-389.

Dharmsathaphorn, K., K. Mandel, J. A. McRoberts, C. A. Cartwright, & H. Masui. 1984. Utilization of a human colonic cell tumor line as a model to study electrolyte transport in intestine. pp.325-333, *in* Intestinal absorption and secretion (Skadhauge, E., & K. Heintze (ed.s)). MTP Press, Lancaster.

Diamond, J. M. 1982. Transcellular cross-talk between epithelial cell membranes. Nature **300**:683-685.

Diamond, J. M., & E. M. Wright. 1969. Biological membranes: the physical basis of ion and nonelectrolyte selectivity. Ann. Rev. Physiol. **31**:581-646.

Dix, J. A., D. A. Ausiello, C. Y. Jung, & A. S. Verkman. 1985. Target analysis studies of red cell water and urea transport. Biochim. Biophys. Acta **821**:243-252.

Donelli, G., & C. Fiorentini. 1992. Cell injury and death caused by bacterial protein toxins. Toxicol. Lett. **64-65**:spec.no.695-699.

Donowitz, M. 1983. Ca^{2+} in the control of active Na and Cl transport: involvement in neurohumoral action. Am. J. Physiol. **245**:G165-G177.

Donowitz, M., R. Fogel, L. Battisti, & N. Asarkof. 1982. The neurohumoral secretagogue carbachol, substance P and neurotensin increase in Ca^{2+} influx and calcium content in rabbit ileum: mechanism of action? Life Sci. **31**:1919-1937.

Donowitz, M., & M. J. Welsh. 1986. Ca^{2+} and cyclic AMP in regulation of intestinal Na, K and Cl transport. Ann. Rev. Physiol. **48**:135-150.

Donowitz, M., & M. J. Welsh. 1987. Regulation of mammalian small intestine electrolyte secretion. pp.1351-1388, *in* Physiology of the gastrointestinal tract (L. R. Johnson (ed.)), **2**. 2nd edition. Raven Press, New York.

Douet, J. P., M. Castroviejo, A. Dodin, & C. Bebear. 1992. Purification and characterization of Kanagawa haemolysin

from *Vibrio parahaemolyticus*. Res. Microbiol. **143**:569-577.

Dougan, D. G. 1986. The role of enterotoxin components in future vaccines against enterotoxigenic *Escherichia coli*-induced diarrhoea and cholera. pp.321-331, *in* Natural toxins: animal, plant, and microbial (Harris, J. B. (ed.)). Clarendon Press, Oxford.

Doyle, M. P. 1990. Pathogenic *Escherichia coli*, *Yersinia enterocolitica*, and *Vibrio parahaemolyticus*. Lancet **336**:1111-1115.

Dressler, V., K. Schwister, C. W. M. Haest, & B. Deuticke. 1983. Dielectric breakdown of the erythrocyte membrane enhances transbilayer mobility of phospholipids. Biochim. Biophys. Acta **732**:304-307.

Drury, R. A. B., & E. A. Wallington. 1967. Carleton's histological technique. 4th edition. Oxford University Press, Oxford.

Duncan, J. L. 1974. Characteristics of streptolysin-O hemolysis: kinetics of hemoglobin and ^{86}Rubidium release. Infect. Immun. **9**:1022-1027.

Duncan, J. L., & R. Schlegel. 1975. Effect of streptolysin-O on erythrocyte membranes, liposomes and lipid dispersions. J. Cell Biol. **67**:160-73.

Dunham, P. B., & J. C. Ellory. 1981. Passive potassium transport in low potassium sheep red cells: dependence on cell volume and chloride. J. Physiol. **318**:511-530.

Dunham, P. B., & J. F. Hoffman. 1978. Na and K transport in red blood cells. pp.252-272, *in* Membrane physiology (Andreoli, T. E., J. F. Hoffman, & D. D. Fanestil (ed.s)). Plenum, New York.

Dunn, M. J. 1969. Alteration of red blood cell sodium transport during malarial infection. J. Clin. Invest. **48**:674-684.

Dwight, J. F. St. J., & B. M. Hendry. 1992. Effects of arachidonic acid on rubidium uptake in human erythrocytes. J. Physiol. **446**:173P.

Dwyer, T. M., D. J. Adams, & B. Hille. 1980. The permeability of the endplate channel to organic cations in frog muscle. J. Gen. Physiol. **75**:469-492.

Dzeniek, R. 1972. Viral and bacterial neuraminidases. Curr. Top. Microbiol. Immunol. **59**:35-74.

Eaton, D. C. 1981. Intracellular sodium ion activity and sodium transport in rabbit urinary bladder. J. Physiol. **316**:527-544.

Eaton, D. C., A. M. Frace, & S. U. Silverthorn. 1982. Active and passive Na fluxes across the basolateral membrane of rabbit urinary bladder. J. Memb. Biol. **67**:219-229.

Eaton, D. C., K. L. Hamilton, & K. E. Johnson. 1984. Intracellular acidosis blocks the basolateral Na-K pump in rabbit urinary bladder. Am. J. Physiol. **247**:F946-F954.

Eckert, R. 1988. Animal energetics and temperature relations. pp.555-605 *in* Animal physiology. Mechanisms and adaptations. 3rd edition. W. H. Freeman and Co., New York.

Edelman, A. M., D. K. Blumenthal, & E. G. Krebs. 1987. Protein serine/threonine kinases. Ann. Rev. Biochem. **56**:567-613.

Ehrenfeld, J., E. J. Cragoe, & B. J. Harvey. 1987. Evidence for a Na^+/H^+ exchanger at the basolateral membranes of the isolated frog skin epithelium: effect of amiloride analogues. Pflug. Archiv. **409**:200-207.

Ehrenstein, G., & H. Lecar. 1977. Electrically gated ion channels in lipid bilayers. Q. Rev. Biophys. **10**:1-34.

Ehrlich, P. 1900. On immunity with special reference to cell life. Proc. Roy. Soc. London **66**:424-428.

Eidels, L., R. L. Proia, & D. A. Hart. 1983. Membrane receptors for bacterial toxins. Microbiol. Rev. **47**:596-620.

Eisenberg, M., J. E. Hall, & C. A. Mead. 1973. The nature of the voltage-dependent conductance induced by alamethicin in black lipid membranes. J. Memb. Biol. **14**:143-176.

Eisenman, G., & R. Horn. 1983. Ionic selectivity revisited: the role of kinetic and equilibrium processes in ion permeation through channels. J. Memb. Biol. **76**:197-225.

Eko, F. O., V. O. Rotimi, & A. O. Coker. 1992. Haemagglutinating and buccal epithelial cell adherence activities of *Vibrio parahaemolyticus*: correlation with virulence. J. Trop. Med. Hyg. **95**:202-205.

Ellory, J. C. 1979. Radiation inactivation for molecular size determinations. Trends Biochem. Sci. **4**:99-100.

Ellory, J. C. 1982. Flux measurements. Tech. Cell. Physiol. **P129**:1-11.

Ellory, J. C. 1987. Amino acid transport systems in mammalian red cells. pp.106-119, *in* Amino acid transport in animal cells (Yudilevich, D. L., & C. A. R. Boyd (ed.s)). Physiological society study guides **2**. Manchester University Press, Manchester.

Ellory, J. C., B. C. Elford, & C. I. Newbold. 1988. Introductory remarks: transport mechanisms across cell membranes. Parasitol. **96**:S5-S9.

Ellory, J. C., & A. C. Hall. 1987. Temperature effects on red cell membrane transport processes. Symp. Soc. Exp. Biol. **41**:53-66.

Ellory, J. C., & A. C. Hall. 1988. Human red cell volume regulation in hypotonic media. Comp. Biochem. Physiol. **90A**:533-537.

Ellory, J. C., A. C. Hall, & J. A. L. Amess. 1987. Passive potassium transport in human red cells. Biomed. Biophys. Acta **46**:31-35.

Ellory, J. C., K. Kirk, S. J. Culliford, B. G. Nash, & J. Stuart. 1992. Nitrendipine is a potent inhibitor of the Ca^{2+}-activated K^+ channel of human erythrocytes. FEBS Lett. **296**:219-221.

Ellory, J. C., & V. L. Lew. 1977. Membrane transport in red cells. Academic Press, London.

Ellory, J. C., & E. M. Tucker. 1983. Cation transport in red blood cells. pp.291-313, *in* Red blood cells of domestic mammals (Agar, N. S., & P. G. Board (ed.s)). Elsevier, Amsterdam.

Ellory, J. C., & M. W. Wolowyk. 1990. Evidence for bumetanide-sensitive, Na^+-dependent, partial Na-K-Cl co-transport in red blood cells of a primitive fish. Can. J. Physiol. Pharmacol. **69**:588-591.

Ellory, J. C., & J. D. Young. 1982. Red cell membranes - a methodological approach. Academic Press, London.

Elson, E. L. 1986. Membrane dynamics studied by fluorescence correlation spectroscopy and photobleaching recovery. Soc. Gen. Physiol. Ser. **40**:367-383.

Endo, Y., K. Tsurugi, T. Yutsoda, Y. Takeda, T. Ogasawara, & K. Igarashi. 1988. Site of action of a vero toxin (VT2) from *Escherichia coli* O157:H7 and of shiga toxin on eukaryotic ribosomes: RNA N-glycosidase activity of the toxins. Eur. J. Biochem. **171**:45-50.

Epidemiological Bulletin. 1992. Cholera situation in the Americas. An update. Epidemiological Bull. **1**:11-13.

Erlij, D., & M. W. Smith. 1973. Sodium uptake by frog skin and its modification by inhibitors of transepithelial sodium transport. J. Physiol. **228**:221-239.

Erlij, D., I. Gersten, G. Sterba, & H. F. Schoen. 1986. Role of prostaglandin release in the response of tight epithelia to Ca^{2+} ionophores. Am. J. Physiol. **250**:C629-C636.

Essig, A., & A. Leaf. 1963. The role of potassium in active transport of sodium by the toad bladder. J. Gen. Physiol. **46**:505-515.

Evans, N. 1986. Bacterial toxins and their effect on the gut. pp.212-236, *in* Natural toxins: animal, plant, and microbial (Harris, J. B. (ed.)). Clarendon Press, Oxford.

Evans, G. S., N. Flint, & C. S. Potten. 1994. Primary cultures for studies of cell regulation and physiology in intestinal epithelium. Ann. Rev. Physiol. **56**:399-417.

Evans, W. H., & J. M. Graham. 1989. Membrane structure and function. Oxford University Press, Oxford.

Facchiano, F., F. Valtorta, F. Benfenati, & A. Luini. 1993. The transglutaminase hypothesis for the action of tetanus toxin. Trends Biochem. Sci. **18**:327-329.

Fagerstam, L. G., J. Lizana, U.-B. Axio-Fredriksson, & L. Wahlstrom. 1983a. Fast chromatofocusing of human serum proteins with special reference to α_1-antitrypsin and Gc-globulin. J. Chromatog. **266**:523-532.

Fagerstam, L. G., L. Wahlstrom, U.-B. Fredriksson, R. M. Muller, & V. Nylund. 1983b. Analysis of human blood plasma by chromatofocusing on Mono P™ followed by pore gradient gel electrophoresis. Protides Biol. Fluids **30**:657-659.

Falkow, S. 1990. The "Zen" of bacterial pathogenicity. pp.3-10, *in* Molecular basis of bacterial pathogenesis (Iglewski, B. H., & V. L. Clark (ed.s)). [The bacteria **11**]. Academic Press, San Diego.

Farmer, J. J., F. W. Hickman-Brenner, & M. T. Kelly. 1985. Chapter 26, *in* Manual of clinical microbiology (Lenette, E. H., A. Balows, W. J. Jr. Hauster, & H. J. Shardarny). 4th edition. American Society for Microbiology, Washington DC.

Farmer, R. E. L., & R. I. Macey. 1972. Perturbation of red cell volume: constancy of membrane transport parameters for certain slow penetrants. Biochim. Biophys. Acta **255**:502-516.

Farmer, R. E. L., & R. I. Macey. 1972. Perturbation of red cell volume: determination of membrane transport parameters for rapid penetrants. Biochim. Biophys. Acta **290**:290-299.

Fasano, A., B. Baudry, J. Galen, J. Ketley, L. Ferraro, J. Michalski, M. Trucksis, S. Guandalini, & J. Kaper. 1992. Further insights into *Vibrio cholerae* diarrheal disease. Gastroenterol. **102**:A209.

Fasano, A., B. Baudry, D. W. Pumplin, S. W. Wasserman, B. D. Tall, J. M. Ketley, & J. B. Kaper. 1991. *Vibrio cholerae* produces a second enterotoxin, which affects intestinal tight junctions. Proc. Nat. Acad. Sci. USA **88**:5242-5246.

Fasano, A., Y. Hokama, R. Russell, & J. Jr. Glen Morris. 1991. Diarrhea in Ciguatera fish poisoning: preliminary evaluation of pathophysiological mechanisms. Gastroenterol. **100**:471-476.

Feachem, R. G. 1981. Environmental aspects of cholera epidemiology. I. A review of selected reports of endemic and epidemic situations during 1961-1980. Trop. Dis. Bull. **78**:675-698.

Feeley, J. C., & M. Pittman. 1963. Studies on the haemolytic activity of El Tor vibrios. Bull. W. H. O. **28**:347-356.

Fehrenbach, F. J., & D. Jurgens. 1991. Cooperative membrane-active (lytic) processes. pp.187-213, *in* Sourcebook of bacterial protein toxins (Alouf, J. E., & J. H. Freer (ed.s)). Academic Press, New York.

Field, M. 1981. Secretion of electrolytes and water by mammalian small intestine. pp.963-982, *in* Physiology of the gastrointestinal tract (Johnson, L. R. (ed.)), **2**. Raven Press, New York.

Field, M. 1993. Intestinal electrolyte secrteion. History of a paradigm. Arch. Surg. **128**:273-278.

Field, M., L. H. Jr. Graff, W. J. Laird, & P. L. Smith. 1978. Heat stable enterotoxin of *Escherichia coli*: *in vitro* effects on guanylate cyclase activity, cyclic GMP concentration and ion transport in small intestine. Proc. Nat. Acad. Sci. USA **75**:2800-2804.

Field, M., M. W. Musch, & J. S. Stoff. 1981. Role of prostaglandins in the regulation of intestinal electrolyte transport. Prostaglandins **21**(S):73-79.

Field, M., M. C. Rao, & E. B. Chang. 1989. Intestinal electrolyte transport and diarrheal disease; part 1. N. Engl. J. Med. **321**:800-806.

Field, M., & C. E. Semrad. 1993. Toxigenic diarrheas, congenital diarrheas, and cystic fibrosis: disorders of intestinal ion transport. Ann. Rev. Physiol. **55**:631-655.

Field, M., P. L. Smith, & J. Bolton. 1980. Ion transport across the isolated intestinal mucosa of the winter flounder, *Pseudopleuronectes americanus*. II. Effects of cyclic AMP. J. Memb. Biol. **55**:157-163.

Finch, C. A., & H. A. Huebers. 1991. Iron absorption. pp.361-372, *in* Handbook of physiology. The gastrointestinal system. Section 6. **4** (Schultz, S. G. (ed.)). American Physiological Society, Bethesda.

Finean, J. B., & R. H. Michell. 1981. Membrane structure. Elsevier, Amsterdam.

Finkelstein, R. A. 1966. Preselection of hemolytic variants of El Tor vibrios. J. Bacteriol. **92**:513-514.

Finkelstein, R. A. 1973. Cholera. CRC Crit. Rev. Microbiol. **2**:553-623.

Finlay, B. B., & S. Falkow. 1989. Common themes in microbial pathogenicity. Microb. Rev. **53**:210-230.

Finlay, B. B., F. Heffron, & S. Falkow. 1989. Epithelial cell surfaces induce *Salmonella* proteins required for bacterial adherence and invasion. Science **243**:940-943.

Finn, A. L. 1975. Action of ouabain on sodium transport in toad urinary bladder. Evidence for two pathways for sodium entry. J. Gen. Physiol. **65**:503-514.

Fiorentini, C., G. Donelli, P. Nicotera, & M. Thelestam. 1993. *Clostridium difficile* toxin A elicits Ca^{2+}-independent cytotoxic effects in cultured normal rat intestinal crypt cells. Infect. Immun. **61**:3988-3993.

Fiorentini, C., & M. Thelestam. 1991. *Clostridium difficile* toxin A and its effects on cells. Toxicon **29**:543-567.

Fisher, M. H., E. L. Kaplan, & L. M. Wannamaker. 1981. Cholesterol inhibition of streptolysin O for myocardial cells in tissue culture. Proc. Soc. Exp. Biol. Med. **163**:233-237.

Fishman, P. H. 1982. Role of membrane gangliosides in the binding and action of bacterial toxins. J. Memb. Biol. **69**:85-97.

Fishman, P. H. & R. O. Brady. 1976. Biosynthesis and function of gangliosides. Gangliosides appear to participate in the transmission of membrane-mediated information. Science **194**:906-915.

Flodin, P. 1962. Dextran gels and their applications in gel filtration. Dissertation. Pharmacia, Uppsala.

Forte, L. R., S. L. Eber, J. T. Turner, R. H. Freeman, K. F. Fok, & M. G. Currie. 1993. Guanylin stimulation of Cl^- secretion in human intestinal T84 cells via cyclic guanosine monophosphate. J. Clin. Invest. **91**:2423-2428.

Frankenhauser, F. B., & A. L. Hodgkin. 1957. The action of calcium on the electrical properties of squid axons. J. Physiol. **137**:218-244.

Freer, J. H. 1986. Membrane damage caused by bacterial toxins: recent advances and new challenges. pp. 189-211, *in* Natural toxins: animal, plant, and microbial (Harris, J. B. (ed.)). Clarendon Press, Oxford.

Friedman, P. A., J. S. Figueiredo, T. Maack, & E. E. Windhaager. 1981. Sodium-calcium interactions in the renal proximal convoluted tubule of the rabbit. Am. J. Physiol. **240**:F558-F568.

Frindt, G., & E. E. Windhager. 1983. Effect of quinidine, low peritubular [Na] or [Ca] on Na transport in isolated perfused cortical collecting tubules. Fed. Proc. **42**:305.

Frindt, G., & E. E. Windhager. 1986. Effect of ionomycin on Na fluxes in perfused cortical collecting tubules of rabbit kidneys. Fed. Proc. **45**:540.

Frizzell, R. A., M. Dugas, & S. G. Schultz. 1975. Sodium chloride transport by rabbit gallbladder. J. Gen. Physiol. **65**:769-795.

Frizzell, R. A., & S. G. Schultz. 1972. Ionic conductances of extracellular shunt pathway in rabbit ileum. J. Gen. Physiol. **59**:318-346.

Frost, L. S. 1992. Bacterial conjugation: everybody's doin' it. Can. J. Microbiol. **38**:1091-1096.

Fuchs, W., E. H. Larsen, & B. Lindemann. 1977. Current-voltage curve of sodium channels and concentration dependence of sodium permeability in frog skin. J. Physiol. **267**:137-166.

Fujino, T. 1974. Discovery of *Vibrio parahaemolyticus*. pp.1-4, *in* International symposium on *Vibrio parahaemolyticus* (Fujino, T., G. Sakaguchi, R. Sakazaki & Y. Takeda (ed.s)). Saikon Publishing, Tokyo.

Fujino, T., Y. Okuno, D. Nakada, A. Aoyama, K. Fukai, T. Mukai, & T. Ueho. 1951. On the bacteriological examination of shirasu food poisoning. J. Jap. Assoc. Infect. Dis. **35**:11[in Japanese].

Fukuta, S., J. L. Magnani, E. M. Twiddy, R. K. Holmes, & V. Ginsburg. 1988. Comparison of the carbohydrate-binding specificities of cholera toxin and *Escherichia coli* heat-labile enterotoxins LTh-I, LT-IIa, and LT-IIb. Infect. Immun. **56**:1748-1753.

Fussle, R., S. Bhakdi, A. Sziegoleit, J. Tranum-Jensen, T. Kranz, & H-J. Wellensiek. 1981. On the mechanism of membrane damage by *Staphylococcus aureus* α-toxin. J. Cell. Biol. **91**:83-94.

Gable, P., J. W. Eaton, & D. L. Confer. 1985. Intoxication of human phagocytes by *Bordetella* adenylate cyclase toxin: implication of a ganglioside receptor. Clin. Res. **33**:844A.

Galen, J. E. 1991. Role of *Vibrio cholerae* neuraminidase in the binding and penetration of cholera toxin [**Thesis**].

Galen, J., J. Ketley, A. Fasano, S. H. Richardson, S. S. Wasserman, & J. B. Kaper. 1992. Role of *Vibrio cholerae* neuraminidase in the function of cholera toxin. Infect. Immun. **60**:406-415.

Ganguly, R., A. K. Ghosh, S. P. De, & D. L. Shrivastava. 1966. Haemolysis in vibrios. Ind. J. Med. Res. **54**:15-23.

Gardos, G. 1958. The function of calcium in the potassium permeability of human erythrocytes. Biochim. Biophys. Acta **30**:653-654.

Garnier, J., D. J. Osguthorpe, & B. Robson. 1978. Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. J. Mol. Biol. **120**:97-120.

Garrahan, P. J., & I. M. Glynn. 1966. Measurement of ^{24}Na and ^{42}K with a liquid-scintillation counting system without added scintillator. J. Physiol. **186**:55P-56P.

Garrahan, P. J., & I. M. Glynn. 1967. Factors affecting the relative magnitudes of the sodium:potassium and sodium:sodium exchanges catalysed by the sodium pump. J. Physiol. **192**:189-216.

Garty, H., & C. Asher. 1985. Ca^{2+}-dependent, temperature-sensitive regulation of Na^{+} channels in tight epithelia. J. Biol.

Chem. **260**:8330-8335.

Garty, H., C. Asher, & O. Yeger. 1987. Direct inhibition of epithelial Na^+ channels by a pH-dependent interaction with calcium and by other divalent ions. J. Memb. Biol. **95**:151-162.

Garty, H., E. D. Civan, & M. M. Civan. 1985. Effects of internal and external pH on amiloride blockable Na transport across toad urinary bladder vesicles. J. Memb. Biol. **87**:67-75.

Geck, P., & E. Heinz. 1989. Secondary active transport: introductory remarks. Kid. Int. **36**:334-341.

Gennis, R. B. 1989. Biomembranes. Molecular structure and function. Springer-Verlag, New York.

Geoffroy, C., J. Mengaud, J. E. Alouf, & P. Cossart. 1990. Alveolysin, the thiol-activated toxin of *Bacillus alvei*, is homologous to listeriolysin-O, perfringolysin-O, pneumolysin, and streptolysin-O and contains a single cysteine. J. Bacteriol. **172**:7301-7305.

Ghijsen, W. E. J. M., M. D. de Jong & C. H. van Os. 1983. Kinetic properties of Na/Ca exchange in basolateral plasma membranes of rat small intestine. Biochim. Biophys. Acta **730**:85-94.

Ghosh, A. K., D. N. Guhamazumder, P. L. Banerjee, K. P. Sengupta, A. K. Bose, & R. N. Majumder. 1974. Studies on mechanism of pathogenicity of *Vibrio parahaemolyticus*. pp.219-216, *in* International symposium on *Vibrio parahaemolyticus* (Fujino, T., G. Sakaguchi, R. Sakazaki, & Y. Takeda (ed.s)). Saikon Publishing, Tokyo.

Gibson, J., J. C. Ellory, S. J. Culliford, & D. A. Fincham. 1993. Volume-sensitive KCl cotransport and taurine influxes in horse red blood cells. Exp. Physiol. **78**:685-695.

Gilly, W. F., & C. M. Armstrong. 1982a. Slowing of sodium opening kinetics in squid axon by extracellular zinc. J. Gen. Physiol. **79**:935-964.

Gilly, W. F., & C. M. Armstrong. 1982b. Divalent cations and the activation kinetics of potassium channels in squid giant axon. J. Gen. Physiol. **79**:965-996.

Gilman, A., E. Koelle, & J. M. Ritchie. 1963. Transport of potassium ions in rat intestine. Nature **197**:1210-1211.

Ginsburg, H. 1988. How and why does the malarial parasite permeabilise its host cell membrane? pp.188-203 *in* Biomembranes: basic and medical research (Berga, G. L., & J. M. Tager (ed.s)). Springer-Verlag, Berlin.

Ginsburg, H., S. Handeli, S. Friedman, R. Gorodetsky, & M. Krugliak. 1986. Effects of red blood cell potassium and hypertonicity on the growth of *Plasmodium falciparum*. Z. Parasitenkd. **72**:185-199.

Ginsburg, H., M. Krugliak, O. Fidelman, & Z. I. Cabantchik. 1983. New permeability pathways induced in membranes of *Plasmodium falciparum* infected erythrocytes. Molec. Biochem. Parasitol. **8**:177-190.

Ginsburg, H., S. Kutner, M. Krugliak, & Z. I. Cabantchik. 1985. Characterisation of permeation pathways appearing in the host membrane of *Plasmodium falciparum* infected red blood cells. Mol. Biochem. Parasitol. **14**:313-322.

Ginsburg, H., & W. D. Stein. 1987. Biophysical analysis of novel transport pathways induced in red blood cell membranes. J. Memb. Biol. **96**:1-10.

Giraldez, F., K. J. Murray, F. V. Sepulveda, & D. N. Sheppard. 1989. Characterization of a phosphorylation-activated Cl^--selective channel in isolated *Necturus* enterocytes. J. Physiol. **416**:517-537.

Girardi, A. J., H. McMichael, & W. Henle. 1956. The use of HeLa cells in suspension for the quantitative study of virus propagation. Virology **2**:532-544.

Glaser, M. 1993. Lipid domains in biological membranes. Curr. Op. Str. Biol. **3**:475-481.

Glass, R. I., M. Libel, & A. D. Brandling-Bennet. 1992. Epidemic cholera in the Americas. Science **256**:1524-1524.

Goldberg, S. L., & J. R. Murphy. 1984. Molecular cloning of the haemolysin determinant from *Vibrio cholerae* El Tor. J. Bacteriol. **160**:239-244.

Goldberg, S. L., & J. R. Murphy. 1985. Cloning and characterization of the hemolysin determinants from *Vibrio cholerae* RV79(HlyA$^+$), RV79(HlyA$^-$), and S69B. J. Bacteriol. **162**:35-41.

Gordon, V. M., W. W. Young, S. M. Lechler, M. C. Lechler, S. H. Leppla, & E. L. Hewlett. 1989. Adenylate cyclase toxins from *Bacillus anthracis* and *Bordetella pertussis*: different processes for interaction with and entry into target cells. J. Biol. Chem. **264**:14792-14796.

Goshima, K., K. Owaribe, H. Yamanaka, & S. Yoshino. 1978. Requirement of calcium ions for cell degeneration with a toxin (vibriolysin) from *Vibrio parahaemolyticus*. Infect. Immun. **22**:821-832.

Goshima, K., T. Honda, M. Hirata, K. Kikuchi, Y. Takeda, & T. Miwitani. 1977. Stopping of the spontaneous beating of mouse and rat myocardial cells in vitro by a toxin from *Vibrio parahaemolyticus*. J. Molec. Cell. Cardiol. **9**:191-213.

Goto, S., K. Ota, & S. Kuwahara. 1959. Studies on purine requirements of V. El Tor. Jpn. J. Microbiol. **3**:223-230.

Gotschlich, F. 1906. Uber Cholera- und choleraahnliche Vibrionen unter den aus Mekka zuruckkenhrenden Pilgern. Z. Hyg. Infektionskr. **53**:281-304.

Gould, G. W., H. M. Thomas, T. J. Jess, & G. I. Bell. 1991. Expression of human glucose transporters in *Xenopus* oocytes: kinetic characterization and substrate specificities of the erythrocyte, liver, and brain isoforms. Biochem. **30**:5139-5145.

Graf, J., & G. Giebisch. 1979. Intracellular sodium activity and sodium transport in *Necturus* gallbladder epithelium. J. Memb. Biol. **47**:327-355.

Grasset, E., P. Gunter-Smith, & S. G. Schultz. 1983. Effects of Na-coupled alanine transport on intracellular K activities and the K conductances of the basolateral membranes of *Necturus* small intestine. J. Memb. Biol. **71**:89-94.

Gray, L. D., & A. S. Kreger. 1985. Purification and characterization of an extracellular cytolysin produced by *Vibrio vulnificus*. Infect. Immun. **48**:62-72.

Gray, G. S., & M. Kehoe. 1984. Primary sequence of the δ-toxin gene from *Staphylococcus aureus*. Infect. Immun. **46**:615-618.

Green, J., A. D. Sharrocks, J. I. Macinnes, & J. R. Guest. 1992. Purification of HlyX, a potential regulator of haemolysin synthesis, and properties of HlyX:FNR hybrids. Proc. R. Soc. Lond. B **248**:79-84.

Green, H., P. Barrow, & B. Goldberg. 1959. Effect of antibody and complement on permeability control in ascites tumor cells and erythrocytes. J. Exp. Med. **110**:699-713.

Green, W. N. 1991. Surface charges and ion channel function. Ann. Rev. Physiol. **53**:341-359.

Greig, E. D. W. 1914. The haemolytic action of Indian strains of cholera and cholera-like vibrios. Ind. J. Med. Res. **2**:623-647.

Griffiths, S. L., & D. R. Critchley. 1991. Characterisation of the binding sites for *Escherichia coli* heat-labile toxin type I in intestinal brush borders. Biochim. Biophys. Acta **1075**:154-161.

Guandalini, S., J. F. Kachur, P. L. Smith, R. J. Miller, & M. Field. 1980. In vitro effects of somatostatin stimulate sodium transport in rabbit intestine. Am. J. Physiol. **238**:G67-G74.

Guerrant, R. G., L. L. Brunton, T. C. Schnaitman, L. I. Rebhun, & A. G. Gilman. 1974. Cyclic adenosine monophosphate and alteration of Chinese Hamster Ovary cell morphology: a rapid, sensitive *in vitro* assay for the enterotoxins of *Vibrio cholerae* and *Escherichia coli*. Infect. Immun. **10**:320-327.

Guharay, F., & F. Sachs. 1984. Stretch-activated single ion channel currents in tissue-cultured embryonic chick skeletal muscle. J. Physiol. **352**:685-701.

Gunter-Smith, P., E. Grasset, & S. G. Schultz. 1982. Sodium-coupled amino acid and sugar transport by *Necturus* small intestine: an equivalent electrical circuit analysis of a rheogenic co-transport system. J. Memb. Biol. **66**:25-39.

Gunther, R. D., & E. M. Wright. 1983. Na^+, Li^+, and Cl^- transport by brush border membranes from rabbit jejunum. J. Memb. Biol. **74**:85-94.

Guroff, G. 1980. Molecular neurobiology. Dekker, New York.

Guy, H. R. 1984. Amino acid side-chain partition energies and distribution of residues in soluble proteins. Biophys. J. **47**:61-70.

Habermann, E. 1989. Palytoxin acts through Na^+,K^+ ATPase. Toxicon **27**:1171-1187.

Hagmann, J., & P. H. Fishman. 1982. Detergent extraction of cholera toxin and gangliosides from cultured cells and isolated membranes. Biochim. Biophys. Acta **720**:181-187.

Hakomori, S. 1984. Ganglioside receptors: a brief overview and introductory remarks. Adv. Exp. Med. Biol. **174**:333-339.

Hakomori, S. 1986. Glycosphingolipids. Sci. Am. **254**:44-53.

Hall, R. H. 1994. Personal communication.

Hall, R. H., & B. S. Drasar. 1990. *Vibrio cholerae* HlyA hemolysin is processed by proteolysis. Infect. Immun. **58**:3375-3379.

Hall, A. C., J. C. Ellory, & R. A. Klein. 1982. Pressure and temperature effects on human red cell cation transport. J. Memb. Biol. **68**:47-56.

Hall, R. H., A. C. Hall, & J. C. Ellory. 1986. The effects of *Vibrio cholerae* haemolysin on cation transport in human erythrocytes. Biochem. Soc. Trans. **14**:120-121.

Hall, R. H., A. C. Hall, J. C. Ellory, C. W. Sutton & D. A. Broadbent. 1985. The molecular mass of *Vibrio cholerae* haemolysin measured by radiation inactivation. Biochem. Soc. Trans. **13**:1251.

Hall, R. H., F. M. Khambaty, M. Kothary, S. P. Keasler, & B. D. Tall. 1993. *Vibrio cholerae* non-O1 associated with *cholera gravis* genetically and physiologically resembles O1 El Tor cholera strains. pp.17-22, *in* Abstracts of the 29th joint conference on cholera and related diarrheal diseases. US-Japan Cooperative Medical Science Program.

Hall, R. H., G. Losonsky, A. P. D. Silveira, R. K. Taylor, J. J. Mekalanos, N. D. Witham, & M. M. Levine. 1991. Immunogenicity of *Vibrio cholerae* O1 toxin-coregulated pili in experimental and clinical cholera. Infect. Immun. **59**:2508-2512.

Hall, A. C., & J. S. Willis. 1986. The temperature dependence of passive potassium permeability in mammalian erythrocytes. Cryobiol. **23**:395-405.

Halle, D., & S. Yedgar. 1988. Mild pressure induces resistance of erythrocytes to hemolysis by snake venom phospholipase A_2. Biophys. J. **54**:393-396.

Halm, D. R., & D. C. Dawson. 1983. Cation activation of the basolateral sodium-potassium pump in turtle colon. J. Gen.

Physiol. **82**:315-329.

Halperin, J. A., C. Brugnara, M. T. Tosteson, T. Van Ha, & D. C. Tosteson. 1989. Voltage-activated cation transport in human erythrocytes. Am. J. Physiol. **257**:C986-C996.

Halperin, J. A., A. Nicholson-Weller, C. Brugnara, & D. C. Tosteson. 1988. Complement induces a transient increase in membrane permeability in unlysed erythrocytes. J. Clin. Invest. **82**:594-600.

Ham, A. W., & D. H. Cormack. 1987. The digestive system. pp.475-517 *in* Ham's histology (Ham, A. W., & D. H. Cormack (ed.s)). J. B. Lippincott Co., Philadelphia.

Hames, B. D., & D. Rickwood. 1985. Gel electrophoresis of proteins. A practical approach. 4th edition. IRL Press, Oxford.

Hansen, M. B., & B. M. Jaffe. 1993. Involvement of eicosanoids and inositol 1, 4, 5-triphosphate in serotonin-induced secretion in rat ileum *in vivo*. Comp. Biochem. Physiol. **105**:77-82.

Hardegree, M. C., & A. T. Tu. 1988. Handbook of natural toxins. **4**. Bacterial toxins. Marcel Dekker, New York.

Harshman, S. 1979. Action of staphylococcal α-toxin on membranes: some recent advances. Molec. Cell. Biochem. **23**:143-152.

Harshman, S., & N. Sugg. 1985. Effect of calcium ions on staphylococcal alpha-toxin-induced hemolysis of rabbit erythrocytes. Infect. Immun. **47**:37-40.

Harris, J. B. 1986. Natural toxins: animal, plant, and microbial. Clarendon Press, Oxford.

Harvey, B. J., & J. E. Ehrenfeld. 1988. Role of Na/H exchange in the control of intracellular pH and membrane conductances in frog skin epithelium. J. Gen. Physiol. **92**:793-810.

Harvey, C. M., & J. C. Ellory. 1989. Identification of amino acid transporters in red blood cells. Met. Enz. **173**:122-159.

Harvey, B. J., & R. P. Kernan. 1984. Intracellular ionic activities in relation to external sodium and effects of amiloride and/or ouabain. J. Physiol. **349**:501-517.

Harvey, B. J., S. R. Thomas, & J. E. Ehrenfeld. 1988. Intracellular pH controls cell membrane Na^+ and K^+ conductances and transport in the frog skin epithelium. J. Gen. Physiol. **92**:767-791.

Hazama, A., & Y. Okada. 1988. Ca^{2+} sensitivity of volume-regulatory K^+ and Cl^- channels in cultured human epithelial cells. J. Physiol. **402**:687-702.

Heck, L. W., K. Morihara, W. B. McRae, & E. J. Miller. 1986. Specific cleavage of human type III and IV collagens by *Pseudomonas aeruginosa* elastase. Infect. Immun. **51**:115-118.

Heeswijk, M. P. E., J. A. M. Geertsen, & C. H. van Os. 1984. Kinetic properties of the ATP-dependent Ca pump and the Na/Ca exchange system in the basolateral membranes from rat kidney cortex. J. Memb. Biol. **79**:19-31.

Helman, S. I., W. Nagel, & R. S. Fisher. 1979. Ouabain on active transepithelial sodium transport in frog skin. Studies with microelectrodes. J. Gen. Physiol. **74**:105-127.

Henkart, P. A. 1985. Mechanism of lymphocyte-mediated cytotoxicity. Ann. Rev. Immunol. **3**:31-58.

Herrington, D. A., R. H. Hall, G. Losonsky, J. J. Mekalanos, R. K. Taylor, & M. M. Levine. 1988. Toxin, toxin-regulated pili, and the *tox*R regulon are essential for *Vibrio cholerae* pathogenesis in humans. J. Exp. Med. **168**:1487-1492.

Heukeshoven, J., & R. Dernik. 1985. Simplified method for silver staining of proteins in polyacrylamide gels and the mechanism of silver staining. Electrophoresis **6**:103-112.

Heyningen, van W. E. 1963. The fixation of tetanus toxin, strychnine and serotonin and other substances by ganglioside. J. Gen. Microbiol. **31**:375-387.

Heyningen, van S. 1973. Cholera toxin: interaction of subunits with ganglioside GM_1. Science **183**:656-657.

Heyningen, van S. 1974. Gangliosides as membrane receptors for tetanus toxin, cholera toxin and serotonin. Nature **249**:415-417.

Heyningen, van S. 1983. The interaction of cholera toxin with gangliosides and the cell membrane. Curr. Top. Memb. Transp. **18**:445-447.

Heyningen, van W. E., C. C. L. Carpenter, N. F. Pierce, & W. B. Greenough III. 1971. Deactivation of cholera toxin by ganglioside. J. Infect. Dis. **124**:415-417.

Hickman, F. W., J. J. III Farmer, D. G. Hollis, G. R. Fanning, A. G. Steigerwalt, R. E. Weaver, & D. J. Brenner. 1982. Identification of *Vibrio hollisae* sp. nov. from patients with diarrhea. J. Clin. Microbiol. **15**:395-401.

Higgins, N. P. 1992. Death and transfiguration among bacteria. Trends Biochem. Sci. **17**:207-211.

Higgins, T. J., B. Gebler, & E. Fromter. 1977. Electrical properties of amphibian urinary bladder epithelium. I. The cell potential profile of *Necturus maculosus*. Pflug. Archiv. **371**:87-97.

Hildebrand, A., M. Pohl, & S. Bhakdi. 1991. *Staphylococcus aureus* α-toxin: dual mechanism of binding to target cells. J. Biol. Chem. **266**:17195-17200.

Hildman, B., A. Schmidt, & H. Murer. 1982. Ca transport across basal-lateral plasma membranes from rat small intestinal epithelial cells. J. Memb. Biol. **65**:55-62.

Hill, J. G., A. Kucksis, & J. M. R. Beveridge. 1965. The effect of diet on the phospholipid composition of the red blood cells of man. J. Am. Oil Chem. Soc. **42**:137-141.

Hille, B. 1984. Ionic channels of excitable membranes. Sinauer Associates, Sunderland MA.

Hirose, R., & E. B. Chang. 1988. Effects of serotonin on Na^+-H^+ exchange and intracellular calcium in isolated chicken enterocytes. Am. J. Physiol. **17**:G891-G897.

Hirschborn, N., & W. B. III Greenough. 1991. Progress in oral rehydration therapy. Sci. Am. **264**:50-56.

Hladky, S. B., & D. A. Haydon. 1972. Ion transfer across lipid membranes in the presence of gramicidin A. Biochim. Biophys. Acta **274**:291-312.

Hoashi, K., K. Ogata, H. Taniguchi, H. Yamashita, K. Tsuji, Y. Mizuguchi, & N. Ohtomo. 1990. Pathogenesis of *Vibrio parahaemolyticus*: intraperitoneal and orogastric challenge experiments in mice. Microbiol. Immunol. **34**:355-366.

Hoffman, E. K. 1985. Role of separate K and Cl channels and of Na/Cl transport in volume regulation in Ehrlich cells. Fed. Proc. **44**:2513-2519.

Hoffman, J. F., & B. III Forbush. 1983. Structure, mechanism and function of the Na/K pump. Curr. Top. Memb. Transp. **19**. Academic Press, New York.

Hoffman, J. F., B. G. Kennedy, & G. Lunn. 1981. Modulation of red cell Na/K pump rates. pp.5-9, *in* Erythrocyte membranes. **2**. Recent clinical and experimental advances. Liss, New York.

Hoffman, E. K., L. O. Simonsen, & I. H. Lambert. 1984. Volume-induced increase of K and Cl permeabilities in Ehrlich ascites tumor cells. J. Memb. Biol. **78**:211-222.

Hoge, C. W., D. Watsky, R. N. Peeler, J. P. Libonati, E. Israel, & J. G. Jr. Morris. 1989. Epidemiology and spectrum of *Vibrio* infections in a Chesapeake Bay community. J. Inf. Dis. **160**:985-993.

Hohne-Zell, B., B. Strecher, & M. Gratzl. 1993. Functional characterization of the catalytic site of the tetanus toxin light chain using permeabilized adrenal chromaffin cells. FEBS Lett. **336**:175-180.

Hojvat, S. A., M. W. Musch, & R. J. Muller. 1983. Stimulation of prostaglandin production in rabbit ileal mucosa by bradykinin. J. Pharmacol. Exp. Ther. **226**:749-755.

Holman, G. D., I. J. Kozka, A. E. Clarke, C. J. Flower, J. Saltis, A. D. Habberfield, I. A. Simpson, & S. W. Cushman. 1990. Cell surface labeling of glucose transporter isoform GLUT4 by bis-mannose photolabel. Correlation with stimulation of glucose transport in rat adipose tissue cells by insulin and phorbol ester. J. Biol. Chem. **265**:18172-18179.

Holmgren, J., H. Elwing, P. Fredman, O. Strannegard, & L. Svennerholm. 1980. Gangliosides as receptors for bacterial toxins and Sendai virus. Adv. Exp. Med. **125**:453-470.

Holmgren, J., P. Fredman, M. Lindblad, A.-M. Svennerholm, & L. Svennerholm. 1982. Rabbit intestinal glycoprotein receptor for *Escherichia coli* heat-labile enterotoxin lacking affinity for cholera toxin. Infect. Immun. **38**:424-433.

Holmgren, J., M. Lindblad, P. Fredman, L. Svennerholm, & H. Myrvold. 1985. Comparison of receptors for cholera and *Escherichia coli* enterotoxins in human intestine. Gastroenterol. **89**:27-35.

Holmgren, J., I. Lonnroth, & L. Svennerholm. 1973. Tissue receptor for cholera exotoxin: postulated structure from studies with GM1 ganglioside and related glycolipids. Infect. Immun. **8**:208-214.

Homaidan, F. R., M. Donowitz, G. A. Weiland, & G. W. G. Shapr. 1989. Two calcium channels in basolateral membranes of rabbit epithelial cells. Am. J. Physiol. **257**:G86-G93.

Homareda, H., and H. Matsui. 1985. Effect of pH on Na^+ and K^+ binding to Na^+, K^+ ATPase. pp.251-254, *in* The sodium pump. 4th International conference on Na^+, K^+ ATPase (Glynn, I. M., & J. C. Ellory (ed.s)). The company of biologists. H. Charlesworth and Co., Huddersfield.

Honda, T., M. Arita, T. Takeda, N. Yoh., & T. Miwatani. 1985. Non-O1 *Vibrio cholerae* produces two newly identified toxins related to *Vibrio parahaemolyticus* hemolysin and *Escherichia coli* heat-stable enterotoxin. Lancet **2**:163-164.

Honda, T., & R. A. Finkelstein. 1979. Purification and characterization of a hemolysin produced by *Vibrio cholerae* biotype El Tor. Another toxic substance produced by cholera vibrios. Infect. Immun. **26**:1020-1027.

Honda, T., K. Goshima, Y. Takeda, Y. Sugino, & T. Miwatani. 1976a. Demonstration of the cardiotoxicity of the thermostable direct hemolysin (lethal toxin) produced by *Vibrio parahaemolyticus*. Infect. Immun. **13**:163-171.

Honda, T., & T. Iida. 1993. The pathogenicity of *Vibrio parahaemolyticus* and the role of the thermostable direct haemolysin and related haemolysins. Rev. Med. Microbiol. **4**:106-113.

Honda, T., & T. Miwatani. 1988. Multi-toxigenicity of *Vibrio cholerae* non-O1. pp.23-32, *in* Advances in research on cholera and related diarrheas (Ohtomo, N. and Sack, R. B. (ed.s)), **6**. KTK Scientific Publishers, Tokyo.

Honda, T., Y. Ni, A. Hata, M. Yoh, T. Miwatani, T. Okamoto, K. Goshima, H. Takakura, S. Tsunasawa, & F. Sakiyama. 1990. Properties of a hemolysin related to the thermostable direct hemolysin produced by a Kanagawa phenomenon-negative, clinical isolate of *Vibrio parahaemolyticus*. Can. J. Microbiol. **36**:395-399.

Honda, T., Y. Ni, & T. Miwatani. 1988. Purification and characterisation of a hemolysin produced by a clinical isolate of Kanagawa phenomenon-negative *Vibrio parahaemolyticus* and related to the thermostable direct haemolysin. Infect. Immun. **56**:961-965.

Honda, T., Y. Ni, T. Miwatani, T. Adachi, & J. Kim. 1992. The thermostable direct hemolysin of *Vibrio parahaemolyticus*

is a pore-forming toxin. Can. J. Microbiol. **38**:1175-1180.

Honda, T., M. Nishibuchi, T. Miwatani, & J. B. Kaper. 1986a. Demonstration of a plasmid-borne gene encoding a thermostable direct haemolysin in *Vibrio cholerae* non-O1 strains. Appl. Env. Microbiol. **52**:1218-1220.

Honda, T., M. Shimuzu, Y. Takeda, & T. Miwatani. 1976b. Isolation of a factor causing morphological changes of chinese hamster ovary cells from the culture filtrate of *Vibrio parahaemolyticus*. Infect. Immun. **14**:1028-1033.

Honda, T., S. Taga, T. Takeda, M. A. Hashibuan, Y. Takeda, & T. Miwitani. 1976c. Identification of lethal toxin with the thermostable direct haemolysin produced by *Vibrio parahaemolyticus*, and some physicochemical properties of the purified toxin. Infect. Immun. **13**:133-139.

Honda, T., Y. Takeda, T. Miwatani, K. Kato, & Y. Nimura. 1976d. Clinical features of patients suffering from food poisoning due to *Vibrio parahaemolyticus* with special reference to changes in electrocardiograms. J. Jpn. Assoc. Infect. Dis. **50**:216-223.

Honda, T., Y. Takeda, T. Miwatani, & N. Nakahara. 1983. Failure of antisera to thermostable direct hemolysin and cholera enterotoxin to prevent accumulation of fluid caused by *Vibrio parahaemolyticus*. J. Infect. Dis. **147**:779.

Honda, T., M. Yoh, I. Narita, T. Miwatani, H. Sima, Y. Xiegen, & L. Xiandi. 1986b. Purification of the thermostable direct haemolysin of *Vibrio parahaemolyticus* by immunoaffinity column chromatography. Can. J. Microbiol. **32**:71-73.

Horiguchi, Y., T. Uemura, S. Kozaki, & G. Sakaguchi. 1986. Effects of Ca^{2+} and other cations on the action of *Clostridium perfringens* enterotoxin. Biochim. Biophys. Acta **889**:65-71.

Hotchkiss, R. D. 1944. Gramicidin, tyrocidine and thyrothricin. Adv. Enzymol. **4**:153-199.

Houslay, M. D., & K. K. Stanley. 1982. Dynamics of biological membranes. Wiley, New York.

Howard, S. P., & J. T. Buckley. 1982. Membrane glycoprotein receptor and hole-forming properties of a cytolytic protein toxin. Biochem. **21**:1662-1667.

Hubel, K. A. 1976. Intestinal ion transport: effect of norepinephrine, pilocarpine and atropine. Am. J. Physiol. **231**:G252-G257.

Hubel, K. A. 1985. Intestinal nerves and ion transport: stimuli, reflexes, and responses. Am. J. Physiol. **248**:G261-G271.

Hughes, J. M., D. G. Hollis, E. J. Gangarosa, & R. E. Weaver. 1978. Non-cholera vibrio infections in the United States: clinical, epidemiologic, and laboratory features. Ann. Intern. Med. **88**:602-606.

Hugo, F., J. Reichweiss, M. Arvand, S. Kramer, & S. Bhakdi. 1986. Use of monoclonal antibody to determine the mode of action of transmembrane pore formation by streptolysin O. Infect. Immun. **54**:641-645.

Huttner, W. B. 1993. Cell biology: snappy exocytoxins. Nature **365**:104-105.

Hynes, W. L., & J. J. Ferretti. 1989. Sequence analysis and expression in *Escherichia coli* of the hyaluronidase gene of *Streptococcus pyogenes* bacteriophage H4489A. Infect. Immun. **57**:533-539.

Ichinose, Y., K. Yamamoto, N. Nakasone, M. J. Tanabe, T. Takeda, T. Miwatani, & M. Iwanaga. 1987. Enterotoxicity of El Tor-like hemolysin of non-O1 *Vibrio cholerae*. Infect. Immun. **55**:1090-1093.

Iglewski, B. H., & V. L. Clark. 1990. Molecular basis of bacterial pathogenesis. [The Bacteria **11**]. Academic Press, San Diego.

Ikigai, H., & T. Nakae. 1987a. Assembly of the α-toxin hexamer of *Staphylococcus aureus* with the liposome membrane. J. Biol. Chem. **262**:2156-2160.

Ikigai, H., & T. Nakae. 1987b. Interaction of the α-toxin of *Staphylococcus aureus* with the liposome membrane. J. Biol. Chem. **262**:2150-2155.

Ilundain, A. 1991. Transport studies in isolated enterocytes. pp.113-130, *in* Cell membrane transport (Yudilevich, D. L., R. Deves, S. Peran, & Z. I. Cabantchik (ed.s)). Plenum, New York.

Inoue, K., Y. Akiyama, T. Kinoshita, Y. Higashi, & T. Amano. 1976. Evidence for a one-hit theory in the immune bactericidal reaction and demonstration of a multi-hit response for hemolysis by streptolysin O and *Clostridium perfringens* theta-toxin. Infect. Immun. **13**:337-344.

Iwamoto, M., Y. Ohno-Iwashita, & S. Ando. 1987. Role of the essential thiol group in the thiol-activated cytolysin from *Clostridium perfringens*. Eur. J. Biochem. **167**:425-430.

Jacobs, M. H. 1932. Osmotic properties of the erythrocyte. III. The applicability of osmotic laws to the rate of hemolysis in hypotonic solutions of non-electrolytes. Biol. Bull. **62**:178-194.

Jain, M. 1988. Introduction to biological membranes. 2nd edition. John Wiley & Sons, New York.

James, G. T. 1978. Inactivation of the protease inhibitor phenylmethylsulfonyl fluoride in buffers. Anal. Biochem. **86**:574-579.

Janda, J. M., C. Powers, R. G. Bryant, & S. L. Abbott. 1988. Current perspectives on the epidemiology and pathogenesis of clinically significant *Vibrio* spp. Clin. Microbiol. Rev. **1**:245-267.

Jay, A. W. L. 1975. Geometry of the human erythrocyte. I. Effect of albumin on cell geometry. Biophys. J. **15**:205-222.

Jay, A. W. L., & S. Rowlands. 1975. The stages of osmotic haemolysis. J. Physiol. **252**:817-832.

Jean-Jacques, W., K. R. Rajashekariah, J. J. III Farmer, F. W. Hickman, J. G. Morris, & C. A. Kallick. 1981. *Vibrio metschnikovii* bacteremia in a patient with cholecystitis. J. Clin. Microbiol. **14**:711-712.

Jennings, M. L., & A. K. Solomon. 1976. Interaction between phloretin and the red blood cell membrane. J. Gen. Physiol. **67**:381-397.

Jensen, M., P. Baumann, M. Mandel, J. V. Lee, & T. N. Bryant. 1980. Characterization of facultatively anaerobic marine bacteria belonging to group F of Lee, Donovan, and Furniss. Curr. Microbiol. **3**:373-375.

Jodal, M., S. Holmgren, O. Lundgren, & A. Sjoqvist. 1993. Involvement of the myenteric plexus in the cholera toxin-induced net fluid secretion in the rat small intestine. Gastroenterol. **105**:1286-1293.

Johnson, D. E., & F. M. Calia. 1976. False-positive rabbit ileal loop reactions attributed to *Vibrio parahaemolyticus* broth filtrates. J. Infect. Dis. **133**:436-440.

Johnson, M. K. 1972. Properties of purified pneumococcal hemolysin. Infect. Immun. **6**:755-760.

Johnson, L. R. 1985. Gastrointestinal physiology. C. V. Mosby Co., St. Louis.

Johnson, M. K., & D. Boese-Marrazzo. 1980. Production and properties of heat-stable extracellular hemolysin from *Pseudomonas aeruginosa*. Infect. Immun. **29**:1028-1033.

Johnstone, A., & R. Thorpe. 1988. Isolation and fractionation of lymphocytes. pp.86-112, *in* Immunochemistry in practice. Blackwell Scientific Publications, Oxford.

Johnstone, R. M., & K. Teng. 1989. Membrane remodelling during reticulocyte maturation. News Physiol. Sci. **4**:37-42.

Jolivet-Reynaud, C., & J. E. Alouf. 1983. Binding of *Clostridium perfringens* ^{125}I-labeled δ-toxin to erythrocytes. J. Biol.

Chem. **258**:1871-1877.

Jolivet-Reynaud, C., J. M. Cavaillon & J. E. Alouf. 1982. Selective cytotoxicity of *Clostridium perfringens* delta toxin on rabbit leukocytes. Infect. Immun. **38**:860-864.

Jolivet-Reynaud, C., J. Estrada, L. A. West, J. E. Alouf, & L. Chedi. 1993. Targeting of GM_2-bearing tumour cells with the cytolytic *Clostridium perfringens* delta toxin. Anticancer Drugs **4**:65-75.

Jolivet-Reynaud, C., B. Hauttecoeur & J. E. Alouf. 1989. Interaction of *Clostridium perfringens* δ toxin with erythrocyte and liposome membranes and relation with the specific binding to the ganglioside G_{M2}.

Jolivet-Reynaud, C., J-M. Launay, & J. E. Alouf. 1988. Damaging effects of *Clostridium perfringens* delta toxin on blood platelets and their relevance to ganglioside G_{M2}. Arch. Biochem. Biophys. **262**:59-66.

Jonge, H. R. de. 1975. The response of small intestinal crypt and villus epithelium to cholera toxin in rat and guinea pig. Evidence against a specific role of crypt cells in choleragen-induced secretion. Biochim. Biophys. Acta **381**:128-143.

Jonge, H. R. de, & M. C. Rao. 1990. Cyclic nucleotide-dependent protein kinases: role in ion transport. pp.191-207, *in* Textbook of secretory diarrhea (Lebenthal, E., & M. E. Duffy (ed.s)). Raven Press, New York.

Jonge, H. R. de, & A. B. Vaandrager. 1987. Signal transduction in mammalian enterocytes: protein phosphorylation and polyphosphoinositide cycling in the brush border membrane. Dig. Dis. Sci. **32**:794A.

Jonson, G., A.-M. Svennerholm, & J. Holmgren. 1989. *Vibrio cholerae* expresses cell surface antigens during intestinal infection which are not expressed during *in vitro* culture. Infect. Immun. **57**:1809-1815.

Joseph, S. W., R. R. Colwell, & J. B. Kaper. 1982. *Vibrio parahaemolyticus* and related halophilic *Vibrio*s. CRC Crit. Rev. Microbiol. **10**:77-124.

Jungworth, A., M. Paulmichl, & F. Lang. 1990. Cadmium enhances potassium conductance in cultured renal epitheloid (MDCK) cells. Kid. Int. **37**:1477-1486.

Kabir, S., N. Ahmad, & S. Ali. 1984. Neuraminidase production by *Vibrio cholerae* O1 and other diarrheagenic bacteria. Infect. Immun. **44**:747-749.

Kabsch, W., & C. Sander. 1983. How good are predictions of protein secondary structure? FEBS Lett. **155**:179-182.

Kalin, M., K. Kanclerski, M. Gandstrom, & R. Mollby. 1987. Diagnosis of pneumococcal pneumonia by enzyme-linked immunosorbent assay of antibodies to pneumococcal hemolysin (pneumolysin). J. Clin. Microbiol. **25**:226-229.

Kalsow, C. M., & F. S. Newman. 1968a. Characterization of hemolysin and hemodigestive enzyme produced by strains of *Vibrio cholerae* and *Vibrio cholerae* type El Tor. Texas Rep. Biol. Med. **26**:493-506.

Kalsow, C. M., & F. S. Newman. 1968b. Comparison of hemolysin-destructive factor and hemodigestive enzyme production by strains of *Vibrio cholerae* and *Vibrio cholerae* type El Tor. Texas. Rep. Biol. Med. **26**:507-515.

Kanbayashi, Y., M. Hotta, & J. Koyama. 1972. Kinetic study on streptolysin O. J. Biochem. **71**:227-237.

Kanclerski, K., M. Gandstrom, & R. Mollby. 1987. Immunological relation between serum antibodies against pneumolysin and against streptolysin O. Acta Pathol. Microbiol. Immunol. Scand. B **95**:241-244.

Kanclerski, K., & R. Mollby. 1987. Production and purification of *Streptococcus pneumoniae* hemolysin (pneumolysin). J. Clin. Microbiol. **25**:222-225.

Kaper, J. B., R. K. Campen, R. J. Seidler, M. M. Baldini, & S. Falkow. 1984. Cloning of the thermostable-direct haemolysin of the Kanagawa phenomenon-associated haemolysin of *Vibrio parahaemolyticus*. Infect. Immun. **45**:290-2.

Kaper, J. B., R. K. Campen, R. J. Seidler, M. M. Baldini, & S. Falkow. 1985. Cloning of the Kanagawa phenomenon associated hemolysin of *Vibrio parahaemolyticus*. pp.229-235, *in* Advances in research on cholera and related diarrheas (Kuwahara, S., & N. F. Pierce (ed.s)), **2**. KTK Scientific Publishers, Tokyo.

Kaper, J. B., & M. M. Levine. 1990. Recombinant attenuated *Vibrio cholerae* O1 strains used as live oral vaccines. Res. Microbiol. **141**:901-906.

Kaper, J. B., H. Lockman, M. M. Baldini, & M. M. Levine. 1984a. A recombinant live oral cholera vaccine. Bio/Technology 2:345-349.

Kaper, J. B., H. Lockman, M. M. Baldini, & M. M. Levine. 1984b. Recombinant nontoxigenic *Vibrio cholerae* strains as attenuated live oral cholera vaccine candidates. Nature **308**:655-658.

Kaplan, M. H., H. Cheml, H. C. Hsieh, A. Stephens, & V. Brinsko. 1986. Importance of exfoliatin toxin A production by *Staphylococcus aureus* strains isolated from clustered epidemics of neonatal pustulosis. J. Clin. Microbiol. **23**:83-91.

Karin, N. J., & J. S. Cook. 1983. Regulation of Na,K-ATPase by its biosynthesis and turnover. Curr. Top. Memb. Transp. **19**:713-751.

Karunasagar, I., I. Karunasagar, G. R. Malathi, & C. N. Nagesha. 1989. Haemolysins of *Vibrio parahaemolyticus* strains passaged in mice. Indian J. Med. Res. **89**:376-380.

Kato, T., Y. Obara, H. Ichinoe, K. Nagashima, S. Akiyama, K. Takizawa, A. Matsushima, S. Yamai, & Y. Miyamoto. 1965. Grouping of *Vibrio parahaemolyticus* (biotype 1) by hemolytic reaction. Skokuhin Eisei Kenkyu **15**:83-86.

Kato, T., Y. Obara, H. Ichinoe, S. Yamai, K. Nagashima, & R. Sakazaki. 1966. Haemolytic activity and toxicity of *Vibrio parahaemolyticus*. Jap. J. Bacteriol. **21**:442-443.

Kato, M., K. Ohno, K. Takeshita, & F. Herz. 1991. Stimulation of human fetal astrocyte proliferation by bacterial lipopolysaccharides and lipid A. Acta Neuropath. **82**:384-388.

Kato, I., & M. Naiki. 1976. Ganglioside and rabbit erythrocyte membrane receptor for staphylococcal alpha-toxin. Infect. Immun. **13**:289-291.

Katsu, T., M. Kuroko, T. Morikawa, K. Sanchika, Y. Fujita, H. Yamamura, & M. Uda. 1989. Mechanism of membrane damage induced by the amphipathic peptides gramicidin S and melittin. Biochim. Biophys. Acta **983**:135-141.

Kaysner, C. A., C. Jr. Abeyta, R. F. Stott, J. L. Lilja, & M. M. Wekell. 1990. Incidence of urea-hydrolysing *Vibrio parahaemolyticus* in Willapa Bay, Washington. Appl. Env. Microbiol. **56**:904-907.

Kennedy, D. J., R. N. Greenberg, J. A. Dunn, R. Abernathy, J. J. Ryerse, & R. L. Guerrant. 1984. Effects of *Escherichia coli* heat-stable enterotoxin STb on intestine of mice, rats, rabbits, and piglets. Infect. Immun. **46**:639-645.

Kennedy, B. G., G. Lunn, & J. F. Hoffman. 1986. Effects of altering the ATP/ADP ratio on pump-mediated Na/K and Na/Na exchanges in resealed human red blood cell ghosts. J. Gen. Physiol. **87**:47-72.

Kimberg, D. V., M. Field, E. Gershon, R. T. Schooley, & A. Henderson. 1973. Effects of cycloheximide on the response of intestinal mucosa to cholera enterotoxin. J. Clin. Invest. **52**:1376-1383.

Kimmich, G. A. 1975. Preparation and characterisation of isolated intestinal epithelial cells and their use in studying intestinal transport. pp.51-115, *in* Methods in membrane biology **4** (Korth, E. (ed.)). Plenum, New York.

Kimmich, G. A. 1981. Gradient coupling in isolated intestinal cells. Fed. Proc. **40**:2474-2479.

Kirk, K., K. J. Ashworth, B. C. Elford, R. A. Pinches, & J. C. Ellory. 1991. Characteristics of $^{86}Rb^{+}$ transport in human erythrocytes infected with *Plasmodium falciparum*. Biochim. Biophys. Acta **1061**:305-308.

Kirk, K., H. A. Horner, B. C. Elford, J. C. Ellory, & C. I. Newbold. 1994. Transport of diverse substrates into malaria-infected erythrocytes via a pathway showing functional characteristics of a chloride channel. J. Biol. Chem. **269**:3339-3347.

Kishita, M., N. Matsuoka, K. Kumagai, S. Yamasaki, Y. Takeda, & M. Nishibuchi. 1992. Sequence variation in the thermostable direct hemolysin-related hemolysin (*trh*) gene of *Vibrio parahaemolyticus*. Appl. Env. Microbiol. **58**:2449-2457.

Kitamura, M., M. Iwamori, & Y. Nagai. 1980. Interaction between *Clostridium botulinum* neurotoxin and gangliosides. Biochim. Biophys. Acta **628**:328-335.

Klein, R. A. 1982. Thermodynamics and membrane processes. Q. Rev. Biophys. **15**:667-757.

Klimpel, K. R., S. S. Molloy, G. Thomas, & S. H. Leppla. 1992. Anthrax toxin protective antigen is activated by a cell surface protease with the sequence specicity and catalytic properties of furin. Proc. Nat. Acad. Sci. U. S. A. **89**:10277-10281.

Klingenberg, M. 1989. Survey of carrier methodology: strategy for identification, isolation, and characterization of transport systems. Met. Enz. **171**:12-23.

Klonk, S., & B. Deuticke. 1992. Involvement of cytoskeletal proteins in the barrier function of the human erythrocyte membrane. I. Impairment of resealing and formation of aqueous pores in the ghost membrane after modification of SH groups. Biochim. Biophys. Acta **1106**:126-136.

Klonk, S., & B. Deuticke. 1992. Involvement of cytoskeletal proteins in the barrier function of the human erythrocyte membrane. II. Formation of membrane leaks in ghost membranes after limited proteolysis of skeletal proteins by trypsin. Biochim. Biophys. Acta **1106**:137-142.

Klonk, S., & B. Deuticke. 1992. Involvement of cytoskeletal proteins in the barrier function of the human erythrocyte membrane. III. Permeability changes of spectrin-depleted inside-out membrane vesicles to hydrophilic nonelectrolytes. Formation of leaks by chemical or enzymatic modification of membrane proteins. Biochim. Biophys. Acta **1106**:143-150.

Klontz, K. C. 1990. Fatalities associated with *Vibrio parahaemolyticus* and *Vibrio cholerae* non-O1 infections in Florida (1981-1988). South Med. J. **83**:500-502.

Klontz, K. C., S. Lieb, M. Schreiber, H. T. Janowski, L. M. Baldy, & R. A. Gunn. 1988. Syndromes of *Vibrio vulnificus* infections. Clinical and epidemiologic features in Florida cases, 1981-1987. Ann. Int. Med. **109**:318-323.

Knickelbein, R., P. S. Aronson, W. Atherton, & J. W. Dobbins. 1983. Sodium and chloride transport across rabbit ileal brush border. I. Evidence for Na-H exchange. Am. J. Physiol. **245**:G504-G510.

Knickelbein, R., P. S. Aronson, & J. W. Dobbins. 1988. Membrane distribution of sodium-hydrogen and chloride-bicarbonate exchangers in crypt and villus cell membranes from rabbit ileum. J. Clin. Invest. **82**:2158-2163.

Knowles, B. H., P. J. White, C. N. Nicholls, & D. J. Ellar. 1992. A broad spectrum cytolytic toxin from *Bacillus thuringiensis* var. *kyushuensis*. Proc. R. Soc. Lond. **248**:1-7.

Knutton, S., D. Jackson, J. M. Graham, K. J. Micklem, & C. A. Pasternak. 1976. Microvilli and cell swelling. Nature **262**:52-54.

Koch, A. L. 1984. Shrinkage of growing *Escherichia coli* cells by osmotic challenge. J. Bacteriol. **159**:919-924.

Koefoed-Johnsen, U., & H. H. Ussing. 1958. The nature of the frog skin potential. Acta Physiol. Scand. **42**:298-308.

Koehler, T. M., & R. J. Collier. 1991. *Anthrax* toxin protective antigen: low-pH-induced hydrophobicity and channnel formation in liposomes. Mol. Microbiol. **5**:1501-1506.

Koski, C. L., L. E. Ramm, C. H. Hammer, M. M. Mayer, & M. L. Shin. 1983. Cytolysis of nucleated cells by complement: cell death displays multi-hit characteristics. Proc. Nat. Acad. Sci. USA **80**:3816-3820.

Kothary, M. H. 1994. Personal communication.

Kothary, M. H., & A. S. Kreger. 1985. Purification and characterization of an extracellular cytolysin produced by *Vibrio damsela*. Infect. Immun. **49**:25-31.

Kothary, M. H., & A. S. Kreger. 1987. Purification and characterization of an elastolytic protease of *Vibrio vulnificus*. J. Gen. Microbiol. **133**:1783-1791.

Krasne, S., G. Eisenman, & G. Szabo. 1971. Freezing and melting of lipid bilayers and the mode of action of nonactin, valinomycin, and gramicidin. Science **174**:412-415.

Krasilnikov, O. V., J. N. Muratkhodjaev, & A. O. Zitzer. 1992. The mode of action of *Vibrio cholerae* cytolysin. The influences on both erythrocytes and planar lipid bilayers. Biochim. Biophys. Acta **1111**:7-16.

Kreger, A. S. 1984. Cytolytic activity and virulence of *Vibrio damsela*. Infect. Immun. **44**:326-331.

Kreger, A. S. 1991. Cytolytic toxins of pathogenic marine *Vibrios*. pp.263-276, *in* Sourcebook of bacterial protein toxins (Alouf, J. E., & J. H. Freer (ed.s)). Academic Press, New York.

Kreger, A. S., A. W. Bernheimer, L. A. Etkin, & L. W. Daniel. 1987. Phospholipase D activity of *Vibrio damsela* cytolysin and its interaction with sheep erythrocytes. Infect. Immun. **55**:3209-3212.

Kreger, A. S., M. H. Kothary, & L. D. Gray. 1988. Cytolytic toxins of *Vibrio vulnificus* and *Vibrio damsela*. Met. Enz. **165**:176-189.

Kudoh, Y., A. Kai, S. Yamada, S. Matsushita, T. Itoh, K. Ohta, & M. Ohashi. 1988. Epidemiological surveys on *Escherichia coli* diarrhea in Tokyo. p.19, *in* Abstracts of the 24th joint conference on cholera and related diarrheal diseases (Takeda, Y., & R. B. Sack (ed.s)). US-Japan Cooperative Medical Science Program, Tokyo.

Kutner, S., W. V. Breuer, H. Ginsburg, S. B. Aley, & Z. I. Cabantchik. 1985. Characterization of permeation pathways in the plasma of human erythrocytes infected with the early stages of *Plasmodium falciparum*. Association with parasite development. J. Cell. Physiol. **125**:521-527.

Kutner, S., H. Ginsburg, & Z. I. Cabantchik. 1983. Permiselectivity changes in malaria (*Plasmodium falciparum*) infected human red blood cell membranes. J. Cell. Physiol. **114**:245-251.

Kwant, W. O., & P. Seeman. 1969. The membrane concentration of a local anaesthetic (chlorpromazine). Biochim. Biophys. Acta **183**:530-543.

Labarca, P., J. Lindstrom, & M. Montal. 1984. Acetylcholine receptor in planar lipid bilayers. J. Gen. Physiol. **83**:473-496.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of the bacteriophage T_4. Nature **227**:680-685.

Lagos, R., A. Avendano, V. Prado, I. Horwitz, S. Wasserman, G. Losonsky, S. Jr. Cryz, J. B. Kaper, & M. M. Levine. 1993. Attenuated live cholera vaccine strain CVD 103-HgR elicits significantly higher serum vibriocidal antibody titres in persons of blood group O. pp. 183-188, *in* Abstracts of the 29th joint conference on cholera and related diarrheal diseases. US-Japan Cooperative Medical Science Program.

Lambert, I. H., E. K. Hoffman, & P. Christensen. 1987. Role of prostaglandins and leukotrienes in volume regulation by Ehrlich ascites tumor cells. J. Memb. Biol. **98**:247-256.

Lau, K. R., R. L. Hudson, & S. G. Schultz. 1984. Cell swelling induces a barium-inhibitable potassium conductance in the basolateral membrane of *Necturus* small intestine. Proc. Nat. Acad. Sci. USA **81**:3591-3594.

Lau, K. R., R. L. Hudson, & S. G. Schultz. 1986. Effect of hypertonicity on the increase in basolateral conductance of *Necturus* small intestine in response to Na-sugar cotransport. Biochim. Biophys. Acta **855**:193-196.

Lauf, P. K., M. L. Parmelee, J. J. Snyder, & D. C. Tosteson. 1971. Enzymatic modification of the L and M antigens in LK and HK sheep erythrocytes and their membranes. J. Memb. Biol. **4**:52-67.

Lauger, P. 1979. Transport of non-interacting ions through channels. pp.17-27, *in* Membrane transport processes, **3** (Stevens, C. F. & R. W. Tsien (ed.s)). Raven Press, New York.

Launay, J. M., & J. E. Alouf. 1979. Biochemical and ultrastructural study of the disruption of blood platelets by streptolysin O. Biochim. Biophys. Acta **556**:278-291.

Laurent, T. C., & J. Killander. 1964. A theory of gel filtration and its experimental verification. J. Chromatog. **14**:317-330.

Lazarovici, P., & E. Yavin. 1985a. Tetanus toxin interaction with human erythrocytes. I. Properties of polysialoganglioside association with the cell surface. Biochim. Biophys. Acta **812**:523-531.

Lazarovici, P., & E. Yavin. 1985b. Tetanus toxin interaction with human erythrocytes. II. Kinetic properties of toxin association and evidence for a ganglioside-toxin macromolecular complex formation. Biochim. Biophys. Acta **812**:532-542.

Lear, J. D., Z. R. Wasserman, & W. F. Degrado. 1988. Synthetic amphiphilic peptide models for protein ion channels. Science **240**:1177-1181.

Ledley, F. D., G. Lee, L. D. Kohn, W. H. Habig, & M. C. Hardegree. 1977. Tetanus toxin interactions with thyroid plasma membranes. J. Biol. Chem. **252**:4049-4055.

Lee, A. G. 1983. p.43, *in* Membrane Fluidity in Biology (Aloia, R. C. (ed.)). Academic Press, New York.

Lee, A. G. 1990. Structure and function of cell membranes. pp.1-43, *in* Comprehensive medicinal chemistry. The rational design, mechanistic study and therapeutic application of chemical compounds. **3**. Membranes and receptors (Hansch, C. (ed.)). Pergamon Press, Oxford.

Lee, A. G., J. M. East, & R. J. Froud. 1986. Are essential fatty acids essential for membrane function? Prog. Lipid Res. **25**:41-46.

Lee, J. V., P. Shread, A. L. Furniss, & T. N. Bryant. 1981. Taxonomy and description of *Vibrio fluvialis* sp. nov. (synonym group F vibrios, group EF6). J. Appl. Bacteriol. **50**:73-94.

Leppla, S. H. 1991. The anthrax toxin complex. pp. 277-302, *in* Sourcebook of bacterial protein toxins (Alouf, J. E., & J. H. Freer (ed.s)). Academic Press, New York.

Le-Tien-Van, & Nguyen-Thi-Nhu-Tuan. 1974. *Vibrio parahaemolyticus* isolated among diarrhea patients. pp.15-20, *in* International symposium on *Vibrio parahaemolyticus* (Fujino, T., G. Sakaguchi, R. Sakazaki, & Y. Takeda (ed.s)). Saikon Publishing, Tokyo.

Levine, M. M., R. E. Black, M. L. Clements, L. Cisneros, A. Saah, D. R. Nalin, D. M. Gill, J. P. Craig, C. R. Young, & P. Ristaino. 1982. The pathogenicity of nonenterotoxigenic *Vibrio cholerae* serogroup O1 biotype El Tor isolated from sewage water in Brazil. J. Infect. Dis. **145**:296-299.

Levine, M. M., J. B. Kaper, D. Herrington, J. Ketley, G. Losonsky, C. O. Tacket, B. Tall, & S. Cryz. 1988a. Safety, immunogenicity, and efficacy of recombinant live oral cholera vaccines, CVD 103 and CVD 103-HgR. Lancet **2**:467-470.

Levine, M. M., J. B. Kaper, D. Herrington, G. Losonsky, J. G. Jr. Morris, M. L. Clements, R. E. Black, B. Tall, & R. Hall. 1988b. Volunteer studies of deletion mutants of *Vibrio cholerae* O1 prepared by recombinant techniques. Infect. Immun. **56**:161-167.

Levitt, D. 1990. Gramicidin, VDAC, porin and perforin channels. Curr. Op. Cell Biol. **2**:689-694.

Lewis, S. A., A. G. Butt, M. J. Bowler, J. P. Leader, & A. D. C. Macknight. 1985. Effects of anions on cellular volume and transepithelial Na transport across toad urinary bladder. J. Memb. Biol. **83**:119-137.

Lewis, S. A., D. C. Eaton, & J. M. Diamond. 1976. The mechanism of Na^+ transport by rabbit urinary bladder. J. Memb. Biol. **28**:41-70.

Lewis, S. A., & N. K. Wills. 1983. Apical membrane permeability and kinetic properties of the sodium pump in rabbit urinary bladder. J. Physiol. **341**:169-184.

Li, J. 1992. Bacterial toxins. Curr. Op. Str. Biol. **2**:545-556.

Li, J. H.-Y., L. G. Palmer, I. S. Edelman, & B. Lindemann. 1982. The role of sodium-channel density in the natriferic response of the toad urinary bladder to antidiuretic hormone. J. Memb. Biol. **64**:91-102.

Liedtke, C. M. 1989. Regulation of chloride transport in epithelia. Ann. Rev. Physiol. **51**:143-160.

Lienhard, G. E., J. W. Slot, D. E. James, & M. M. Mueckler. 1992. How cells absorb glucose. Sci. Am. **266**:86-91.

Linder, R., & A. W. Bernheimer. 1991. Comparative toxinology of bacterial and invertebrate cytotoxins. pp.101-116, *in* Sourcebook of bacterial protein toxins (Alouf, J. E., & J. H. Freer (eds.)). Academic Press, New York.

Link, E., L. Edelmann, J. H. Chou, T. Binz, S. Yamasaki, U. Eisel, M. Baumert, T. C. Sudhof, H. Niemann, & R. Jahn. 1992. Tetanus toxin action: inhibition of neurotransmitter release linked to synaptobrevin proteolysis. Biochem. Biophys. Res. Comm. **189**:1017-1023.

Liu, P. V. 1974. Extracellular toxins of *Pseudomonas aeruginosa.* J. Infect. Dis. **130**:S94-S99.

Liu, P. V. 1979. Extracellular toxins of *Pseudomonas aeruginosa.* pp.63-68, *in Pseudomonas aeruginosa*: clinical manifestations of infection and current therapy (Doggett, R. G. (ed.)). Academic Press, New York.

Ljungh, A., & T. Wadstrom. 1983. Toxins of *Vibrio parahaemolyticus* and *Aeromonas hydrophila.* J. Toxicol. Toxin Rev. **1**:257-307.

Lockwood, D. E., A. S. Kreger, & S. H. Richardson. 1982. Detection of toxins produced by *Vibrio fluvialis.* Infect. Immun. **35**:702-708.

Loewenstein, W. R. 1984. Channels in the junctions between cells. pp.221-252, *in* Ion channels: molecular and physiological aspects (Srein, W. D. (ed.)). Academic Press, Orlando.

London, E., N. D. Ulbrandt, D. Tortorella, J. X. Jiang, & F. S. Abrams. 1993. Insights into membrane protein folding and translocation from the bahaviour of bacterial toxins: models for membrane translocation. Soc. Gen. Physiol. Ser. **48**:45-61.

Lorenzen, M., C. O. Lee, & E. E. Windhager. 1984. Cytosolic Ca^{2+} and Na^+ activities in perfused proximal tubules of *Necturus* kidney. Am. J. Physiol. **247**:F93-F102.

Love, M., D. Teebken-Fisher, J. E. Hose, J. J. III Farmer, F. W. Hickman, & G. R. Fanning. 1981. *Vibrio damsela*, a marine bacterium, causes skin ulcers on the damselfish *Chromis punctipinnus.* Science **214**:1139-1140.

Low, J. C., R. C. Davies, & W. Donachie. 1992. Purification of listeriolysin O and development of an immunoassay for diagnosis of listeric infections in sheep. J. Clin. Microbiol. **30**:2705-2708.

Lowry, O. H., N. J. Rosebrough, A. L. Farr, & R. J. Randall. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. **193**:265-275.

Lucio, F. J., B. M. Hendry, & J. C. Ellory. 1991. The effects of cholesterol depletion on the sodium pump in human red cells. Exp. Physiol. **76**:437-443.

Ludens, J. H. 1978. Studies on the inhibition of Na^+ transport in toad bladder by the ionophore A-23187. J. Pharmacol. Exp. Ther. **206**:414-422.

Macey, R. I., J. S. Adorante, & F. W. Orme. 1978. Erythrocyte membrane potentials determined by hydrogen ion distribution. Biochim. Biophys. Acta **512**:284-295.

Machen, T. E., & W. L. McLennan. 1980. Na^+-dependent H^+ and Cl^- transport in vitro frog gastric mucosa. Am. J. Physiol. **238**:G403-G413.

Machleidt, H., S. Roth, & P. Seeman. 1972. The hydrophobic expansion of erythrocyte membranes by the phenol anaesthetics. Biochim. Biophys. Acta **255**:178-189.

MacRobbie, E. A. C., & H. H. Ussing. 1961. Osmotic behavior of the epithelial cells of frog skin. Acta Physiol. Scand. **53**:348-365.

Madara, J. L. 1991. Functional morphology of epithelium of the small intestine. pp.83-112, *in* Handbook of physiology. Gastrointestinal physiology. Section 6. **4** (Schultz, S. G. (ed.)). American Physiological Society, Bethesda.

Madara, J. L., & K. Dharmsathaphorn. 1985. Occluding junction structure-function relationships in a cultured epithelial monolayer. J. Cell. Biol. **101**:2124-2133.

Madden, J. M., B. A. McCardell, & D. B. Shah. 1984. Cytotoxin production by members of the genus *Vibrio*. Lancet **1**:1217-1218.

Maddy, A. H. 1976. Biochemical analysis of membranes. Chapman and Hall, London.

Maeda, N., K. Aono, M. Sekiya, T. Suda, & T. Shiga. 1977. A computerized method for the determination of the osmotic fragility curve of erythrocytes. Anal. Biochem. **83**:149-161.

Manning, P. A., M. H. Brown, & M. W. Heuzenroeder. 1984. Cloning of the structural gene (*hly*) for the haemolysin of *Vibrio cholerae* El Tor strain O17. Gene **31**:225-231.

Marai, K., & A. Kucksis. 1973. Molecular species of glycerolipids of adenosine triphosphatase and sarcotubular membranes of rabbit skeletal muscle. Can. J. Biochem. **51**:1248-1261.

Marchlewicz, B. A., & J. L. Duncan. 1981. Lysis of erythrocytes by a hemolysin produced by a Group B *Streptococcus* sp. Infect. Immun. **34**:787-794.

Marne, C., & R. Aznar. 1988. *Vibrio parahaemolyticus*-associated diarrhoea in Spain **[letter]**. European J. Clin. Microbiol. Infect. Dis. **7**:439-440.

Martonosi, A. N. 1985. The enzymes of biological membranes. 2nd edition. Plenum Press, New York.

Marusic, E., J. P. Hayslett, & H. J. Binder. 1981. Corticosteroid binding studies in cytosol of colonic mucosa of the rat. Am. J. Physiol. **240**:G417-G423.

Maruyama, Y., D. V. Gallacher, & O. H. Petersen. 1983. Voltage and Ca^{2+}-activated K^+ channel in basolateral acinar cell membranes of mammalian salivary glands. Nature **302**:827-829.

Matsudaira, P. 1993. A practical guide to protein and peptide purification for microsequencing. 2nd edition. Academic Press, San Diego.

McCartney, C., & J. P. Arbuthnott. 1978. Mode of action of membrane-damaging toxins produced by staphylococci.

pp.89-122, *in* Bacterial toxins and cell membranes (Jeljaszewicz, J., & T. Wadstrom (ed.s)). Academic Press, New York.

McEwan, G. T. A., C. D. A. Brown, B. H. Hirst, & N. L. Simmons. 1993. Characterisation of volume-activated ion transport across monolayers of human intestinal T_{84} cells. Pflug. Archiv. **423**:213-220.

McEwan, G. T. A., C. D. A. Brown, B. H. Hirst, & N. L. Simmons. 1994. Kanagawa haemolysin of *Vibrio parahaemolyticus* stimulates transepithelial Cl^- secretion in human intestinal T_{84} epithelial cell layers. J. Physiol. **475**:92P

Mekada, E., H. Senoh, R. Iwamoto, Y. Okada, & T. Uchida. 1991. Purification of diphtheria toxin receptor from Vero cells. J. Biol. Chem. **266**:20457-20462.

Mekalanos, J. J. 1992. Environmental signals controlling expression of virulence determinants in bacteria. J. Bacteriol. **174**:1-7.

Melloni, E., F. Salamino, B. Sparatore, M. Michetti, S. Pontremoli, & B. L. Horecker. 1984. Regulation of Ca^{2+}-dependent neutral proteinases from rabbit liver by an endogenous inhibitor. Arch. Biochem. Biophys. **232**:513-519.

Melnik, E., R. Latorre, J. E. Hall, & D. C. Tosteson. 1977. Phloretin-induced changes in ion transport across lipid bilayer membranes. J. Gen. Physiol. **69**:243-257.

Menestrina, G. 1986. Ion channels formed by *Staphylococcus aureus* alpha toxin: voltage-dependent inhibition by divalent and trivalent cations. J. Memb. Biol. **90**:177-190.

Menestrina, G., C. L. Bashford, & C. A. Pasternak. 1990. Pore-forming toxins: experiments with *S. aureus* toxin, *C. perfringens* θ toxin and *E. coli* haemolysin in lipid bilayers, liposomes and intact cells. Toxicon **28**:477-491.

Merrell, B. R., R. I. Walker, & S. W. Joseph. 1984. *In vitro* and *in vivo* pathologic effects of *Vibrio parahaemolyticus* on human epithelial cells. Can. J. Microbiol. **30**:381-388.

Messner, G., W. Wang, M. Paulmichl, H. Oberleithner, & F. Lang. 1985. Ouabain decreases apparent potassium conductance in proximal tubules of the amphibian kidney. Pflug. Archiv. **405**:131-137.

Michalski, J., J. E. Galen, A. Fasano, & J. B. Kaper. 1993. CVD110, an attenuated *Vibrio cholerae* O1 El Tor live oral vaccine strain. Infect. Immun. **61**:4462-4468.

Middlebrook, J. L., & R. B. Dorland. 1984. Bacterial toxins: cellular mechanisms of action. Microbiol. Rev. **48**:199-221.

Miller, J. H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, New York.

Miller-Podraza, H., R. O. Bradley, & P. H. Fishman. 1982. Biosynthesis and localisation of gangliosides in cultured cells. Biochem. **21**:3260-3265.

Misaki, H., & M. Matsumoto. 1978. Purification of lysophospholipase of *Vibrio parahaemolyticus* and its properties. J. Biochem. **83**:1395-1405.

Mitsui, K., T. Sekiya, Y. Nozawa, & J. Hase. 1979a. Alteration of human erythrocyte plasma membranes by perfringolysin O as revealed by freeze-fracture electron microscopy: studies on *Clostridium perfringens* exotoxins. V. Biochim. Biophys. Acta **554**:68-75.

Mitsui, K., T. Sekiya, S. Okamura, Y. Nozawa, & J. Hase. 1979b. Ring formation of perfringolysin O as revealed by negative stain electron microscopy. Biochim. Biophys. Acta **558**:307-313.

Mittal, C. K., & F. Murad. 1982. Guanylate cyclase: regulation of cyclic GMP metabolism. pp.225-260, *in* Handbook of experimental pharmacology (Nathanson, J. A., & J. W. Kebabian (ed.s)). Springer-Verlag, Berlin.

Miwitani, T., & Y. Takeda. 1976. *Vibrio parahaemolyticus*: a causative bacterium of food poisoning. Saikon Publishing,

Tokyo.

Miyake, M., T. Honda, & T. Miwatani. 1988. Purification and characterization of *Vibrio metschnikovii* cytolysin. Infect. Immun. **56**:954-960.

Miyake, M., T. Honda, & T. Miwatani. 1989. Effects of divalent cations and saccharides on *Vibrio metschnikovii* cytolysin-induced hemolysis of rabbit erythrocytes. Infect. Immun. **57**:158-163.

Miyamoto, Y., T. Kato, Y. Obara, S. Akiyama, K. Takizawa, & S. Yamai. 1969. *In vitro* hemolytic characteristics of *Vibrio parahaemolyticus*: its close correlation with human pathogenicity. J. Bacteriol. **100**:1147-1149.

Miyamoto, Y., Y. Obara, T. Nikkawa, S. Yamai, T. Kato, Y. Yamada, & M. Ohashi. 1980. Simplified purification and biophysicochemical characteristics of Kanagawa phenomenon-associated haemolysin of *Vibrio parahaemolyticus*. Infect. Immun. **28**:567-576.

Miyoshi, S., H. Yamanaka, N. Miyoshi, & S. Shinoda. 1985. Non-thiol-activated property of a cholesterol-binding hemolysin produced by *Vibrio vulnificus*. FEMS Microbiol. Lett. **30**:213-216.

Mizoguchi, T. 1963. Clinical features of food poisoning due to *Vibrio parahaemolyticus*. pp.367-395, *in Vibrio parahaemolyticus* (Fujino, T., & H. Fukumi (ed.s)). Isseido, Tokyo.

MMWR. [Morbidity and Mortality Weekly Report]. 1969a. An outbreak of acute gastroenteritis during a tour of the orient - Alaska. MMWR **18**:150.

MMWR. [Morbidity and Mortality Weekly Report]. 1969b. Follow-up of an outbreak of gastroenteritis during a tour of the orient - Alaska. MMWR. **18**:168.

MMWR. [Morbidity and Mortality Weekly Report]. 1969c. Acute gastroenteritis among tour groups to the Orient - United States. MMWR **18**:301-303.

Montecucco, C., & G. Schiavo. 1993. Tetanus and botulism neurotoxins: a new group of zinc proteases. Trends Biochem. Sci. **18**:324-327.

Moos, M. Jr., N. Y. Nguyen & T. Y. Liu. 1988. Reproducible high yield sequencing of proteins electrophoretically separated and transferred to an inert support. J. Biol. Chem. **263**:6005-6008.

Moritz, M., & A. H. Womelsdorf. 1973. Rabbit cholera: inhibitory effect of tenuazonic acid on cholera-induced secretion of water and electrolytes. Gastroenterol. **65**:259-264.

Morris, J. G. Jr., & R. E. Black. 1985. Cholera and other vibrioses in the United States. N. Engl. J. Med. **312**:343-350.

Morris, J. G. Jr., H. G. Miller, R. Wilson, C. O. Tacket, D. G. Hollis, F. W. Hickman, R. E. Weaver, & P. E. Blake. 1982. Illness caused by *Vibrio damsela* and *Vibrio hollisae*. Lancet **1**:1294-1296.

Morris, J. G. Jr., R. Wilson, B. R. Davies, I. K. Wachsmuth, C. F. Riddle, H. G. Wathen, R. A. Pollard, & P. A. Blake. 1981. Non-O1 group *Vibrio cholerae* gastroenteritis in the United States: clinical, epidemiologic, and laboratory aspects of sporadic cases. Ann. Intern. Med. **94**:656-658.

Moss, D. E., & D. Fahrney. 1978. Kinetic analysis of differences in brain acetylcholinesterase from fish or mammalian sources. Biochem. Pharmacol. **27**:2693-2698.

Moss, J., & M. Vaughan. 1986. Amino acid specific ADP-ribosylation: substrate specificty of an ADP-ribosylarginine hydrolase from turkey erythrocytes. Biochem. **25**:5408-5414.

Moss, J., & M. Vaughan. 1988a. ADP-ribosylation of guanyl nucleotide-binding regulatory proteins by bacterial protein toxins. Adv. Enzymol. **61**:303-379.

Moss, J., & M. Vaughan. 1988b. Cholera toxin and *E. coli* enterotoxins and their mechanisms of action. pp.39-87, *in* Handbook of natural toxins. **4**: bacterial toxins (Hardegree, M. C., & A. T. Tu (ed.s)).

Moss, J., & M. Vaughan. 1989. Guanine-nucleotide-binding proteins (G-proteins) in activation of adenyl cyclase: lessons learned from cholera and "travellers diarrhea." J. Lab. Clin. Med. **113**:258-268.

Muller-Eberhard, H. J. 1985. Transmembrane channel-formation by five complement proteins. Biochem. Soc. Symp. **50**:235-246.

Munch-Petersen, E., & R. Christie. 1947. On the effect of the interaction of staphylococcal β-toxin and group B streptococcal substance on red blood corpuscles and its use as a test for the identification of *Streptococcus agalactiae*. J. Pathol. Bacteriol. **59**:367-371.

Murer, H., U. Hopfer, & R. Kinne. 1976. Sodium/proton antiport in brush border membrane vesicles isolated from rat small intestine and kidney. Biochem. J. **154**:597-604.

Musch, M. W., J. F. Kachur, R. J. Miller, M. Field, & J. S. Stoff. 1983. Bradykinin stimulated electrolyte secretion in rabbit and guinea pig intestine: involvement of arachidonic acid metabolites. J. Clin. Invest. **71**:1073-1083.

Mynott, T. L., D. S. Chandler, & R. K. J. Luke. 1991. Efficacy of enteric-coated protease in preventing attachment of enterotoxigenic *Escherichia coli* and diarrheal disease in the RITARD model. Infect. Immun. **59**:3708-3714.

Mynott, T. L., S. Guandalini, & A. Fasano. 1993. Protease treatment of intestinal mucosa inhibits toxin-induced secretion *in vitro*. p.237, *in* Abstracts of the 29th joint conference on cholera and related diarrheal diseases. US-Japan Cooperative Medical Science Program.

Nagayama, K., M. Arita, & T. Honda. 1993. Cell-associated hemagglutinin of *Vibrio parahaemolyticus* and its role for intestinal adherence. pp.169-171 *in* Abstracts of 29th joint conference on cholera and related diarrheal diseases. US-Japan Cooperative Medical Science Program.

Nair, G. B., S. K. Bhattachrya, T. Ramamurthy, A. K. Mukhopadhyay, S. Garg, B. C. Deb, M. J. Albert, R. B. Sack, M. Chongsanguan, W. Chaicumpa, P. Moolasart, P. Kandhasingha, T. Shimada, T. Takeda, T. Yamamoto, T. Ohashi, J. Tada, T. Nakayama, S. Fukushima, H. Kurazono, J. Okuda, A. Pal, T. Karasawa, Y. Uesaka, H. Shirai, & Y. Takeda. 1993. Origin, spread, and characteristics of *Vibrio cholerae* O139 Bengal. pp.9-11, *in* Abstracts of the 29th joint conference on cholera and related diarrheal diseases. US-Japan Cooperative Medical Science Program.

Nair, G. B., & S. C. Pal. 1989. Enterotoxin induced diarrhoea - an update. Trop. Gastroenterol. **10**:133-141.

Nakasone, N., & M. Iwanaga. 1990. Pili of *Vibrio parahaemolyticus* strain as a possible colonization factor. Infect. Immun. **58**:61-69.

Nakasone, N., & M. Iwanaga. 1991. Purification and characterisation of pili isolated from *Vibrio parahaemolyticus* Na2. Infect. Immun. **59**:726-728.

Nakasone, N., & M. Iwanaga. 1992. The role of pili in colonization of the rabbit intestine by *Vibrio parahaemolyticus* Na2. Microbiol. Immunol. **36**:123-130.

Nath, K., J. W. Shay, & A. P. Bollon. 1978. Relationship between dibutyryl cyclic AMP and microtubule organization in contracting heart muscle cells. Proc. Nat. Acad. Sci. USA **75**:319-323.

Nellans, H. N., & J. E. Popovitch. 1981. Calmodulin-regulated, ATP-driven calcium transport by basolateral membranes of rat small intestine. J. Biol. Chem. **256**:9932-9936.

Neutra, M., & D. Louvard. 1989. Differentiation of intestinal cells in vitro. pp.363-398, *in* Functional epithelial cells in culture (Matlin, K. S., & J. D. Valentich (ed.s)). Liss, New York.

Nicholls, P. J., & R. J. Youle. 1992. The structure of *Pseudomonas* exotoxin A as a guide to rational design. Target. Diagn. Ther. 7:439-446.

Nicotera, P., G. Bellomo, & S. Orrenius. 1992. Calcium-mediated mechanisms in chemically induced cell death. Ann. Rev. Pharmacol. Toxicol. **32**:449-470.

Niedermeyer, W. 1985. Interaction of streptolysin-O with biomembranes: kinetic and morphological studies on erythrocyte membranes. *Toxicon* **23**:425-439.

Nielson, R. 1979. A 3 to 2 coupling of the Na-K pump responsible for the transepithelial Na transport in frog skin disclosed by the effect of Ba. Acta Physiol. Scand. **107**:189-191.

Nikkawa, T., Y. Obara, S. Yamai, & Y. Miyamoto. 1972. Purification of a hemolysin from *Vibrio parahaemolyticus*. Jap. J. Med. Sci. Biol. **25**:197-200.

Nishibuchi, M., A. Fasano, R. G. Russell, & J. B. Kaper. 1992. Enterotoxigenicity of *Vibrio parahaemolyticus* with and without genes encoding thermostable direct haemolysin. Infect. Immun. **60**:3539-3545.

Nishibuchi, M., M. Ishibashi, Y. Takeda, & J. B. Kaper. 1985. Detection of the thermostable direct haemolysin gene and related DNA sequences in *Vibrio parahaemolyticus* and other *Vibrio* species by the DNA colony hybridisation technique. Infect. Immun. **49**:481-486.

Nishibuchi, M., & J. B. Kaper. 1985. Nucleotide sequence of the thermostable direct haemolysin gene of *Vibrio parahaemolyticus*. J. Bacteriol. **162**:558-564.

Nishibuchi, M., & J. B. Kaper. 1990. Duplication and variation of the thermostable direct haemolysin (tdh) gene in *Vibrio parahaemolyticus*. Molec. Microbiol. **4**:87-99.

Nishibuchi, M., V. Khaeomanee-Iam, T. Honda, J. B. Kaper, & T. Miwatani. 1990. Comparative analysis of the hemolysin genes of *Vibrio cholerae* non-O1, *V. mimicus*, and *V. hollisae* that are similar to the *tdh* gene of *V. parahaemolyticus*. FEMS Microbiol. Lett. **67**:251-256.

Nishibuchi, M., & R. J. Seidler. 1983. Medium-dependent production of extracellular enterotoxins by non-O1 *Vibrio cholerae*, *Vibrio mimicus*, and *Vibrio fluvialis*. Appl. Env. Microbiol. **45**:228-231.

Nishibuchi, M., R. J. Seidler, D. M. Rollins, & S. W. Joseph. 1983. Vibrio factors cause rapid fluid accumulation in suckling mice. Infect. Immun. **40**:1083-1091.

Nishibuchi, M., T. Taniguchi, T. Misawa, V. Khaeomanee-Iam, T. Honda, & T. Miwatani. 1989. Cloning and nucleotide sequence of the gene (*trh*) encoding the haemolysin related to the thermostable direct haemolysin of *Vibrio parahaemolyticus*. Infect. Immun. **57**:2691-2697.

Oakley, B. R., D. R. Kirsch, & N. R. Morris. 1980. A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. Anal. Biochem. **105**:361-363.

Obara, Y. 1971. Studies on hemolytic factors of *Vibrio parahaemolyticus*. II. The extraction of hemolysin and its properties. J. Jap. Ass. Infect. Dis. **45**:392-398.

Obara, Y., S. Yamai, T. Nikkawa, Y. Miyamoto, M. Ohashi, & T. Shimada. 1974. Histopathological changes in the small intestine of suckling mice challenged orally with purified hemolysin from *Vibrio parahaemolyticus*. pp.253-257, *in* International symposium on *Vibrio parahaemolyticus* (Fujino, T., G. Sakaguchi, R. Sakazaki & Y. Takeda (ed.s)). Saikon Publishing, Tokyo.

Oberleithner, H., M. Meight, H.-J. Westphal, & W. Wang. 1987. Aldosterone activates Na^+/H^+ exchange and raises cytoplasmic pH in target cells of the amphibian kidney. Proc. Nat. Acad. Sci. USA **84**:1464-1468.

Oberley, T. D., & J. L. Duncan. 1971. Characteristics of streptolysin O action. Infect. Immun. **4**:683-687.

O'Brien, A. D., M. E. Chen, R. K. Holmes, J. B. Kaper, & M. M. Levine. 1984. Environmental and human isolates of *Vibrio cholerae* and *Vibrio parahaemolyticus* produce a *Shigella dysenteriae* 1 (Shiga-like) cytotoxin. Lancet **1**:77-78.

Ogata, M., V. K. Chaudhary, I. Pastan, & D. J. Fitzgerald. 1990. Processing of *Pseudomonas* exotoxin by a cellular protease results in the generation of a 37000-Da toxin fragment that is translocated to the cytosol. J. Biol. Chem. **265**:20678-20685.

Ohno-Iwashita, Y., M. Iwamoto, K. Mitsui, S. Ando, & K. Nagai. 1988. Protease-nicked θ-toxin of *Clostridium perfringens*, a new membrane probe with no catalytic effect reveals two classes of cholesterol as toxin-binding sites on sheep erythrocytes. Eur. J. Biochem. **176**:95-101.

Ohno-Iwashita, Y., M. Iwamoto, K. Mitsui, H. Kawasaki, & S. Ando. 1986. Cold-labile hemolysin produced by limited proteolysis of θ-toxin from *Clostridium perfringens*. Biochem. **25**:6048-6053.

Ojcius, D. M., & J. D.-E. Young. 1991. Cytolytic pore-forming proteins and peptides: is there a common structural motif? Trends Biochem. Sci. **16**:225-229.

Op den Kamp, J. A. F. 1979. Lipid asymmetry in membranes. Ann. Rev. Biochem. **48**:47-71.

Ornstein, L. 1964. Disc electrophoresis-I: background and theory. Ann. N. Y. Acad. Sci. **121**:321-349.

Ostroff, R. M., B. Wretlind, & M. L. Vasil. 1989. Mutations in the hemolytic-phospholipase C operon result in decreased virulence of *Pseudomonas aeruginosa* PAO1 grown under phosphate-limiting conditions. Infect. Immun. **57**:1369-1373.

Ota, K. 1990. *Vibrio* induced food-poisoning. Food Chem. **6**:52-58.

Overath, P., & L. Thilo. 1978. Membrane structure. pp.1-44, *in* Biochemistry of cell walls and membranes II (Metcalfe, J. C. (ed.)). University Park Press, MD.

Owen, J. D., M. Steggall, & E. M. Eyring. 1974. The effect of phloretin on red cell nonelectrolyte permeability. J. Memb. Biol. **19**:79-92.

Palmer, L. G. 1985. Modulation of apical Na permeability of the toad urinary bladder by intracellular Na, Ca and H. J. Memb. Biol. **83**:57-69.

Palmer, L. G., & G. Frindt. 1987. Effects of cell Ca and pH on Na channels from rat cortical collecting tubule. Am. J. Physiol. **253**:F333-F339.

Parker, M. W., F. Pattus, A. D. Tucker, & D. Tsernoglou. 1989. Structure of the membrane-pore-forming fragment of colicin-A. Nature **337**:93-96.

Pasternak, C. A. 1986. Effect of pore-formers on intracellular calcium. Cell Calcium **7**:387-397.

Pasternak, C. A. 1991. Effect of divalent cations on membranes: reversible and irreversible closure of channels induced by membrane-inserting proteins and other agents. Roman. J. Biophys. **1**:3-11.

Pasternak, C. A., G. M. Alder, C. L. Bashford, Y. E. Korchev, & C. Pederzolli. 1992. Membrane damage: common mechanisms of induction and prevention. FEMS Microbiol. Immunol. **5**:83-92.

Paton, J. C., P. W. Andrew, G. J. Boulnois, & T. J. Mitchell. 1993. Molecular analysis of the pathogenicity of *Streptococcus pneumoniae*: the role of pneumococcal proteins. Ann. Rev. Microbiol. **47**:89-115.

Pattus, F., D. Massote, H. U. Wilmsen, J. Lakey, A. Tsernoglou, & M. W. Parker. 1990. Colicins: procaryotic killer pores. Experientia **46**:180-192.

Petersen, O. H., & Y. Maruyama. 1984. Calcium-activated potassium channels and their role in secretion. Nature **307**:693-696.

Pezard, C., P. Berche, & M. Mock. 1991. Contribution of individual toxin components to virulence of *Bacillus anthracis*. Infect. Immun. **59**:3472-3477.

Pharmacia. 1991. PhastSystem. Development technique file number 210. Sensitive silver staining. Pharmacia, Uppsala, Sweden.

Pierce. 1993. BCA. Protein assay reagent. Instructions. Pierce, Rockford, IL.

Pierce, N. F., & A. Mondal. 1974. Clinical features of cholera. pp.209-220, *in* Cholera (Barua, D., & W. Burrows (ed.s)). W. B. Saunders, Philadelphia.

Plotkin, B. J., S. G. Kilgore, & L. MacFarland. 1990. Polyvibrio infections: *Vibrio parahaemolyticus* and *Vibrio vulnificus* dual wound and multiple site infections **[letter]**. J. Infect. Dis. **161**:364-365.

Pollack, L. R., E. H. Tate, & J. S. Cook. 1981. Na^+/K^+ ATPase in HeLa cells after prolonged growth in low K^+ or ouabain. J. Cell. Physiol. **106**:85-97.

Ponder, E. 1948. Hemolysis and related phenomena. Grune & Stratton, New York.

Porath, J. 1963. Some recently developed fractionation procedures and their application to peptide and protein hormones. J. Appl. Chem. **6**:233-244.

Porath, J., & P. Flodin. 1959. Gel filtration: a method for desalting and group separation. Nature **183**:1657-1659.

Powell, D. W. 1987. Intestinal water and electrolyte transport. pp.1267-1305, *in* Physiology of the gastrointestinal tract (L. R. Johnson (ed.)). Raven Press, New York.

Poznansky, M., S. Tong, P. C. White, J. M. Milgram, & A. K. Solomon. 1976. Nonelectrolyte diffusion across lipid bilayer systems. J. Gen. Physiol. **67**:45-66.

Prosser, C. L., & J. E. Heath. 1991. Temperature. pp. 109-165, **in** Environmental and metabolic animal physiology. Comparative animal physiology (Prosser, C. L. (ed.)). 4th edition. Wiley-Liss, New York.

Quinn, P. J. 1976. The molecular biology of cell membranes. Macmillan, Oxford.

Racker, E. 1985. Reconstitutions of transporters, receptors, and pathological states. Academic Press, New York.

Racusen, L., & H. J. Binder. 1980. Effect of prostaglandin on ion transport across isolated colonic mucosa. Dig. Dis. Sci. **25**:900-904.

Raftery, M. A., S. M. J. Dunn, B. M. Conti-Tronconi, D. S. Middlemas, & R. D. Crawford. 1983. The nicotinic acetylcholine receptor: subunit structure, functional binding sites and ion transport properties. Cold Spring Harbor Symp. Quant. Biol. **48**:21-33.

Ramamoorthy, S., D. R. Cool, V. B. Mahesh, F. H. Leibach, H. E. Melikian, R. D. Blakely, & V. Ganapathy. 1993. Regulation of the human serotonin transporter. Cholera toxin-induced stimulation of serotonin uptake in human placental choriocarcinoma cells is accompanied by increased serotonin transporter mRNA levels and serotonin transporter-specific ligand binding. J. Biol. Chem. **268**:21626-21631.

Ramamurthy, T., S. Garg, R. Sharma, S. K. Bhattacharya, G. B. Nair, T. Shimada, T. Takeda, T. Karasawa, H. Kurazono, A. Pal, & Y. Takeda. 1993. Emergence of a novel strain of *Vibrio cholerae* with epidemic potential in southern and eastern India. Lancet **341**:703-704.

Rand, R. P. 1964. Mechanical properties of the red cell membrane. II. Viscoelastic properties of the membrane. Biophys. J. **4**:303-316.

Rand, R. P., & A. C. Burton. 1963. Area and volume changes in hemolysis of single erythrocytes. J. Cell. Comp. Physiol. **61**:245-253.

Rao, M. C., & M. Field. 1983. Role of calcium and cyclic nucleotides in the regulation of intestinal ion transport. pp.227-239, *in* Intestinal transport: fundamental and comparative aspects (Gilles-Baillien, M., & R. Gilles (ed.s)). Springer-Verlag, Berlin.

Rao, M. C., & H. R. de Jonge. 1990. Ca and phospholipid-dependent protein kinases: role in ion transport. pp.209-232, *in* Textbook of secretory diarrhea (Lebenthal, E., & M. E. Duffy (ed.s)). Raven Press, New York.

Rappuoli, R., & M. Pizza. 1991. Structure and evolutionary aspects of ADP-ribosylating toxins. pp.1-22, *in* Sourcebook of bacterial protein toxins (Alouf, J. E., & J. H. Freer (ed.s)). Academic Press, London.

Read, R. J., & P. E. Stein. 1993. Toxins. Curr. Op. Str. Biol. **3**:853-860.

Rees, D. C., L. DeAntonio, & D. Eisenberg. 1989. Hydrophobic organisation of membrane proteins. Science **245**:510-513.

Reithmeier, R. A. F. 1985. p.503, *in* Biochemistry of lipids and membranes (Vance, D. E., & J. E. Vance (ed.s)). Benjamin/Cummings, California.

Rich, D. H., M. S. Bernatowicz, N. S. Agarwal, M. Kawai, & F. G. Salituro. 1985. Inhibition of aspartic proteases by pepstatin and 3-methylstatine derivatives of pepstatin. Evidence for collected-substrate inhibition. Biochem. **24**:3165-3173.

Rick, R., G. Spancken, & D. Dorge. 1988. Effects of aldosterone and ADH on intracellular electrolytes in the toad urinary bladder epithelium. J. Memb. Biol. **101**:275-282.

Robertis, E. D. P. de, & E. M. F. Jr. de Robertis. 1987. Cell membrane and permeability. pp.103-129, *in* Cell and molecular biology. 8th edition. Lea & Febiger, Philadelphia.

Roberts, W. G., R. N. Fedorak, & E. B. Chang. 1988. In vitro effects of the long-acting somatostatin analog SMS 201-995 on electrolyte transport by the rabbit ileum. Gastroenterol. **94**:1343-1350.

Robertson, D. L., M. T. Tippetts, & S. H. Leppla. 1988. Nucleotide sequence of the *Bacillus anthracis* edema factor gene (*cya*): a calmodulin-dependent adenylate cyclase. Gene **73**:363-371.

Roggin, G. M., J. G. Banwell, J. G. Yardley, & T. H. Hendrix. 1972. Unimpaired response of rabbit jejunum to cholera after selective damage to villus epithelium. Gastroenterol. **63**:981-989.

Rodgers, W., & M. Glaser. 1993. Distributions of proteins and lipids in the erythrocyte membrane. Biochem. **32**:12591-12598.

Roos, A., & W. Boron. 1981. Intracellular pH. Physiol. Rev. **61**:296-434.

Rothman, J. E., & J. Lenard. 1977. Membrane asymmetry. Science **195**:743-753.

Rottem, S., K. Groover, W. H. Habig, M. F. Barile, & M. C. Hardegree. 1990. Transmembrane diffusion channels in *Mycoplasma galliseptum* induced by tetanolysin. Infect. Immun. **58**:598-602.

Roy, C., S. Mukerjee, & S. J. W. Tanamal. 1963. Haemolytic and non-haemolytic El Tor vibrios. Ann. Biochem. Exp. Med. **23**:553-558.

Sa, G., & P. L. Fox. 1994. Basic fibroblast growth factor-stimulated endothelial cell movement is mediated by a *pertussis* toxin-sensitive pathway regulating phospholipase A2 activity. J. Biol. Chem. **269**:3219-3225.

Sachs, F. 1986a. Mechanical transduction: unification? News Physiol. Sci. **1**:98-100.

Sachs, F. 1986b. Mechanotransducing ion channels. pp.181-193, *in* Ionic channels in cells and model systems (Latorre, R. (ed.)). Plenum, New York.

Sachs, G., & G. Muallem. 1989. Site and mechanisms of Ca^{2+} movement in non-excitable cells. Cell Calcium **10**:265-273.

Sacks, G., & S. Fleischer. 1989. Transport machinery: an overview. Met. Enz. **171**:3-12.

Sahyoun, N., T. Shatila, H. III LeVine, & P. Cuatrecasas. 1981. Cytoskeletal association of the cholera toxin receptor in rat erythrocytes. Biochem. Biophys. Res. Comm. **102**:1216-1222.

Sakazaki, R. 1968. Proposal of *Vibrio alginolyticus* for the biotype 2 of *Vibrio parahaemolyticus*. Jap. J. Med. Sci. Biol. **21**:359.

Sakazaki, R., & A. Balows. 1981. The genera *Vibrio*, *Pleisiomonas* and *Aeromonas*. pp.1272-1301, *in* The prokaryotes (Starr, M. D., H. Stolp & H. G. Schlegel (ed.s)). Springer-Verlag, Berlin.

Sakazaki, R., S. Iwanami, & H. Fukumi. 1963. Studies on the enteropathogenic facultatively halophilic bacteria, *Vibrio parahaemolyticus*. I. Morphological, cultural and biochemical properties and its taxonomical position. Jap. J. Med. Sci. Biol. **16**:161-188.

Sakazaki, R., K. Tamura, T. Kato, Y. Obara, S. Yamai, & K. Hobo. 1968. Studies on the enteropathogenic, facultatively halophilic bacteria, *V. parahaemolyticus*. III. Enteropathogenicity. Jpn. J. Med. Sci. Biol. **21**:325-331.

Sakazaki, R., K. Tamura, A. Nakamura, T. Kurata, A. Ghoda, & Y. Kazuno. 1974a. Enteropathogenic activity of *Vibrio parahaemolyticus*. pp.231-235, *in* International symposium on *Vibrio parahaemolyticus* (Fujino, T., G. Sakaguchi, R. Sakazaki & Y. Takeda (ed.s)). Saikon Publishing, Tokyo.

Sakazaki, R., K. Tamura, A. Nakamura, T. Kurata, A. Ghoda, & Y. Kazuno. 1974b. Studies on the enteropathogenic activity of *Vibrio parahaemolyticus* using ligated gut loop model in rabbits. Jap. J. Med. Sci. Biol. **27**:35-43.

Sakurai, J., M. A. Bahavar, Y. Jinguji, & T. Miwatani. 1975. Interaction of thermostable direct haemolysin of *Vibrio parahaemolyticus* with human erythrocytes. Biken J. **18**:187-192.

Sakurai, J., T. Honda, Y. Jinguji, M. Arita, & T. Miwatani. 1976. Cytotoxic effect of the thermostable direct haemolysin produced by *Vibrio parahaemolyticus* on FL cells. Infect. Immun. **13**:876-883.

Sakurai, J., A. Matsuzaki, & T. Miwatani. 1973. Purification and characterisation of thermostable direct hemolysin of *Vibrio parahaemolyticus*. Infect. Immun. **8**:775-780.

Sanyal, S. C. 1986. Live oral cholera vaccines: a new toxin produced by candidate strains. pp.304-319, *in* Natural toxins: animal, plant, and microbial (Harris, J. B. (ed.)). Clarendon Press, Oxford.

Sanyal, S. C., P. K. B. Neogi, K. Alam, M. I. Huq, & K. A. Al-Mahmud. 1984. A new enterotoxin produced by *Vibrio cholerae* O1. J. Diarrh. Dis. Res. **2**:3-12.

Sanyal, S. C., & P. C. Sen. 1974. Human volunteer study on the pathogenicity of *Vibrio parahaemolyticus*. pp.227-230, *in* International symposium on *Vibrio parahaemolyticus* (Fujino, T., G. Sakaguchi, R. Sakazaki & Y. Takeda (ed.s.)). Saikon Publishing, Tokyo.

Sanyal, S. C., J. Sil, & R. Sakazaki. 1973. Laboratory infection by *Vibrio parahaemolyticus*. J. Med. Microbiol. **6**:121-122.

Sarkar, B. L., R. Kumar, S. P. De, & S. C. Pal. 1987. Hemolytic activity of and lethal toxin production by environmental strains of *Vibrio parahaemolyticus*. Appl. Env. Microbiol. **53**:2696-2698.

Sathyamoorthy, V. 1994. Personal communication.

Schantz, E. J., & E. A. Johnson. 1992. Properties and use of botulinum toxin and other microbial neurotoxins in medicine. Microbiol. Rev. **56**:80-99.

Schaufuss, P., R. Sting, W. Schaeg, & H. Blobel. 1989. Isolation and characterization of hyaluronidase from *Streptococcus uberis*. Int. J. Med. Microbiol. **271**:46-53.

Schengrund, C. L., B. R. DasGupta, & N. J. Ringler. 1991. Binding of botulium and tetanus toxins to ganglioside G_{T1b} and derivatives thereof. J. Neurochem. **57**:1024-1032.

Schultz, S. G. 1981. Homocellular regulatory mechanisms in sodium-transporting epithelia: avoidance of extinction by 'flush-through'. Am. J. Physiol. **241**:F579-F590.

Schultz, S. G. 1986. Cellular models of epithelial ion transport. pp. 519-534, *in* Physiology of membrane disorders (Andreoli, T. F., J. F. Hoffmann, D. D. Fanestil, & S. G. Schultz (ed.s)). 2nd edition. Plenum, New York.

Schultz, S. G. 1991. Intestinal absorption and secretion. Handbook of Physiology. Section 6. **4**. The gastrointestinal system. American Physiological Society, Bethesda.

Schultz, S. G., R. E. Fuisz, & P. F. Curran. 1966. Amino acid and sugar transport in rabbit ileum. J. Gen. Physiol. **49**:849-866.

Schultz, S. G., & R. L. Hudson. 1991. Biology of sodium-absorbing epithelial cells: dawning of a new era. pp. 45-81 *in* Handbook of Physiology. Gastrointestinal physiology. Section 6. (Schultz, S. G. (ed.)). American physiological society, Bethesda.

Schultz, S. G., & R. Zalusky. 1964. Ion transport in isolated rabbit ileum. I. Short-circuit current and Na^+ fluxes. J. Gen. Physiol. **47**:567-584.

Schulz, S., C. K. Green, P. S. T. Yuen, & D. L. Garbers. 1990. Guanylyl cyclase is a heat-stable enterotoxin receptor. Cell **63**:941-948.

Schwarz, W., & H. Passow. 1983. Ca^{2+}-activated K^+ channels in erythrocytes and excitable cells. Ann. Rev. Physiol. **45**:359-374.

Seelig, J., & N. Waespe-Sarcevic. 1978. Molecular order in cis and trans unsaturated phospholipid bilayers. Biochem. **17**:3310-3315.

Sellin, J. H., & M. Field. 1981. Physiologic and pharmacologic effects of glucocorticoids on ion transport across rabbit ileal mucosa in vitro. J. Clin. Invest. **67**:770-779.

Sellin, J. H., H. Oyarzabai, E. J. Cragoe, & G. D. Potter. 1989. Phenamil inhibits electrogenic sodium absorption in rabbit ileum. Gastroenterol. **96**:997-1003.

Semrad, C., & E. B. Chang. 1987. Calcium-mediated cyclic AMP inhibition of Na/H exchange in the small intestine. Am. J. Physiol. **21**:C315-C322.

Semrad, C., & E. B. Chang. 1990. Inhibition of Na/H exchange in avian intestine by atrial natriuretic factor. J. Clin. Invest. **86**:585-591.

Semrad, C. E., E. J. Cragoe, & E. B. Chang. 1990. Inhibition of Na^+/H^+ exchange in avian intestine by atrial natriuretic factor. J. Clin. Invest. **86**:585-591.

Sepulveda, F. V. 1991. Cell culture in the study of epithelial development and function. pp.131-141, *in* Cell membrane transport (Yudilevich, D. L., R. Deves, S. Peran, & Z. I. Cabantchik (ed.s)). Plenum, New York.

Seyama, I., H. Irisawa, T. Honda, Y. Takeda, & T. Miwatani. 1977. Effect of hemolysin produced by *Vibrio parahaemolyticus* on membrane conductance and mechanical tension of rabbit myocardium. Jap. J. Physiol. **27**:43-56.

Shandera, W. X., J. M. Johnston, B. R. Davis, & P. A. Blake. 1983. Disease from infection with *Vibrio mimicus*, a newly recognised *Vibrio* species. Ann. Intern. Med. **99**:169-171.

Shapiro, A. L., D. Vinuela, & J. V. Jr. Maizel. 1967. Molecular weight estimation of polypeptide chains by electrophoresis in SDS-polyacrylamide gels. Biochim. Biophys. Res. Commun. **28**:815-820.

Shaw, R., R. D. Hufstrader, & P. Reed. 1976. Personal communication *in* Joseph *et al.*, 1982.

Shigematsu, Y., J. Vaughn, E. D. Frohlich, & F. E. Cole. 1994. Adenosine 5'-triphosphate, phorbol ester, and pertussis toxin effects on atrial natriuretic peptide stimulation of guanylate cyclase in a human renal cell line. Life Sci. **54**:213-221.

Shinoda, S., S. Miyoshi, H. Yamanaka, & N. Miyoshi-Nakahara. 1985. Some properties of *Vibrio vulnificus* hemolysin. Microbiol. Immunol. **29**:583-590.

Shoshan, M. C., C. Fiorentini, & M. Thelestam. 1993. Signal transduction pathways and cellular intoxication with *Clostridium difficile* toxins. J. Cell. Biochem. **52**:107-115.

Sidel, V. W., & A. K. Solomon. 1957. Entrance of water into human red cells under an osmotic pressure gradient. J. Gen. Physiol. **41**:243-257.

Siebens, A. W. 1985. Cellular volume control. pp.91-115, *in* The kidney, physiology and pathophysiology (Seldin, D. W., & G. Giebisch (ed.s)). Raven Press, New York.

Sigma Chemical Co. 1992. p. 513, *in* Sigma manual. Sigma Chemical Co., St. Louis, Mo.

Simon, M. I., M. P. Strathman, & N. Gautam. 1991. Diversity of G proteins in signal transduction. Science **252**:802-808.

Simons, T. J. B. 1976. Calcium-dependent potassium exchange in human red cell ghosts. J. Physiol. **256**:209-225.

Simons, T. J. B. 1982. A method for estimating free Ca within human red blood cells, with an application to the study of their Ca-dependent K permeability. J. Memb. Biol. **66**:235-247.

Simmons, N. L. 1993. Renal epithelial Cl^- secretion. Exp. Physiol. **78**:117-137.

Simpson, L. L., & M. M. Rapport. 1971. The binding of botulinum toxin to membrane lipids: sphingolipids, steroids and fatty acids. J. Neurochem. **18**:1751-1759.

Singer, S. J. 1974. The molecular organization of membranes. Ann. Rev. Biochem. **43**:805-833.

Singer, S. J. 1992. The structure and function of membranes a personal memoir. J. Memb. Biol. **129**:3-12.

Singer, S. J., & G. L. Nicolson. 1972. The fluid mosaic model of the structure of cell membranes. Science **175**:720-731.

Skerman, V. B. D. 1949. A mechanical key for the generic identification of bacteria. Bacteriol. Rev. **13**:175-188.

Skou, J. C., & J. G. Norby. 1979. (Na,K)ATPase structure and kinetics. Academic Press, New York.

Smart, T. G. 1990. Uncultured lobster muscle, cultured neurons and brain slices: the neurophysiology of zinc. J. Pharm. Pharmacol. **42**:377-387.

Smith, P. K., R. I. John, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenanzo, E. K. Fujimoto, N. M. Goeke, B. J. Olson, & D. C. Klenk. 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. **150**:76-85.

Snyder, J. D., & M. H. Merson. 1982. The magnitude of the global problem of acute diarrheal disease: a review of acute surveillance data. Bull. WHO **60**:605-613.

Soderberg, L., L. Wahlstrom, & J. Bergstrom. 1983. Fast protein separations on Mono Q, Mono S and Mono P. Protides Biol. Fluids **30**:635-640.

Spiegel, S. 1988. Insertion of ganglioside G_{M1} into rat glioma C6 cells renders them susceptible to growth inhibition by the B subunit of cholera toxin. Biochim. Biophys. Acta **969**:249-256.

Spiegel, S., R. Blumenthal, P. H. Fishman, & J. S. Handler. 1985. Gangliosides do not move from apical to basolateral plasma membrane in cultured epithelial cells. Biochim. Biophys. Acta **821**:310-318.

Spira, W. M., R. R. Daniel, Q. S. Ahmed, A. Huq, A. Yusuf, & D. A. Sack. 1978. Clinical features and pathogenicity of O group 1 non-agglutinating *Vibrio cholerae* and other vibrios isolated from cases in diarrhea in Dacca, Bangladesh. pp. 137-153 *in* Symposium on cholera: Karatsu, 1978; Proceedings of the 14th Joint conference on cholera and related diarrheal disease. U.S.-Japan cooperative medical science program.

Spira, W. M., & P. J. Fedorka-Cray. 1983. Production of cholera toxin-like toxin by *Vibrio mimicus* and non-O1 *Vibrio cholerae*: batch culture conditions for optimum yields and isolation of hypertoxigenic lincomycin-resistant mutants. Infect. Immun. **42**:501-509.

Spira, W. M., P. J. Fedorka-Cray, & P. Pettebone. 1983. Colonization of the rabbit small intestine by clinical and environmental isolates of *Vibrio cholerae* and *Vibrio mimicus*. Infect. Immun. **41**:1175-1183.

Spriggs, D. R., & R. B. Sack. 1990. Summary of the 25th United States-Japan joint conference on cholera and related diarrheal diseases. J. Infect. Dis. **162**:584-590.

Squire, P. G. 1964. A relationship between the molecular weights of macromolecules and their elution volumes based on a model for Sephadex gel filtration. Arch. Biochem. Biophys. **107**: 471-478.

Stein, W. D. 1967. The movement of molecules across cell membranes. Academic Press, New York.

Stein, W. D. 1986. Transport and diffusion across cell membranes. Academic Press, London.

Stein, W. D. 1989. The cotransport systems. Curr. Op. Cell Biol. **1**:739-745.

Stein, W. D. 1990. Carriers, channels and pumps. An introduction to membrane transport. Academic Press, London.

Stein, J. M., J. C. Ellory, & E. M. Tucker. 1987. The effect of sodium periodate treatment on the modulation of the sodium pump in low-potassium type (LK) sheep red cells by the L antigen. Biochim. Biophys. Acta **904**:330-336.

Stepanov, A. S. 1991. The molecular nature of *Bacillus anthracis* toxin. Mol. Gen. Mikrobiol. Virusol. **1**:3-10.

Stephen, J., & R. A. Pietrowski. 1986. Bacterial toxins. 2nd edition. American Society for Microbiology, Washington D. C.

Sterzik, B., & F. J. Fehrenbach. 1985. Reaction components influencing CAMP factor induced haemolysis. J. Gen. Microbiol. **131**:817-820.

Stevens, R. C., M. L. Evenson, W. Tepp, & B. R. DasGupta. 1991. Crystallization and preliminary X-ray analysis of botulinum neurotoxin Type A. J. Mol. Biol. **222**:877-880.

Sting, R., P. Schaufuss, & H. Blobel. 1990. Isolation and characterization of hyaluronidases from *Streptococcus dysgalactiae, S. zooepidemicus* and *S. equi*. Int. J. Med. Microbiol. **272**:276-282.

Streuli, C. H., B. Patel, & D. R. Critchley. 1981. The cholera toxin receptor GM_1 remains associated with Triton X-100

cytoskeletons of BALB/c-3T3 cells. Exp. Cell. Res. **136**:247-254.

Stryer, L. 1988. Biochemistry. 3rd edition. W. H. Freeman and Co., New York.

Sugii, S., & T. Tsuji. 1989. Studies on the binding substances on human erythrocytes for the heat-labile enterotoxin isolated from chicken enterotoxigenic *Escherichia coli.* Biochim. Biophys. Acta **983**:30-34.

Sullivan, S. K., & M. Field. 1991. Ion transport across mammalian small intestine. pp.287-301 *in* Handbook of physiology. Gastrointestinal physiology. Section 6. **4** (Schultz, S. G. (ed.)).

Sutton, C. W., R. H. Hall, & D. A. Broadbent. 1985. Purification and characterization of haemolysin from *Vibrio cholerae* biotype El Tor. J. Med. Microbiol. **19**:Siii.

Suttorp, N., M. Fuhrmann, S. Tannert-Otto, F. Grimminger, & S. Bhakdi. 1993. Pore-forming bacterial toxins potently induce release of nitric oxide in porcine endothelial cells. J. Exp. Med. **178**:337-341.

Swatek, F. E. 1967. Textbook of microbiology. C. V. Mosby Co., St. Louis.

Sweadner, K. J., & S. M. Goldin. 1980. Active transport of sodium and potassium ions. N. Engl. J. Med. **302**:777-783.

Switzer, R. C., C. R. Merril, & S. Shifrin. 1979. A highly sensitive silver stain for detecting proteins and peptides in polyacrylamide gels. Anal. Biochem. **98**:231-237.

Tacket, C. O., G. Losonsky, J. P. Nataro, S. J. Cryz, R. Edelman, J. B. Kaper, & M. M. Levine. 1992. Onset and duration of protective immunity in challenged volunteers after vaccination with live oral cholera vaccine CVD 103-HgR. J. Infect. Dis. **166**:837-841.

Takeda, T., T. Honda, Y. Takeda, & T. Miwatani. 1980. Pathogenesis of *Vibrio parahaemolyticus*, *in* Natural toxins (Eaker, D., & T. Wadstrom (ed.s)). Pergamon Press, Oxford.

Takeda, Y. 1983. Thermostable direct haemolysin of *Vibrio parahaemolyticus*. Pharmac. Ther. **19**:123-146.

Takeda, Y. 1988. Thermostable direct haemolysin of *Vibrio parahaemolyticus*. Met. Enz. **165**:189-193.

Takeda, Y., S. Taga, & T. Miwatani. 1978. Evidence that thermostable direct hemolysin of *Vibrio parahaemolyticus* is composed of two subunits. FEMS Microbiol. Lett. **4**:271-274.

Takeda, Y., T. Takeda, T. Honda, & T. Miwatani. 1976. Inactivation of the biological activities of the thermostable direct hemolysin of *Vibrio parahaemolyticus* by ganglioside G_{T1}. Infect. Immun. **14**:1-5.

Takeda, Y., T. Takeda, T. Honda, J. Sakurai, N. Ohtomo, & T. Miwatani. 1975. Inhibition of hemolytic activity of the thermostable direct hemolysin of *Vibrio parahaemolyticus* by ganglioside. Infect. Immun. **12**:931-933.

Takeuchi, H., Y. Shibano, K. Morihara, J. Fukushima, S. Inami, B. Keil, A. M. Gilles, S. Kawamoto, & K.Okuda. 1992. Structural gene and complete amino acid sequence of *Vibrio alginolyticus* collagenase. Biochem. J. **281**:703-708.

Takizawa, I. 1956. Studies on pathogenic halophilic bacteria. Yokohama Med. Bull. **2**:313-322.

Tamura, N., S. Kobayashi, H. Hashimoto, & S.-I. Hirose. 1993. Reactive arthritis induced by *Vibrio parahaemolyticus*. J. Rheumatol. **20**:1062-1063.

Taniguchi, H., H. Hirano, S. Kubomara, K. Higashi, & Y. Mizuguchi. 1986. Comparison of the nucleotide sequences of the genes for the thermostable and the thermolabile hemolysin from *Vibrio parahaemolyticus*. Microb. Path. **1**:425-432.

Taniguchi, H., S. Kubomara, H. Hirano, K. Mizue, M. Ogawa, & Y. Mizuguchi. 1990. Cloning and characterization of gene encoding a new thermostable haemolysin from *Vibrio parahaemolyticus*. FEMS Microbiol. Lett. **67**:339-345.

Tauxe, R. V., & P. A. Blake. 1992. Epidemic cholera in Latin America. J. A. M. A. **267**:1388-1390.

Taylor, A. 1975. Effect of quinidine on the action of vasopressin. Fed. Proc. **34**:285.

Taylor, A. 1981. Role of cytosolic calcium and sodium-calcium exchange in regulation of transepithelial sodium and water absorption. pp.233-259, *in* Ion transport by epithelia (Schultz, S. G. (ed.)). Raven Press, New York.

Taylor, A. 1989. The role of sodium-calcium exchange in sodium-transporting epithelia. pp.298-323, *in* Sodium-calcium exchange (Allen, T. J. A., D. Noble, & H. Reuter (ed.s)). Oxford University Press, Oxford.

Taylor, A., E. Eich, M. Pearl, A. S. Brem, & E. Q. Peeper. 1987. Cytosolic calcium and the action of vasopressin in toad urinary bladder. Am. J. Physiol. **252**:F1028-F1041.

Taylor, A., & L. G. Palmer. 1982. Hormonal regulation of sodium chloride and water transport in epithelia. pp.253-298, *in* Biological regulation and development. **3A** (Goldberger, R. F., & K. Yamamoto (ed.s)). Plenum, New York.

Taylor, A., & E. E. Windhager. 1985. Cytosolic calcium and its role in the regulation of transepithelial ion and water transport. pp.1297-1379, *in* Physiology and pathophysiology of electrolyte metabolism (Seldin, D., & G. Giebisch (ed.s)). Raven Press, New York.

Terai, A., K. Baba, H. Shirai, O. Yoshida, Y. Takeda, & M. Nischibuchi. 1991. Evidence for insertion sequence-mediated spread of the thermostable direct haemolysin gene among *Vibrio* species. J. Bacteriol. **173**:5036-5046.

Terai, A., H. Shirai, O. Yoshida, Y. Takeda, & M. Nishibuchi. 1990. Nucleotide sequence of the thermostable hemolysin gene (*tdh* gene) of *Vibrio mimicus* and its evolutionary relationship with the *tdh* genes of *Vibrio parahaemolyticus*. FEMS Microbiol. Lett. **71**:319-323 (published erratum appears *in* (1991) FEMS Microbiol. Lett. **64**:341).

Tesh, V. L., & A. D. O'Brien. 1991. The pathogenic mechanisms of shiga toxin and the shiga-like toxins. Mol. Microbiol. **5**:1817-1822.

Thelestam, M., J. E. Alouf, C. Geoffroy, & R. Mollby. 1981. Membrane-damaging action of alveolysin from *Bacillus alvei*. Infect. Immun. **32**:1187-1192.

Thielman, N. M., G. D. Fang, L. J. Barrett, M. C. Fonteles, & R. L. Guerrant. 1993. Inhibition of cholera toxin effects on intestinal secretion and Chinese Hamster Ovary cell elongation by platelet activating factor antagonists. pp.172-176, *in* Abstracts of the 29th joint conference on cholera and related diarrheal diseases. US-Japan Cooperative Medical Science Program.

Thomas, R. C. 1984. Experimental displacement of intracellular pH and the mechanism of its subsequent recovery. J. Physiol. **354**:3-22.

Thomas, S. R., Y. Suzuki, S. M. Thompson, & S. G. Schultz. 1983. Electrophysiology of *Necturus* urinary bladder. I. "Instantaneous" current-voltage relations in the presence of varying mucosal sodium concentrations. J. Memb. Biol. **73**:157-175.

Thompson, M. R., J. Forristal, P. Kauffman, T. Madden, K. Kozac, R. E. Morris, & C. B. Saelinger. 1991. Isolation and characterization of *Pseudomonas aeruginosa* exotoxin A binding glycoprotein from mouse LM cells. J. Biol. Chem. **266**:2390-2396.

Thompson, T. E., & T. W. Tillack. 1985. Organisation of glycosphingolipids in bilayers and plasma membranes of mammalian cells. Ann. Rev. Biophys. Biophys. Chem. **14**:361-386.

Tison, D. L., & M. T. Kelly. 1984. *Vibrio* species of medical importance. Diagn. Microbiol. Infect. Dis. **2**:263-276.

Toda, M., S. Okuba, H. Ikigai, & T. Shimamura. 1990. Antibacterial and antihaemolysin activities of tea catechins and their structural relatives. Nippon-Saikingaku-Zasshi **45**:561-566.

Toda, M., S. Okuba, R. Ohnishi, & T. Shimamura. 1989. Antibacterial and bacteriocidal activities of Japanese green tea. Nippon-Saikingaku-Zasshi **44**:669-672.

Toda, H., F. Sakiyama, M. Yoh, T. Honda, & T. Miwatani. 1991. Tryptophan 65 is essential for haemolytic activity of the thermostable direct hemolysin from *Vibrio parahaemolyticus*. Toxicon **29**:837-844.

Tosco, M., M. N. Orsenigo, G. Esposito, & A. Faelli. 1988. Na^+/H^+ exchange mechanism in the basolateral membrane of the rat enterocyte. Biochim. Biophys. Acta **944**:473-476.

Tosteson, M. T., J. A. Halperin, Y. Hishi, & D. C. Tosteson. 1991. Palytoxin induces an increase in the cation conductance of red cells. J. Gen. Physiol. **98**:969-985.

Tosteson, M. T., S. J. Holmes, M. Razin, & D. C. Tosteson. 1985. Melittin lysis of red cells. J. Memb. Biol. **87**:35-44.

Trucksis, M., J. E. Galen, J. Michalski, A. Fasano, & J. B. Kaper. 1993. Accessory cholera enterotoxin (Ace), the third toxin of a *Vibrio cholerae* virulence cassette. Proc. Nat. Acad. Sci. USA **90**:5267-5271.

Tsunasawa, S., A. Sugihara, T. Masaki, F. Sakiyama, Y. Takeda, T. Miwatani, & K. Narita. 1987. Amino acid sequence of thermostable direct haemolysin produced by *Vibrio parahaemolyticus*. J. Biochem. **101**:111-121.

Turini, P., S. Kurooka, M. Steer, A. N. Corbascio, & T. P. Singer. 1969. The action of phenylmethylsulfonyl fluoride on human acetylcholinesterase, chymotrypsin and trypsin. J. Pharmacol. Exp. Ther. **167**:98-104.

Turnberg, L. 1971. Potassium transport in the human small bowel. Gut **12**:811-818.

Turnberg, L. 1991. Cellular basis of diarrhoea. The Croonian lecture 1989. J. Roy. Coll. Physic. **25**:53-62.

Turnbull, P. C. B. 1981. *Bacillus cereus* toxins. Pharmacol. Ther. **13**:453-505.

Turnheim, K. 1991. Intrinsic regulation of apical sodium entry in epithelia. Physiol. Rev. **71**:429-445.

Turnheim, K., R. A. Frizzell, & S. G. Schultz. 1978. Interaction between cell sodium and the amiloride-sensitive sodium entry step in rabbit colon. J. Memb. Biol. **39**:233-256.

Turnheim, K., S. M. Thompson, & S. G. Schultz. 1983. Relation between intracellular sodium and active sodium transport in rabbit colon: current-voltage relations of the apical sodium entry mechanism in the presence of varying luminal sodium concentrations. J. Memb. Biol. **76**:299-309.

Twedt, R. M., & D. F. Brown. 1974. Studies on the enteropathogenicity of *Vibrio parahaemolyticus* in the ligated rabbit ileum. pp.211-217, *in* International symposium on *Vibrio parahaemolyticus*. (Fujino, T., G. Sakaguchi, R. Sakazaki, & Y. Takeda (ed.s)). Saikon Publishing, Tokyo.

Umesaki, Y., K. Takamizawa, & M. Ohara. 1989. Structural and compositional difference in the neutral glycolipids between epithelial and non-epithelial tissue of the mouse small intestine. Biochim. Biophys. Acta **1001**:157-162.

Urry, D. W. 1971. The gramicidin A transmembrane channel: a proposed $\pi_{(L,D)}$ helix. Proc. Nat. Acad. Sci. USA **68**:672-676.

Ussing, H. H. 1982. Volume regulation of frog skin epithelium. Acta Physiol. Scand. **114**:363-369.

Ussing, H. H., & K. Zerahn. 1951. Active transport of sodium as the source of electrical current in the short-circuited frog skin. Acta Physiol. Scand. **23**:110-127.

Vann, J. M., & R. A. Proctor. 1988. Cytotoxic effects of ingested *Staphylococcus aureus* on bovine endothelial cells: role of *S. aureus* α-hemolysin. Microb. Path. **4**:443-453.

Veatch, W. R., & L. Stryer. 1977. The dimeric nature of the gramicidin A transmembrane channel: conductance and fluorescence energy transfer studies of hybrid channels. J. Molec. Biol. **113**:89-102.

Venerando, B., B. Cestaro, A. Fiorilli, R. Ghidoni, A. Preti, & G. Tettamanti. 1982. Kinetics of *Vibrio cholerae* sialidase action on gangliosidic substrates at different supramolecular-organisational levels. Biochem. J. **203**:735-742.

Venerando, B., S. Roberti, S. Sonnino, A. Fiorilla, & G. Tettamanti. 1982. Interactions of ganglioside GM1 with human and fetal calf sera. Formation of ganglioside-serum albumin complexes. Biochim. Biophys. Acta **692**:18-26.

Verkleij, A. J., R. F. A. Zwaal, B. Roelofsen, P. Comfurius, D. Kastelijn, & L. L. M. van Deenen. 1973. The asymmetric distribution of phospholipids in the human red cell membrane. A combined study using phospholipases and freeze-etch electron microscopy. Biochim. Biophys. Acta **323**:178-193.

Waddell, T., A. Cohen, & C. A. Lingwood. 1990. Induction of verotoxin sensitivity in receptor-deficient cell-lines using the receptor glycolipid globotriosylceramide. Proc. Nat. Acad. Sci. USA **87**:7898-7901.

Wagatsuma, S. 1968. On a medium for haemolytic reaction. Media Circle **13**:159-162.

Waldman, D. B., J. D. Gardner, A. M. Zfass, & G. M. Makhlouf. 1977. Effects of vasoactive intestinal peptide, secretin, and related peptides on rat colonic transport and adenylate cyclase activity. Gastroenterol. **73**:518-523.

Walker, R. I., & R. L. Owen. 1990. Intestinal barriers to bacteria and their toxins. Ann. Rev. Med. **41**:393-400.

Wallace, B. A., & K. Ravikumar. 1988. The gramicidin pore: crystal structure of a cesium complex. Science **241**:182-187.

Walling, M. W., A. K. Mircheff, C. H. van Os, & E. M. Wright. 1978. Subcellular distribution of nucleotide cyclases in rat intestinal epithelium. Am. J. Physiol. **235**:E539-E545.

Walsh, J. A., & K. S. Warren. 1979. Selective primary health care. New Engl. J. Med. **301**:967-974.

Watanabe, K., S. Hakomori, M. E. Powell, & M. Yokota. 1980. The amphipathic membrane proteins associated with gangliosides: the Paul-Bunnell antigen is one of the gangliophilic proteins. Biochem. Biophys. Res. Comm. **92**:638-646.

Watanabe, Y., & G. R. Seaman. 1962. Purification and properties of the hemolysin from El Tor vibrio. Arch. Biochem. Biophys. **97**:393-398.

Watanabe, Y., & W. F. Verwey. 1966. The biological characterization of the haemolysin from the El Tor variety of *Vibrio cholerae*. J. Infect. Dis. **116**:363-371.

Weber, K., & M. Osborn. 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. **224**:4406-4412.

Weer, P. de. 1985. Cellular sodium-potassium transport. pp.31-48 *in* The kidney: physiology and pathophysiology (Seldin, D. W., & G. Giebisch (ed.s)). Raven Press, New York.

Weer, P. de., D. C. Gadsby, & R. F. Rakowski. 1988. Voltage-dependence of the Na-K pump. Ann. Rev. Physiol. **50**:225-241.

Weiner, R. N., E. Schneider, C. W. M. Haest, B. Deuticke, R. Benz, & M. Frimmer. 1985. Properties of the leak permeability induced by a cytotoxic protein from *Pseudomonas aeruginosa* (PACT) in rat erythrocytes and black lipid membranes. Biochim. Biophys. Acta **820**:173-182.

Wellisch, G., E. Cohen, Z. Cahane, & J. Horowitz. 1984. Simple method for collagenase determination in 38 *Pseudomonas aeruginosa* strains. J. Clin. Microbiol. **20**:1020-1021.

Welsh, M. J. 1987. Electrolyte transport by airway epithelium. Physiol. Rev. **67**:143-184.

Welsh, M. J., & J. D. McCann. 1985. Intracellular calcium regulates potassium channels in a chloride-secreting epithelium. Proc. Nat. Acad. Sci. USA **82**:8823-8826.

Welsh, M. J., P. L. Smith, & R. A. Frizzell. 1982. Chloride secretion by canine tracheal epithelium. II. The cellular electrical potential profile. J. Memb. Biol. **70**:227-238.

Welsh, M. J., P. L. Smith, M. Fromm, & R. A. Frizzell. 1982. Crypts are the site of intestinal fluid and electrolyte secretion. Science **218**:1219-1221.

Wicken, A. J., & K. W. Knox. 1980. Bacterial cell surface amphiphiles. Biochim. Biophys. Acta **604**:1-26.

Wiechelman, K., R. Braun, & J. Fitzpatrick. 1988. Investigation of the bicinchoninic acid protein assay: identification of the groups responsible for color formation. Anal. Biochem. **175**:231-237.

Wiesmann, W., S. Sinha, & S. Klahr. 1977. Effects of ionophore A23187 on baseline and vasopressin-stimulated sodium transport in the toad bladder. J. Clin. Invest. **59**:418-425.

Will, P. C., J. L. Lebowitz, & U. Hopfer. 1980. Induction of amiloride-sensitive sodium transport in the rat colon by mineralocorticoids. Am. J. Physiol. **238**:F261-F268.

Williams, D. P., Z. Wen, R. S. Watson, J. Boyd, T. B. Strom, & J. R. Murphy. 1990. Cellular processing of the interleukin-2 fusion toxin DAB_{486}-IL-2 and efficient delivery of diphtheria fragment A to the cytosol of target cells requires Arg^{194}. J. Biol. Chem. **265**:20673-20677.

Wills, N. K., & S. A. Lewis. 1980. Intracellular Na activity as a function of Na transport across a tight epithelium. Biophys. J. **30**:181-186.

Wilmsen, H. U., F. Pattus, & J. T. Buckley. 1990. Aerolysin, a hemolysin from *Aeromonas hydrophila*, forms voltage-gated channels in planar lipid bilayers. J. Memb. Biol. **115**:71-81.

Wilson, R., S. Lieb, A. Roberts, S. Stryker, H. Janowski, R. Gunn, B. Davis, C. F. Riddle, T. Barrett, J. G. Jr. Morris, & P. A. Blake. 1981. Non-O group 1 *Vibrio cholerae* gastroenteritis associated with eating raw oysters. Am. J. Epidemiol. **114**:293-298.

Windhager, E. E., & A. Taylor. 1983. Regulatory role of intracellular calcium ions in epithelial Na transport. Ann. Rev. Physiol. **45**:519-532.

Wingender, H. 1974. Proteinase inhibitors of microbial origin. pp.548-559, *in* Proteinase inhibitors: proceedings of the second international research conference (Fritz, H., H. Tschesche, L. J. Greene, & E. Truschett (ed.s)). Bayer Symposium V. Springer-Verlag, Berlin.

Wiseman, G. M., & J. D. Caird. 1967. The nature of staphylococcal beta hemolysin. I. Mode of action. Can. J. Microbiol. **13**:369-376.

Wolosin, J. M., & J. G. Forte. 1985. K^+ and Cl^- conductance in apical membrane from secreting oxyntic cells are concurrently inhibited by divalent cations. J. Memb. Biol. **83**:261-272.

Wong, S. M., & H. S. Jr. Chase. 1986. Role of intracellular calcium in cellular volume regulation. Am. J. Physiol. **250**:C841-C852.

Wood, J. D. 1991. Communication between minibrain in gut and enteric immune system. News Physiol. Sci. **6**:64-69.

Woodhull, A. M. 1973. Ionic blockage of sodium channels in nerve. J. Gen. Physiol. **61**:687-708.

Wyn Jones, R. G., & O. R. Lunt. 1967. The function of calcium in plants. Bot. Rev. **33**:407-426.

Xu-Hy. 1989. Study on 438 strains of pathogenic *Vibrio* and their resistance to drugs. Chung-Hua-Liu-Hsing-Ping-Hsueh-Tsa-Chi **10**:337-340.

Yamamoto, K., M. Al-Omani, T. Honda, Y. Takeda, & T. Miwatani. 1984. Non-O1 *Vibrio cholerae* hemolysin: purification, partialcharacterization, and immunological relatedness to El Tor hemolysin. Infect. Immun. **45**:192-196.

Yamamoto, T., K. Fujita, & Y. Yokota. 1990a. Piliated *Vibrio parahaemolyticus* adherent to human ureteral mucosa. J. Infect. Dis. **161**:361-362.

Yamamoto, K., Y. Ichinose, N. Nakasone, M. Tanabe, M. Nagahama, J. Sakurai, & M. Iwanaga. 1986. Identity of hemolysins produced by *Vibrio cholerae* non-O1 and *V. cholerae* O1, biotype El Tor. Infect. Immun. **51**:927-931.

Yamamoto, K., Y. Ichinose, H. Shinagawa, K. Makino, A. Nakata, M. Iwanaga, T. Honda, & T. Miwatani. 1990b. Two-step processing for activation of the cytolysin/hemolysin of *Vibrio cholerae* O1 biotype El Tor: nucleotide sequence of the structural gene (*hly*A) and characterization of the processed products. Infect. Immun. **58**:4106-4116.

Yamamoto, K., A. C. Wright, J. B. Kaper, & J. G. Jr. Morris. 1990c. The cytolysin gene of *Vibrio vulnificus*: sequence and relationship to the *Vibrio cholerae* El Tor haemolysin gene. Infect. Immun. **58**:2706-2709.

Yamamoto, T., & T. Yokota. 1989. Adherence targets of *Vibrio parahaemolyticus* in human small intestines. Infect. Immun. **57**:2410-2419.

Yamasaki, S., H. Shirai, Y. Takeda, & M. Nishibuchi. 1991. Analysis of the gene of *Vibrio hollisae* encoding the hemolysin similar to the thermostable direct hemolysin of *Vibrio parahaemolyticus*. FEMS Microbiol. Lett. **80**:259-263.

Yanagase, Y., K. Inoue, M. Ozaki, T. Ochi, T. Amano, & M. Chazono. 1970. Hemolysins and related enzymes of *Vibrio parahaemolyticus*. I. Identification and partial purification of enzymes. Biken J. **13**:77-92.

Yang, F. Y., Y. G. Huang, & Y. P. Tu. 1993. Divalent cations and lipid-protein interactions of biomembranes. Biosci. Rep. **13**:143-157.

Yang, J. M., C. O. Lee, & E. E. Windhager. 1988. Regulation of cytosolic free calcium in isolated perfused proximal tubules of *Necturus*. Am. J. Physiol. **225**:F787-F799.

Yeagle, P. 1992. The structure of biological membranes. CRC Press, London.

Yingst, D. R. 1988. Modulation of the Na,K-ATPase by Ca and intracellular proteins. Ann. Rev. Physiol. **50**:291-303.

Yoh, M., T. Honda, & T. Miwatani. 1985. Production by non-O1 *Vibrio cholerae* of hemolysin related to thermostable direct hemolysin of *Vibrio parahaemolyticus*. FEMS Microbiol. Lett. **29**:197-200.

Yoh, M., T. Honda, & T. Miwatani. 1986a. Purification and partial characterization of a non-O1 *Vibrio cholerae* hemolysin that cross-reacts with thermostable direct hemolysin of *Vibrio parahaemolyticus*. Infect. Immun. **52**:319-322.

Yoh, M., T. Honda, & T. Miwatani. 1986b. Purification and partial characterization of a *Vibrio hollisae* hemolysin that relates to thermostable direct hemolysin of *Vibrio parahaemolyticus*. Can. J. Microbiol. **32**:632-636.

Yoh, M., T. Honda, T. Miwatani, S. Tsunasawa, & F. Sakiyama. 1989. Comparative amino acid sequence analysis of hemolysins produced by *Vibrio hollisae* and *Vibrio parahaemolyticus*. J. Bacteriol. **171**:6859-6861.

Yoshida, H., T. Honda, & T. Miwatani. 1991. Purification and characterization of a hemolysin of *Vibrio hollisae* that relates to the thermostable direct hemolysin of *Vibrio parahaemolyticus*. FEMS Microbiol. Lett. **84**:249-253.

Yoshikawa, H., I. Kawamura, M. Fujita, H. Tsukada, M. Arakawa, & M. Mitsuyama. 1993. Membrane damage and interleukin-1 production in murine macrophages exposed to listeriolysin O. Infect. Immun. **61**:1334-1339.

PUBLICATIONS

Papers arising from this investigation:

Huntley, J. S., A. C. Hall, & K. Kirk. 1993. Kanagawa haemolysin induces a cation leak in human erythrocyte membranes. J. Physiol. **459**:291P.

Huntley, J. S., & A. C. Hall. 1993. The effects of membrane active agents on the leak induced in erythrocyte membranes by Kanagawa haemolysin. Biochem. Soc. Trans. **21**(2[2]):219S.

Huntley, J. S., & A. C. Hall. 1993. Kanagawa haemolysin of *Vibrio parahaemolyticus* induces a nitrendipine-sensitive K^+ influx in human erythrocytes. J. Physiol. **467**:383P.

Huntley, J. S., & A. C. Hall. 1993. Human erythrocytes: a model membrane system for analysis of bacterial toxin mechanisms. p.49, *in* Abstracts 1993 Red Blood Cell Meeting. The Wellcome Trust.

Huntley, J. S., A. C. Hall, V. Sathyamoorthy, & R. H. Hall. 1993. Thermostable direct hemolysin of *Vibrio parahaemolyticus* increases the cation permeability of erythrocyte membranes. p.45[B108], *in* Abstracts of the 93rd General Meeting of the American Society for Microbiology. American Society for Microbiology, Washington.

Huntley, J. S., A. C. Hall, V. Sathyamoorthy, & R.H.Hall. 1993. Cation flux studies on the lesion induced in human erythrocyte membranes by the thermostable direct hemolysin of *Vibrio parahaemolyticus*. Infect. Immun. **61**(10):4326-4332.

Huntley, J. S., & A. C. Hall. 1993. Factors affecting erythrocyte membrane susceptibility to Kanagawa haemolysin of *Vibrio parahaemolyticus*. p.225, *in* Abstracts of the twenty-ninth joint conference on cholera and related diarrheal diseases. The United States-Japan Cooperative Medical Science Program.

Huntley, J. S., & A. C. Hall. 1994. Aspects of the haemolytic reaction induced by Kanagawa haemolysin of *Vibrio parahaemolyticus*. Toxicon (in press).

Huntley, J. S., & A. C. Hall. The dynamics of action of Kanagawa haemolysin of *Vibrio parahaemolyticus*. (in preparation).

Huntley, J. S., A. C. Hall, V. Sathyamoorthy, & R. H. Hall. Nature of cation leaks induced in cell membranes by *Vibrio* toxins. (in preparation).

Huntley, J. S., & A. C. Hall. The role of Ca^{2+} in the perturbation of membrane function by Kanagawa haemolysin of *Vibrio parahaemolyticus*. (in preparation).

Huntley, J. S., A. C. Hall, V. Sathyamoorthy, & R. H. Hall. The haemolytic action of purified thermostable direct haemolysin (TDH) of *Vibrio parahaemolyticus*. (in preparation).