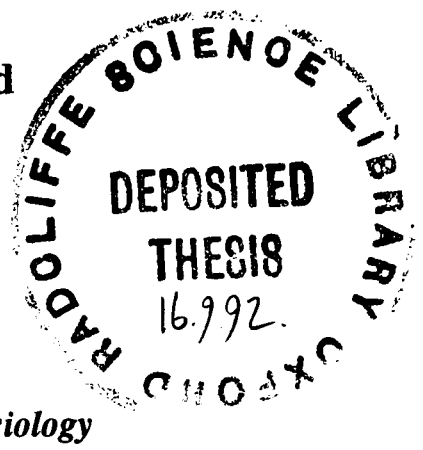
ZINC TRANSPORT ACROSS CELL MEMBRANES

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Thesis submitted for the degree of Doctor of Philosophy

University of Oxford



University Laboratory of Physiology

Trinity Term 1992

ABSTRACT

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The mechanism of zinc transport has been investigated in red cells from normal humans, lampreys, sheep, sickle cell anaemia patients and in bovine chondrocytes. In all the cell types investigated except for lamprey red cells, zinc transport is mainly via the anion exchanger (band 3), which accounts for over 80% of total measured zinc uptake, when the medium contains no zinc binding ligands. Zinc uptake via the band 3 pathway is stimulated by the presence of bicarbonate (5mM) and inhibited by treatment with DIDS or SITS (10μM). This anion-dependent mechanism represents the major route for zinc transport across the cell membrane *in vitro*. The presence of the zinc binding ligands albumin and histidine in the media greatly reduced the uptake of zinc via the anion exchanger due to the decrease in free zinc concentration. Histidine, in addition to its chelating effect, shows a specific facilitating effect on zinc uptake in all the cell types. This stimulating effect of histidine was stereospecific (significantly different between L-, and D-histidine) in red cells from normal humans and sickle cell anaemia patients, but not in red cells from lampreys, sheep, and bovine chondrocytes. Evidence from all cell types strongly suggests that the stimulus is due to the cotransport of zinc and histidine via the histidine transport systems, which are system L, and y^+ in normal human and sickle red cells; a non-stereospecific L-like system in lamprey red cells and bovine chondrocytes; system C or unknown specific histidine transporter in sheep red cells. The amino acid linked zinc uptake may represent a physiologically significant mechanism for zinc transport into cells.

Dedication

To my Lord Jesus Christ and my dear parents

for their everlasting love and support

"What we call the beginning is often the end
And to make an end is to make a beginning....
We shall not cease from exploration
And the end of all our exploring
Will be to arrive where we started
And know the place for the first time..."

from Little Gidding by T.S. Eliot

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

CHAPTER ONE: GENERAL INTRODUCTION

Pg

1.1 Importance of Zinc in Biology 1

1.2 Metabolism of Zinc 2

1.3 Red Cells as a Model System 5

1.4 Concept of Mediated Transport 8

1.5 Band 3 Anion Transporter 10

1.6 Amino Acid Transport Systems 11

1.6.1 System L 12

1.6.2 System y^+ 13

1.6.3 System N 13

1.7 Structure of this Thesis 14

CHAPTER TWO: MATERIALS AND METHODS

2.1 Incubation Solutions and Chemicals 15

2.2 Preparation of Cells 16

2.3 Flux Measurement 16

2.3.1 Rapid Wash Technique 16

2.3.1.1 Separation 17

2.3.1.2 Scintillation Counting 17

2.3.1.3 Calculation 18

2.3.2 Cell Counting 20

2.3.2.1 Erythrocytes 20

2.3.2.2 Chondrocytes 21

2.4 General Experimental Protocol 21

2.4.1 Zero-trans Flux 21

2.4.2 Time course for Initial Uptake 22

2.4.3 Concentration-dependent Uptake 22

2.4.4 Estimation of Kinetic Parameters, K_m and V_{max} 23
2.4.5 Other Characteristics 24
2.4.5.1 Na^+-dependence 24
2.4.5.2 Inhibitor Experiments 25
2.4.5.3 *Trans*-stimulation 25
2.4.5.4 Substrate Stereospecificity 25
2.4.6 Estimation of ionic zinc, (zinc-histidine) complex concentrations 26
2.5 Statistical Methods 26

CHAPTER THREE: ZINC TRANSPORT IN HUMAN RED CELLS

Introduction 28
Materials and Methods 29
Results 30
3.1 Time Course 30
3.2 Effect of DIDS (SITS) & Bicarbonate 30
3.3 Effect of Albumin 31
3.4 Effect of Histidine 32
3.4.1 Concentration-dependent Uptake 32
3.4.2 Effect of Albumin 33
3.4.3 Temperature Effects 34
3.4.4 Na^+-dependence 34
3.4.5 *Cis*- & *Trans*-effect of L-leucine & L-lysine 35
3.5 Histidine Transport in Human Red Cells 36
3.5.1 Time Course 36
3.5.2 Concentration-dependent Histidine Uptake 36
3.5.3 Na^+-dependence 36
3.5.4 Investigation of Histidine Pathways 37
Discussion 37

CHAPTER FOUR: ZINC TRANSPORT IN LAMPREY RED CELLS

Introduction 40

Materials and Methods 41

Results 42

4.1 Zinc Uptake in Lamprey Red Cells 42

4.1.1 Time Course 42

4.1.2 Effect of DIDS & Bicarbonate 42

4.1.3 Concentration-dependent Zinc Uptake 43

4.2 Effect of Histidine on Zinc Uptake in Lamprey Red Cells 43

4.3 L-Histidine Uptake in Lamprey Red Cells 44

4.3.1 Time Course 44

4.3.2 Investigation of L-histidine Transport Pathways 44

4.3.3 Substrate Stereospecificity of L-histidine Transport Systems 45

4.4 *Trans*-effect of Leucine and Lysine on (Zinc-histidine) Uptake 46

4.5 Na^+-dependence 47

Discussion 47

CHAPTER FIVE: ZINC TRANSPORT IN SHEEP RED CELLS

Introduction 51

Materials and Methods 51

Results 52

5.1 Time Course 52

5.2 Effect of SITS & Bicarbonate 53

5.3 Effect of L,D-histidine on SITS-insensitive Component 54

5.4 L-Histidine Uptake 54

5.4.1 Time Course 55

5.4.2 Concentration-dependent Uptake 55

5.4.3 Effect of SITS on L-histidine Uptake 56

Discussion 56

CHAPTER SIX: ZINC TRANSPORT IN HUMAN SICKLE CELLS

Introduction 60

Materials and Methods 61

Results 62

6.1 Time Course 62

6.2 Effect of DIDS & Bicarbonate 62

6.3 Effect of Histidine on Non-Band 3 Pathways 63

6.4 Effect of BSA on the Histidine Effect 64

Discussion 64

CHAPTER SEVEN: ZINC TRANSPORT IN BOVINE CHONDROCYTES

Introduction 67

Materials and Methods 68

Results 69

7.1 Time Course 69

7.2 Surface Binding 69

7.3 Concentration-dependent Uptake 70

7.4 Effect of Na^+-replacement on Zinc Uptake 70

7.5 Effect of SITS & Bicarbonate 70

7.6 Effect of Histidine 71

Discussion 72

CHAPTER EIGHT: GENERAL DISCUSSION

8.1 Introduction 76

8.2 Zinc Entry via the Band 3 Anion Exchanger 76

8.3 Zinc Entry via Amino Acid Transport Systems 77

8.4 Physiological Relevance 79

BIBLIOGRAPHY 81

ACKNOWLEDGEMENT

"One knows nobody so well as one's 'fellow'. Every step of the common journey tests his metal; and the test are tests we fully understand because we are undergoing them ourselves... Sometimes he wonders what he is doing there among his betters. He is lucky beyond desert to be in such company." from The Four Loves by C.S. Lewis

I would like to express my most sincere gratitude to the following people who made the completion of this thesis possible.

Dr Clive Ellory, my supervisor, for his expert supervision and endless support.

Dr Kiaran Kirk, Dr Andrew Hall, and all other members from Clive's group, for their genuine concern and help.

The Pollards, The Caves, The Spences, The Kirks, for their constant encouragement.

Friends from Linacre College, Wesley MemorialChurch and Physiology Department.

Finally, I particularly thank Jim Huntley, Robert Wilkins and Bryony Rooper for their help in proof-reading.

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Importance of Zinc in Biology

In 1869, Raulin was the first to describe that zinc was essential for the growth of a fungus Aspergillus niger (Endre *et al.*, 1990). Since then the clinical manifestations of zinc deficiency have been found in many animal species including rats, pigs, chickens, turkeys, Japanese quail, cattle, sheep, goats, guinea pigs, dogs, squirrel monkeys, pheasants and humans (Endre *et al.*, 1990). The symptoms of inadequate zinc metabolism vary slightly between different animal species but the most common features include (1) lesions of the skin, (2) abnormalities of the skeleton and (3) defects in the reproductive organs, particularly testicular development in the male (Evans, 1986). Zinc is important at all levels of metabolism since it is a crucial part of metalloenzymes; zinc has been identified as a cofactor for over 200 enzymes. It can also serve as a component in conjunction with metal-activated enzymes (Cousins, 1986). Many important enzymes such as carbonic anhydrase, alkaline phosphatase, and alcohol dehydrogenase require zinc for their maximum catalytic activity (Cousins, 1986). The diverse role of zinc is not just restricted to its association with enzymes. Zinc is also involved in maintaining structural integrity of membranes (Bettger and O'Dell, 1981). For example, zinc can prevent membrane damage caused by cytotoxins (Bashford *et al.*, 1988; Mahadevan *et al.*, 1990). Dietary supplementation of zinc decreases the osmotic fragility of cells (Rifkind, 1987). Recently it was reported that zinc can

mediate directly the binding of human growth hormone (hGH) to the prolactin (PRL) receptor (Cunningham *et al*, 1990) by forming a 'zinc sandwich' complex of one Zn^{+} (per hGH. hPRL) receptor. In humans, zinc deficiency has been linked with many disease states (see Table 1.1) (Endre *et al.*, 1990) and the supplementation of zinc has become an increasingly important remedy in treating these diseases clinically (for reviews see Evans, 1986; Endre *et al.*, 1990).

Despite its significant role in biology (as described above), it is surprising that our knowledge of zinc metabolism and transport is still relatively limited, particularly at a cellular level. This study is devoted to the investigation of zinc transport mechanisms across the cell membrane. As we appreciate the fact that a sufficient zinc supply is "vital" for the welfare of the cells or the whole organism, it is clearly important to understand how cells have evolved an efficient transport mechanism for this element.

1.2 Metabolism of Zinc

The human body is able to respond to large variations in dietary zinc yet maintain a relatively constant body content of zinc (Jackson, 1988). It is generally accepted that the status of zinc is regulated homeostatically by a sophisticated mechanism, which has not yet been clearly defined (Cousins, 1985). The homeostasis of zinc is achieved by regulation of both gastrointestinal absorption and excretion of the element (Figure 1.1; Cousins, 1985). In human adults the RDA (recommended daily allowance) is 15mg/day, of which about 1/3 of daily intake is absorbed. The absorption occurs mainly in the small intestine under close hormonal regulation and is further affected by many other factors (food composition and physiological status) (see reviews for more details, Cousins, 1985; Jackson, 1988). Zinc is excreted mainly by way of the faeces. When the

Table 1.1 The Clinical Spectrum of Human Zinc Deficiency

Severe Zinc Deficiency

Causes:

- -Acrodermatitis enteropathica
- -Total parenteral nutrition with zinc replacement
- -Excess alcohol intake
- -Prolonged penicilliamine or histidine therapy

Clinical manifestations:

- -Dermatitis; diarrhoea; weight loss
- -Neurosensory disorders; emotional disorders
- -Recurrent infections due to cell-mediated immune dysfunction
- -Hypogonadism in males
- -Delayed healing of ulcers

Moderate Zinc Deficiency

Causes:

- -Sickle cell disease
- -Malnutrition, due to dietary factors
- -Malabsorption syndromes
- -Liver cirrhosis
- -Alcoholism

Clinical manifestations:

- -Growth retardation
- -Hypogonadism (in males)
- -Rough, dry skin; delayed wound healing; poor appetite; decreased taste acuity; abnormal dark adaptation; mental lethargy; cell-mediated immune dysfunctions

Mild Zinc Deficiency

Causes:

- -Elderly subjects
- -Relative increase in zinc requirement

Clinical manifestations:

- -Decreased serum testosterone levels in males
- -Abnormal dark adaptation
- -Weight loss, decreased lean body mass
- -Hyperammonemia
- -Decreased activity of serum thymulin and IL-2
- -Serum zinc concentration may be normal

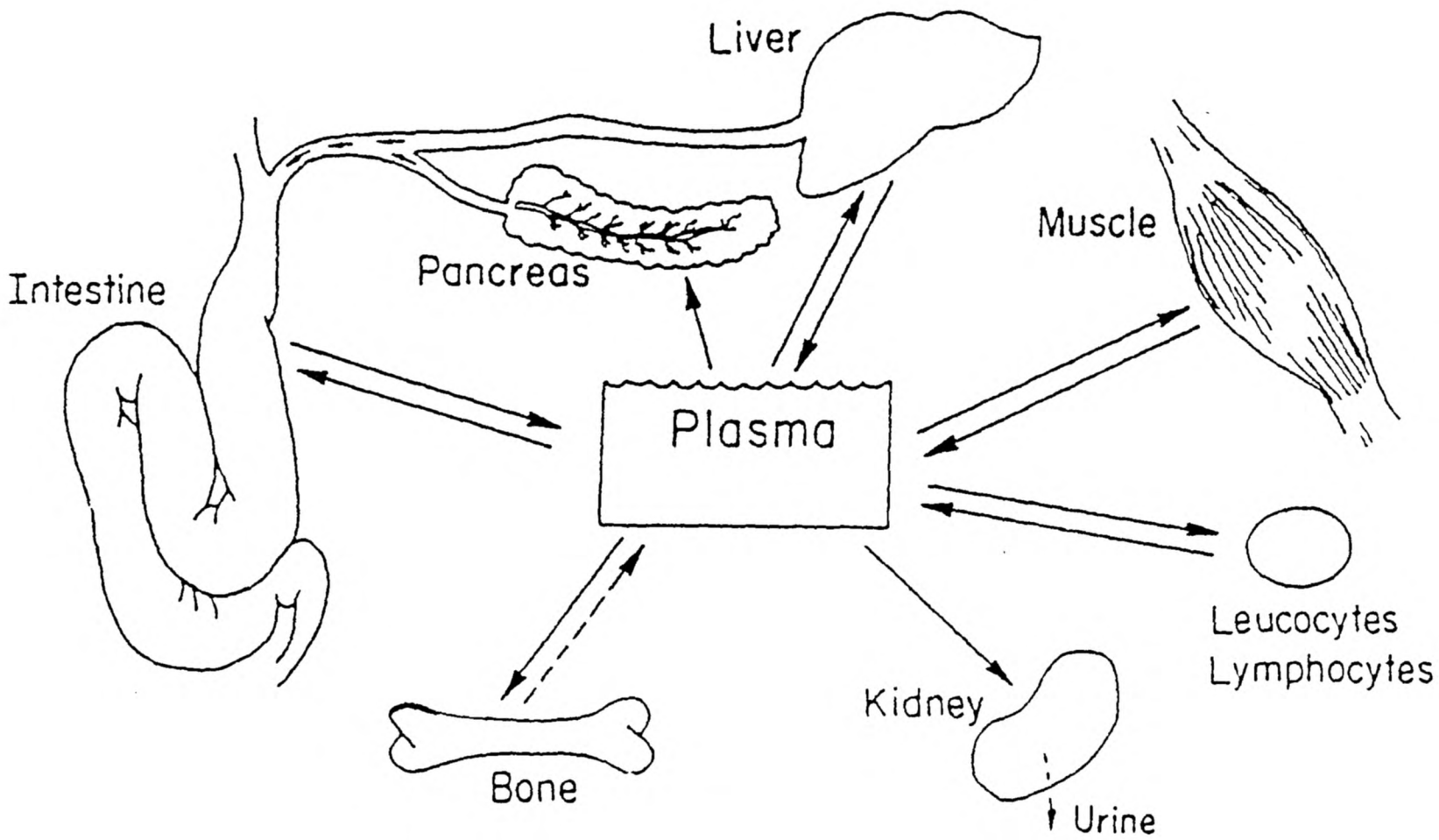


Figure 1.1

Basic aspects of mammalian zinc metabolism (taken from Cousin, 1986).

dietary supply is low, the absorption is increased by increased intestinal transport and reduced secretion of endogenous zinc into the intestine. Endogenous zinc is secreted into the intestine from pancreatic and biliary secretions. Newly absorbed zinc is then transported in portal plasma and redistributed in other tissues very quickly after the absorption (Cousins, 1985). Table 1.2 lists the amount of zinc present in different major tissues (Jackson, 1988). On the whole, muscle and bone represent the largest pools, which account for more than 80% of total body zinc. The pool is only returned to plasma when bone is mobilized to maintain calcium homeostasis (Cousins, 1985). During increased muscle catabolism, body zinc may be lost in urine (Cousins, 1985). It is apparent from the figures shown in Table 1.2 that zinc is primarily an 'intracellular' ion with intracellular zinc contributing well over 95% of total body zinc. Inside the cells, the majority of zinc is found to be bound to metallothionein (a regulatory protein for zinc and copper) or enzymes (Cousins, 1985).

The blood contains about 0.1% of total body zinc (Cousins, 1985). The majority of zinc in blood is found within erythrocytes and leucocytes, which represent 85% and 3% of the blood zinc content respectively (Dennes *et al.*, 1962). Plasma zinc accounts for less than 10% of blood zinc and is mostly protein-bound (>95%; Cousins, 1985). The total zinc concentration in plasma is about 15μM, of which 20% is tightly bound to α_2-macroglobulin (with an binding constant of 10^{10}). This fraction of zinc complex is not metabolically active (Jackson, 1988). Albumin, which binds more than 80% of plasma zinc, has been identified as a carrier for zinc in portal plasma (Cousins, 1985). The small residual fraction of non protein bound plasma zinc (2-5%) binds to small molecular-weight-ligands, mainly amino acids (histidine, cysteine, glutamine, and lysine etc) and peptides (Cousins, 1985). Virtually all the reactive groups of amino acids in peptide linkage serve as ligands for zinc: in particular the carboxyl group of glutamic acid, the ε-amino group of

Table 1.2 Approximate zinc content of Major Organs and Tissues in Normal Adult Man (70 kg)

Tissue	Total Zn content (g)	% of total body Zn
Skeletal muscle	1.53	57
Bone	0.77	29
Skin	0.16	6
Liver	0.13	5
Brain	0.04	1.5
Kidneys	0.02	0.7
Heart	0.01	0.4
Hair	<0.01	0.1(approx)
Blood Plasma	<0.01	0.1(approx)

Modified from Jackson (1988).

lysine, the imidazole group of histidine, the phenoxy group of tyrosine, and the sulphhydryl group of cysteine (O'Dell and Campbell, 1977).

From the facts that the newly absorbed zinc is redistributed into the major tissues very rapidly and over 95% of the body zinc is found within the cells, it is reasonable to suspect that the body must have a highly specific mechanism for transporting zinc across cell membranes in all tissues. However, to date such a transport process is poorly defined. The chemical form of zinc which is transported is also unknown. Zinc transport across the cell membrane has been studied in several isolated cell types including intestinal cells (Cousins, 1985), endothelial cells (Bobilya *et al.*, 1992), hepatocytes (Pattison and Cousins, 1986), fibroblasts (Ackland *et al.*, 1988), synaptosomes (Wensink *et al.*, 1988), trophoblast cells (Mas and Sarkar, 1991), leucocytes (Jones *et al.*, 1980) and erythrocytes (Alda Torrubia, and Garay, 1989; Kalfakakou and Simons, 1990; Aiken *et al.*, 1992). Two kinds of transport mechanisms were reported in most of the studies: a non-saturable uptake of zinc which represents passive diffusion and a saturable carrier-mediated process. Unfortunately, most of these studies *in vitro* have used micromolar concentrations of $ZnCl_2$ as a source of zinc and did not include physiological ligands in the medium. It cannot be overemphasized that under physiological conditions, tissues are exposed only to plasma zinc which largely exists either in protein-bound or amino acid-bound form and the concentration of free zinc is negligible. Therefore, for most of the experiments carried out in ligand-free media, though the kinetic data seems accurate, the results cannot be representative of what happens *in vivo*. To understand the mechanism by which tissues or cells can take up zinc from the systemic circulation, it is essential to examine the effect and the role of physiological ligands on zinc transport.

1.3 Red Cells as a Model System

"I have been told that 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....."

Eric Ponder, cited in Schatzmann, 1989.

In this thesis, red cells from normal humans, sickle cell anaemia patients, normal and genetically defective sheep, and lamprey have been used, in addition to the use of bovine chondrocytes. Each of these cell types represents a unique model system for understanding the mechanism of zinc transport. The characteristics and background of each cell type will be explained in more detail in later chapters.

As a dead-end cell type, red cells represent a largely homogenous cell type with relatively less metabolism to complicate transport processes (Ellory, 1987). Their advantage of being easily attainable have made them the preferred model system for membrane transport studies of all kinds. In the study of zinc transport, there are two significant roles of red cells which make it a physiologically relevant cell type to work on. First, red cells represent the largest zinc storage pool in the systemic circulation. In the blood, over 90% of zinc is accumulated within the red cells. Studies *in vitro* have shown that there is a rapid exchange of zinc between red cells and plasma, but no detectable transference of zinc from leucocytes to plasma (Dennes *et al.*, 1962). Hence, it is quite possible that red cells can serve as a vehicle to provide zinc to the major tissues.

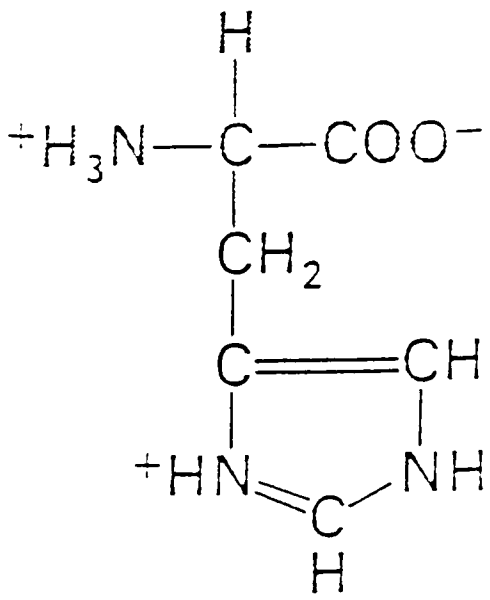
Second, a decreased erythrocyte zinc concentration is commonly observed in zinc deficiency and used as one of the indices for the purpose of diagnosis (Cousins, 1985). However, very little is known about the mechanism for such a decrease in erythrocyte zinc content in terms of changes in transport properties.

Studies *in vitro* using $ZnCl_2$ as the source of Zn have reported that the transport of zinc across the red cell membrane depends on the free zinc concentration in the medium and the presence of any binding ligands (protein or amino acids) has a great inhibitory effect on the apparent uptake rate of zinc (Kruckeberg and Brewer, 1978). The inhibitory effect of ligands is explained by the binding of zinc which subsequently causes a decrease in Zn^{2+} concentration in the medium. Other metal ions, such as Co^{2+}, Mn^{2+}, Cd^{2+}, and Cu^{2+} have no effect on Zn^{2+} uptake whereas Fe^{2+} has a slight inhibitory effect (Rifkind, 1983). The uptake of zinc into the red cell when no ligand is present can be very rapid. Kruckeberg *et al.* reported the initial uptake rate of zinc at 37^0C pH 7.4 is 9.8 μ mol zinc. ml^{-1} red cells. hr^{-1} (Kruckeberg and Brewer, 1978). The quantity of zinc which can be taken up by the red cell is also very large and can reach saturating levels higher than that of intracellular haemoglobin (Rifkind, 1983). The efflux of zinc from the red cell is much slower, as a result of the relatively tight binding of zinc to haemoglobin or other intracellular molecules once it has been taken in (Rifkind, 1983; Kalfakakou and Simons, 1990). The uptake of zinc is much more rapid than the passive transport of calcium, when the calcium pump is arrested (Rifkind, 1983). Furthermore, zinc uptake is inhibited by trinitrocresol (TNC), which accelerates Ca^{2+} influx, suggesting a different pathway for Ca^{2+} and Zn^{2+} (Rifkind, 1983). Studies in human red cells have reported that Zn^{2+} can form anionic complexes with Cl^- and HCO_3^- and enter via the anion exchanger; this represents the major route for zinc uptake into red cells (Alda Torrubia and Garay, 1989; Kalfakakou and Simons, 1990). However, these studies, like those

in other isolated cell types, did not add zinc binding ligands in the medium. Thus, the physiological relevance of such a transport mechanism remains to be established. Another report using rat erythrocytes has observed that the zinc-binding ligand histidine showed a stereospecific stimulating effect on zinc uptake (Aiken *et al.*, 1991) and the same authors have proposed the possibility of the cotransport of zinc and histidine via the amino acid transport system. The precise mechanism by which this occurs and the amino acid transport systems involved remain uncertain.

In this research, both the anionic and amino acid linked mechanism for zinc transport were investigated. More importantly, the physiological significance of these transport pathways was evaluated by testing the effects of the presence of the plasma zinc-binding ligands. Albumin and histidine were the two ligands chosen to serve this purpose. The reason for choosing albumin is obvious. As the most important binding ligand in plasma, the ability of albumin to give up zinc and allow it to be transported across cell membranes plays a significant role in determining zinc bioavailability. There are several reasons for the use of histidine. Amongst all the small-molecular-weights binding ligands, histidine and cysteine are the most potent because of their high capacity and affinity for binding zinc (Jackson, 1988). Histidine is the simpler compound to work with than cysteine because the latter is easily oxidized to cystine (Figure 1.2; Stryer, 1975), which can cause unnecessary complications in flux experiments. Also, cysteine is found to be transported via the band 3 anion exchanger (Young *et al.*, 1987) but histidine is not (see Chapter 3). It is one of the main purposes of this study to distinguish the different transport pathways for zinc. Therefore, histidine seems a better model substrate in avoiding the overlapping contribution from the band 3 pathway. In their study on rat erythrocytes, Aiken *et al.* (1992) used histidine rather than cysteine. The choice of histidine in the present research also allows comparisons with their data to be made.

(A)



(B)

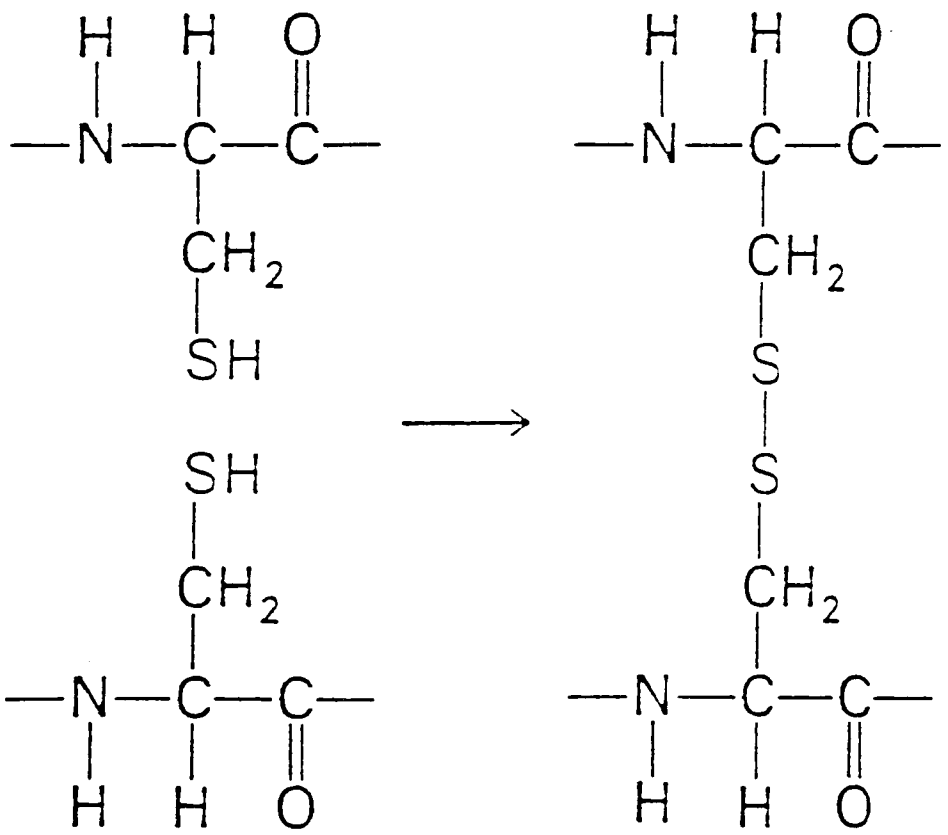


Figure 1.2 (A) & (B)

(a) Structure of histidine, (b) a disulphide bridge (-S-S-) is formed from the sulphhydryl groups (-SH) of two cysteine residues. The product is a cystine residue (Stryer,1975).

1.4 Concept of Mediated Transport

All biological membranes share a common structure: assemblies of lipid and protein molecule held together by noncovalent interactions (Figure 1.3). The membranes then serve as a barrier that can maintain a well-regulated concentration of ions on either side and via transporters allow nutrients to enter and waste products to leave the cell. Because of its hydrophobic interior, the lipid bilayer of cell membranes is highly impermeable to charged molecules (ions), no matter how small. However, it is now known that specific membrane proteins can transfer specific small molecules across cell membranes. The main routes by which ions and molecules cross membranes are simple diffusion, permeation through channels, facilitated diffusion, and secondary and primary active transport (Figure 1.4). The results presented in this thesis show that facilitated diffusion is the principal mechanism involved in zinc transport. Conventionally the characterization of a facilitated transport system is carried out by studying the kinetics, effects of specific inhibitors , ion-dependence, substrate specificity and substrate stereospecificities.

Kinetics

A carrier protein specifically binds and transfers a solute molecule across the lipid bilayer. This process is similar to an enzyme-substrate reaction, and the carriers behave like membrane-bound enzymes. The carrier protein has a specific binding site for the substrate. When the carrier is saturated ,ie. when all the binding sites are occupied, the rate of transport is maximal. This rate, referred to as V_{max}, is characteristic of the specific carrier. Each carrier protein also has a specific binding constant for its substrate, K_m, equal to the concentration of substrate when the transport rate is half its maximal value (Figure 1.5).

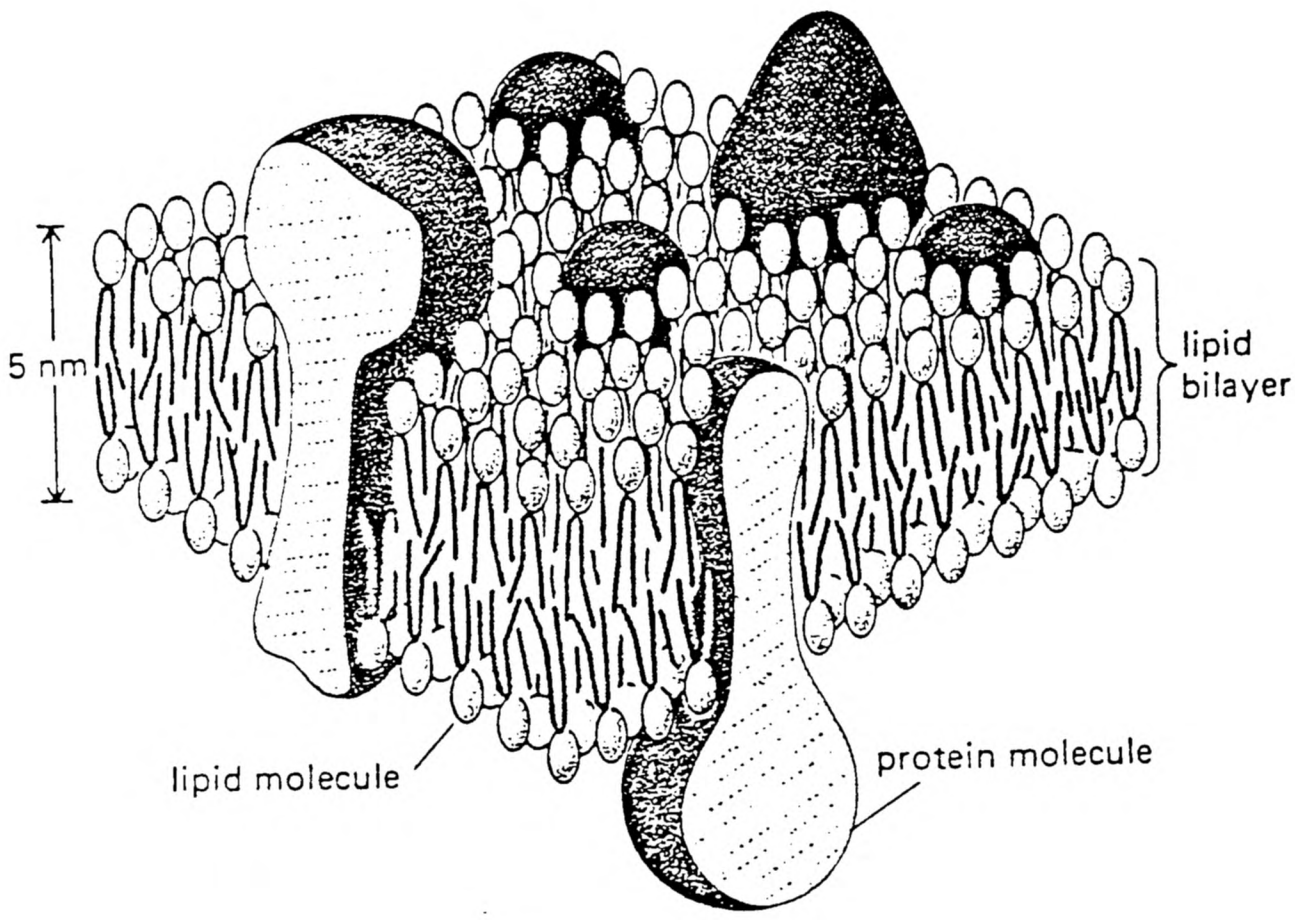


Figure 1.3

Schematic three dimensional view of a small section of a cell membrane.

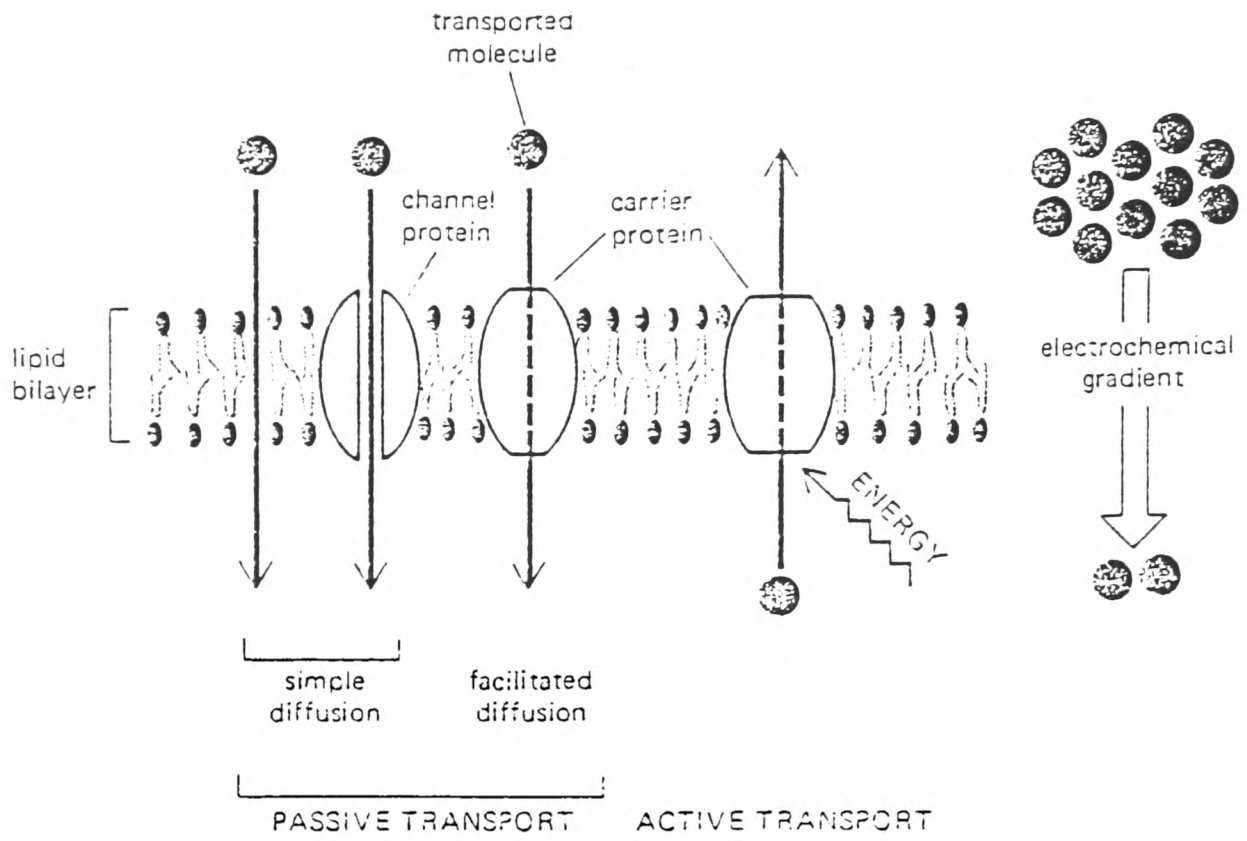


Figure 1.4

Schematic diagram of passive transport down an electrochemical gradient and active transport against an electrochemical gradient.

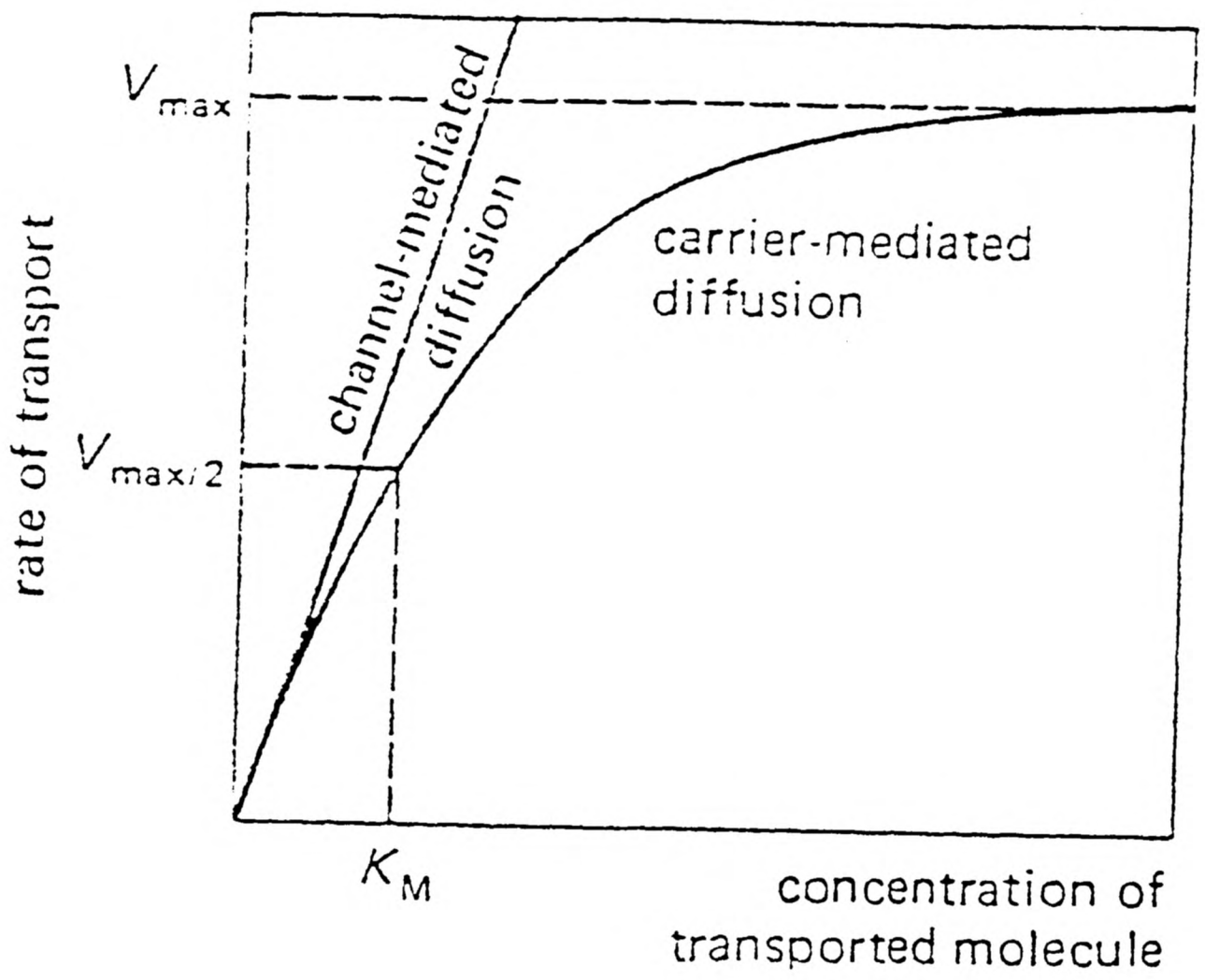


Figure 1.5

Kinetics of channel-mediated diffusion compared to carrier-mediated transport. Whereas the rate of the former is always proportional to the substrate concentration, the rate of the latter reaches a maximum (V_{max}) when the carrier is saturated. The concentration of the substrate when transport is at half its maximal value is defined to be the binding constant (K_m) of the carrier for the substrate.

Inhibitors

Inhibitors change the normal operation of the membrane transporter and can provide fundamental information about its characateristics. There are three kinds of inhibition (competitive, uncompetitive, and noncompetitive) according to the different way in which the kinetic parameters (V_{max} and K_m) of the transporters are altered by the addition of inhibitors (Stein, 1990). In this study, a specific inhibitor DIDS (or SITS) was used to block zinc transport via the anion exchanger. Excess concentrations of paradigm amino acids were employed as competitive inhibitors to inhibit the transport of histidine via defined amino acid transport systems.

Trans-Stimulation

Figure 1.6 shows a diagram of a carrier model. In this model, E represent a carrier molecule that can exist in two conformations E_1 and E_2 which have their binding sites for the substrate S facing sides 1 and 2 of the membrane. In many cases, the conformational changes of the loaded carrier are faster than are those of the unloaded carrier. Thus, a carrier system will often show trans-acceleration of the uptake of radioactive label due to the presence of substrate at the trans face. In contrast, a channel will always show trans inhibition, caused by blockage of the channel in both directions as the substrate concentration builds up at one or other face of the membrane. Hence, the effect of trans-stimulation is a very effective way to distinguish a carrier-mediated process from a ion channel.

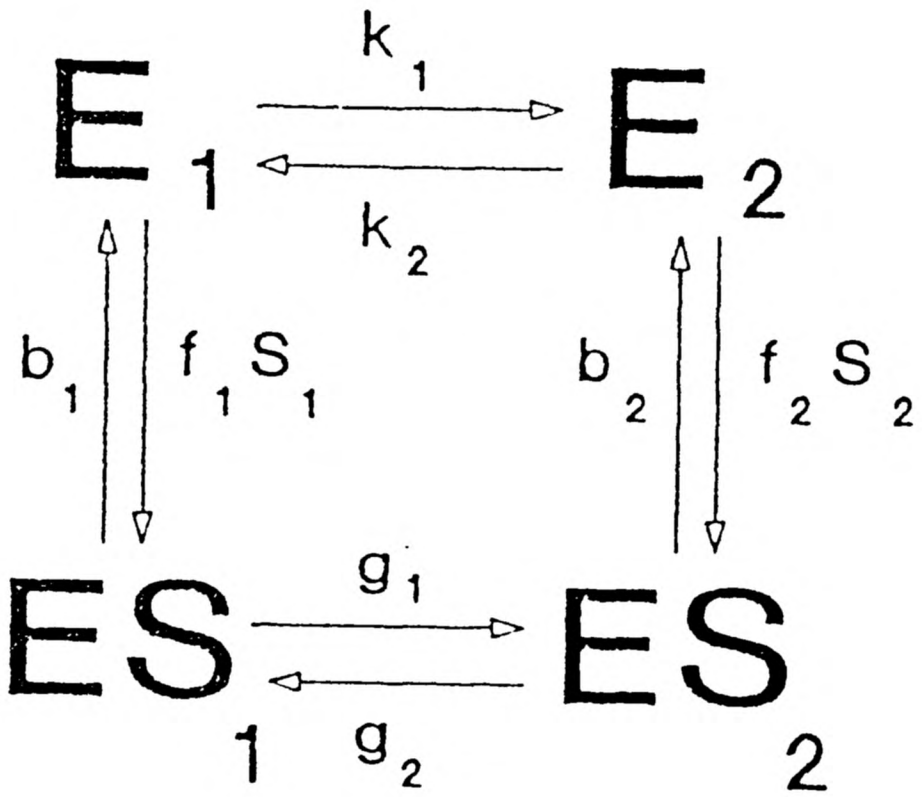


Figure 1.6

Schematic diagram of the carrier model.

Substrate Stereospecificity

Substrate stereospecificity is one of the characteristics of carrier-mediated transport (i.e. to have a preference for a particular stereo-isomer of the same substrate). For example, the glucose transporter prefers D-glucose over L-glucose whereas most of amino acid transporters prefer L-form substrates. It should be noted that although the stereospecificity of substrates is often observed, it is not universal for all known transporters. For example, a L-like system which is found in lamprey red cells and chondrocytes (Chapters 4 & 7) shows no stereospecific preference for leucine. Nevertheless, this characteristic is still useful in ruling out transport by channels and distinguishing between different transport systems.

1.5 Band 3 Anion Transporter

The anion transport protein (band 3), which is abundant in the red cell membrane (10^6 copies per cell), has been very well studied and characterised since its discovery in late 1970s (for reviews see Jennings, 1985 & 1989) (Figure 1.7). The major physiological function of the anion exchanger is to exchange chloride ions for bicarbonate ions across the red cell membrane. The band 3 protein has a molecular mass of 97,000 Da (Stein, 1990) and is a typical intrinsic membrane protein, being firmly bound to the membrane and released only by treating membranes with detergents. The anion transporter has a very wide substrate specificity including bicarbonate, chloride ions, some inorganic anions (nitrate, thiocyanate, sulfate), and many organic anions such as malonate and phthalate. They are chemically varied but ,interestingly, share very similar affinity for the anion transporter and have very different maximal velocities (Stein, 1990). It is

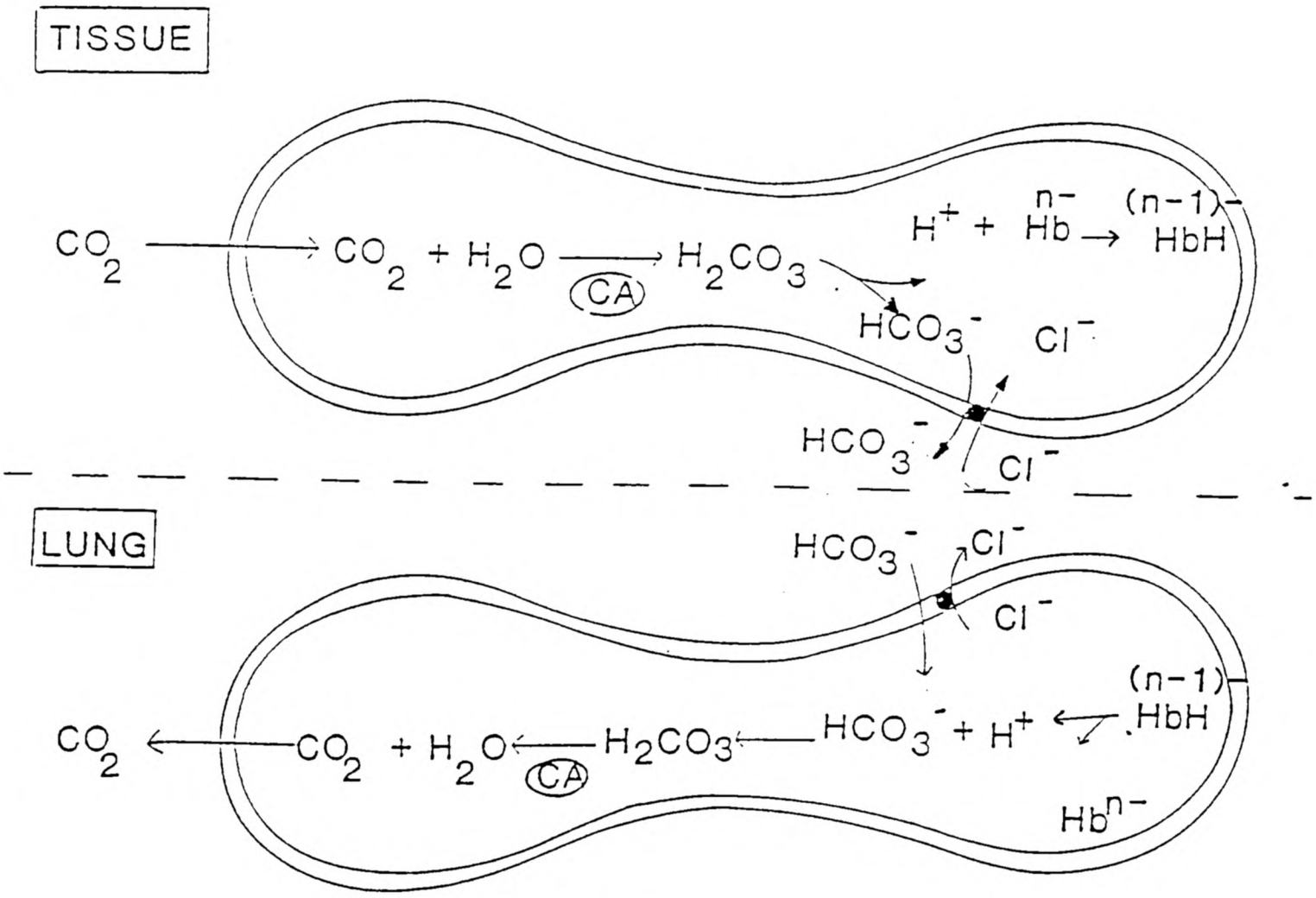


Figure 1.7

In the tissue, CO_2 is soluble in the membrane and crosses by diffusion. Subsequent hydration by carbonic anhydrase (CA) leads to the formation of HCO_3^- and H^+. The proton is buffered by haemoglobin and the HCO_3^- is exchanged with extracellular Cl^- by the band 3 anion exchanger. In the lung, the process is reversed and the band 3 anion exchanges extracelluar HCO_3^- for intracellular Cl^-. The HCO_3^- combines with a proton from the haemoglobin, and in the presence of CA, the CO_2 so produced diffuses out of the red cell. The process is also known as the Chloride shift and the Jacobs-Stewart cycle.

also reported that the band 3 transporter can even transport some cations such as lithium, sodium, potassium (Bernhard, 1979), lead (Simons, 1986), zinc (Alda Torrubia and Garay, 1989; Kalfakakou and Simons, 1990) by forming anionic complexes, in addition to some amino acids (glycine, cysteine, and serine) (Young *et al.*, 1981). The characteristics of this pathway are its anion (Cl^- and HCO_3^-) dependence and its sensitivity to specific inhibitors such as DIDS (4, 4'-Diisothiocyanatostilbene-2, 2'disulphonic Acid) or SITS (4-Acetamido-4'isothiocyanatostilbene-2,2'-disulphonic Acid). In this present research, these two major characteristics of the band 3 transporter have been used in all the cell types studied to identify the involvement of the anion exchanger in transporting zinc.

1.6 Amino Acid Transport Systems

In 1913, Van Slyke and Meyer first reported that animal tissues, in particular the liver, could accumulate amino acids at higher concentrations than the surrounding fluid (Christensen *et al*, 1948). Since then, amino acid transport in different tissues and cells has been studied extensively and many distinct amino acid transport systems have been identified (Christensen, 1984; Ellory, 1987). The identification of a distinct system is achieved by studying the substrate specificity, kinetics, ion dependence, exchange properties, and substrate stereospecificity (Christensen, 1984). One of the major difficulties in defining amino acid transporters, as opposed to, for example, the nucleoside transporter, or the sodium pump, is the lack of any high affinity, specific inhibitors (Ellory, 1987; Barker and Ellory, 1990). In addition to this, the broad overlapping of substrates (one transport system can transport several amino acids with different affinities; several amino acid transport systems contribute to the transport of one amino acid) complicates the identification of

amino acid transport systems. For example, in their work on glycine transport, Ellory *et al* have reported the existence of at least five transport system (Figure 1.8). Table 1.3 lists the major amino acid transport systems identified in mammalian cells.

The results of this thesis suggest that zinc transport is via amino acid transport systems in a form of zinc-histidine complex. In most cell types used, histidine transport has never been studied. Investigation has therefore been carried out in most of these cell types to identify the histidine transport pathways. There are three amino acid transport systems have been of particular interest in their participation in histidine transport, namely system L, y^+, and N. These transport systems are described below in more detail.

1.6.1 System L

System L was first described in the Ehrlich ascites tumour cell by Winter and Christensen (1964) and later confirmed by other groups (Young *et al.*, 1980; Rosenberg, 1981). This L system is most active for amino acids with large apolar branched-chain and aromatic amino acids including leucine, isoleucine, tyrosine, tryptophan, valine, phenylalanine, methionine and glutamine. There is usually a high transport capacity, so that even a poor substrate may achieve a significant flux via system L (Ellory, 1987). System L is ubiquitous, being found in many tissues and among many species (Barker and Ellory, 1990). The substrate stereospecificity of this system is only partial or none (see Chapter 3, 4, & 7).

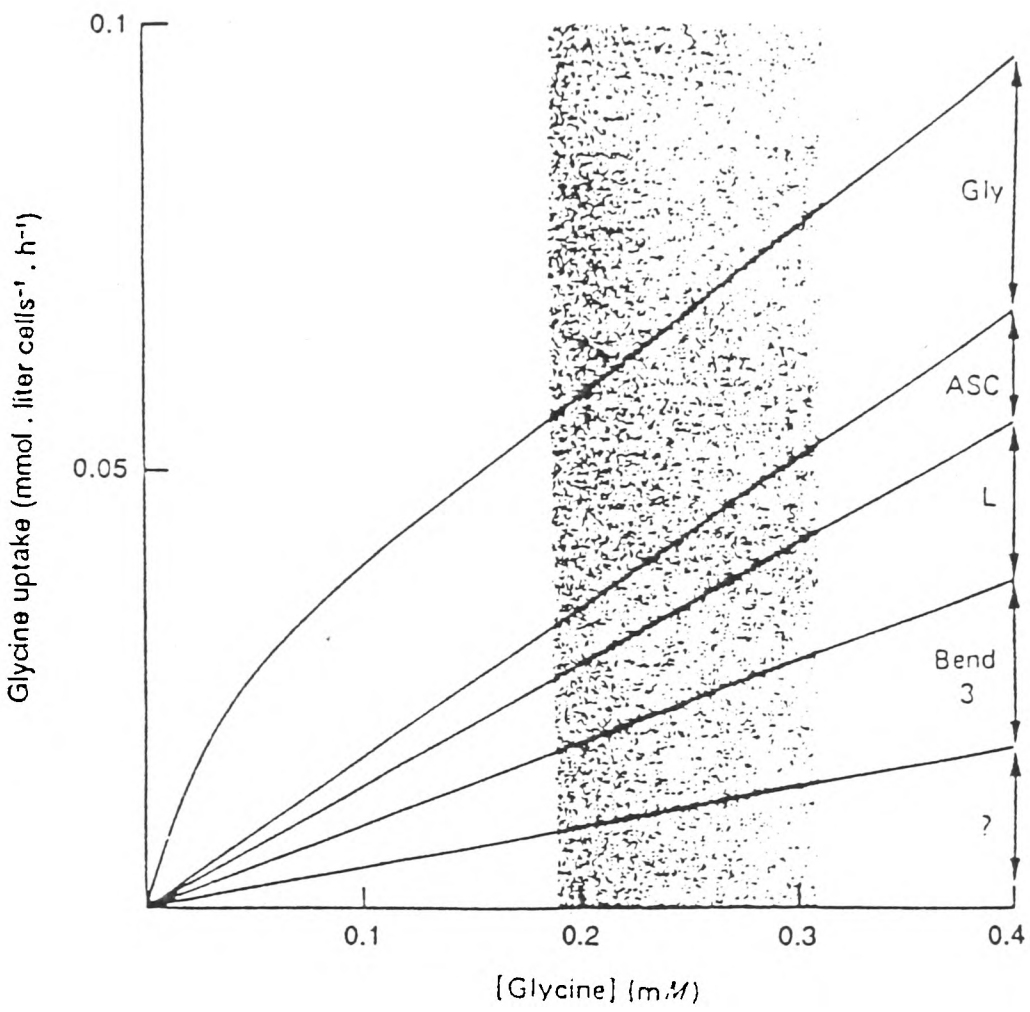


Figure 1.8

Five routes of glycine uptake into human red cells. The upper curve shows total glycine uptake as a function of glycine concentration (at 37^{0}C). The routes marked Gly, ASC, and L represent uptake by three specific amino acid transporters. The route marked 'bend 3' designated glycine that is transported on the anion exchanger band 3, while the route marked with a question mark (?) is by a route which is yet to be identified. Taken, with kind permission, from J.C. Ellory *et al.* (1981).

Table 1.3 Classification of the Amino Acid Transporters

Name of system	Preferred substrates	Na^+-dependency
A	Ala, aminoisobutyric	Yes
ASC	Ala, ser, cys, thr	Yes
Gly	Gly, sarcosine	Yes
L	Leu, phe, trp	No
T	Trp, phe	No
N	Gln, his, asn	Yes
y^+	Arg, lys, orithine	No
x^-	Glu, asp	Yes

Modified from Stein (1990).

1.6.2 System y^+

System y^+ was first described in Ehrlich tumour cells and then identified in human red cells (Gardner and Levy , 1972). It is a very specific transport system for cationic amino acids (lysine, arginine, and ornithine), and shows exceptionally high affinity. For example, system y^+ has an apparent K_mm for arginine of 50μM. As a Na^+-independent system, system y^+ can operate in an exchange mode. Therefore, the flux of histidine via system y^+ is usually found to be strongly *trans*-stimulated by internal substrates (lysine). It is highly stereoselective in favour of the L-isomer of its substrate. Conventionally, it is accepted that system y^+ is the dominant pathway responsible for cationic amino acid entry into cells. However, it has recently been reported that there might be a new, distinct pathway which could also contribute to lysine transport in human red cells (Deves *et al.*, 1991).

1.6.3 System N

Kilberg, Handlogten and Christensen (1980) identified a novel system in hepatocytes which was specific for glutamine, asparagine and histidine. It was called system N to indicate the presence of a nitrogen side chain of its substrates. System N is characterized in the hepatocyte by its sodium dependence, and high sensitivity to changes in pH, and is subject to adaptive regulation (Kilberg *et al.*, 1980). The presence of system N has been reported in human red cells by using kinetic studies with glutamine as the principal substrate (Ellory *et al.*, 1983).

There is slight species variation in amino acid transport systems. Nevertheless, there are at least

two characteristics which have been used in this present study to distinguish between these three amino acid transport systems (L, y^+ and N). They are testing the dependence on Na^+ and substrate stereospecificity.

1.7 Structure of This Thesis

The remainder of this thesis is divided into seven chapters. Chapter Two is a description of materials and methods. Chapters three, four, five, and six deal with the identification of the zinc transport pathways in red cells from different animal species. Each chapter is devoted to a particular species; chapter three, human; chapter four, lamprey; chapter five; sheep; chapter six, human sickle red cells. Chapter Seven presents the result from a study of bovine chondrocytes. Chapter Eight is a general discussion.

CHAPTER TWO

MATERIALS AND METHOD

2.1 Incubation Solutions & Chemicals

In most of my experiments cells were washed and preincubated in isosmotic saline which contained (in mM): NaCl (150), MOPS (3-[N-Morpholino]propanesulfonic acid) (10), KCl (5), D-glucose (5), pH 7.4 at room temperature. When appropriate, 5mM $KHCO_3$ was added to test bicarbonate dependence. In experiments where Na-dependence was studied, NMDG (N-methyl-D-glutamine) was used to replace Na^+. After incubation, fluxes were stopped and cells washed with ice-cold washing solutions containing (in mM): $MgCl_2$ (107), MOPS (10) or NaCl (150), EDTA (Ethylenediaminetetraacetic acid) (10), HEPES (N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]) (10), pH 7.4. Except for lamprey Ringer which has a pH of 7.6, the pH value of all solutions was adjusted to 7.4 with 1M Trizma Base solution (Tris[hydroxymethyl]aminomethane) or concentrated NaOH. Osmolarity of solutions was adjusted by adding sucrose and checked by using a Wescor 5100c Vapour Pressure Osmometer.

Radioisotopes ^{65}Zn, ^{14}C or ^{3}H-amino acid were purchased from The Radiochemical Centre, Amersham or NEN Research Products Division of DuPont. Non-radioactive amino acids and chemicals were obtained either from Sigma Chemical Company, Dorset or BDH Chemicals Limited, Warwickshire.

2.2 Preparation of Cells

Different cell types studied in this thesis consist of fresh red cells from healthy volunteers, out patients with homozygous sickle cell disease, Finnish landrace sheep, river lampreys and bovine chondrocytes. The preparation of different cell types will be described in more detail in individual chapters.

After isolation, cells were washed three times with saline in which the experiment was carried out then resuspended to a haematocrit of 5-10%. Cell suspensions were then incubated for 2-3 hours at 37^{0}C to deplete intracellular amino acids prior to the experiment (Harvey, 1990). The saline medium was changed every half hour during this process.

2.3 Flux Measurement

Radioactive tracer methods (Ellory, 1982) were employed in this thesis to measure the movement of zinc and amino acids across cell membranes. Flux measurements were carried out by incubating isolated cells in the appropriate medium containing the desired radioisotope, at the required temperature and length of time, after which the cells were separated from the medium. The technique employed in flux studies presented in this thesis is a rapid wash technique.

2.3.1 Rapid Wash Technique

Using the rapid wash technique, fluxes under each condition were performed in triplicate. The substance in question and its radioisotope plus any inhibitors under investigation (or equal

amounts of isosmotic sucrose for controls) were added to 1.5ml polypropylene microtubes (Eppendorf). Cell suspensions and stock solutions containing test substrate were usually preincubated in the water bath at the required temperature for at least 5 minutes to allow temperature equilibration. The flux was started by adding stock solutions into tubes containing the cell suspension to a final haematocrit of 5-10%. Fluxes were stopped by taking samples out of the waterbath at the prerequisite time and washing the cells 5 or 6 times immediately with ice-cold washing solution. For incubation periods of more than five minutes the Eppendorf tubes were shaken periodically to prevent settling of cells.

2.3.1.1 Separation

After the incubation period the cells were centrifuged immediately, and supernatant was aspirated. One wash consisted of the addition of 1 ml of washing solution to the pellet; resuspension of the pellet by agitatition using a vortex mixer; rapid centrifugation at 14,000 g for approximately 10 seconds; and aspiration of the supernatant. The cells were washed 5 or 6 times after removal of the original supernatant in order to remove all remaining extracellular tracer. The whole procedure for washing 12 samples 5 times was usually carried out within ten minutes.

2.3.1.2 Scintillation Counting

After the final aspiration the cell pellets were resuspended in 0.5 ml of 0.1% (v/v) Triton X-100 in order to lyse the cells. 0.5ml of 5% trichloroacetic acid was then added to acidify the sample and to precipitate the proteins, and the samples were centrifuged (14000 g) for five minutes. The

supernatant was then transferred to scintillation vials containing 3ml of scintillation fluid (Packard Pico-fluor 40). The samples were counted in a Packard Tri Carb 2000CA Liquid Scintillation Analyzer over the energy spectrum 0-18.6 Kev for ^{3}H isotopes, 0-156 Kev for ^{14}C isotopes and 0-327 Kev for ^{65}Zn isotopes. Counts from a background sample containing 3ml scintillation fluid, 0.5ml 0.1% Triton X-100, and 0.5ml TCA were automatically subtracted from the standards and sample counts. Standards were prepared by adding a known dilution of the tracer to scintillation vials with contents identical to the background samples. The presence of equal amounts of Triton X-100 and trichloroacetic acid in the background, standards, and samples ensured constant quenching.

2.3.1.3 Calculations

The specific activity of the extracellular medium was calculated from the standards as follows: the amount of amino acid (in mmoles) or zinc (in μmoles) in the standard was divided by the counts per minute (CPM) of the standard. The result is the specific activity in mmole CPM^{-1} or μmol CPM^{-1}. This value is then multiplied by the sample CPM giving a value for the total amount of amino acid or zinc crossing the membrane in time, t (min). In the case of red cells, the rate of flux is expressed in terms of mmol (or μmol). l $cells^{-1}$. min^{-1}. Therefore, the above can be summarized in equation form as shown in Equation 2.1.

$$\text{Flux rate} = \frac{CPM_{sample} * [\text{Standard}] * \text{Standard Volume}}{CPM_{standard} * \text{Cell Volume} * \text{Flux Time (min)}}$$

(Eq. 2.1)

For chondrocytes, the rate of flux is usually expressed in mmol (or μmol). 10^6 cells^{-1}. min^{-1}. The above can be equated as follows:

$$\text{Flux rate} = \frac{CPM_{sample} * [\text{Standard}] * \text{Standard Volume} * 10^6 \text{ cells}}{CPM_{standard} * \text{Cell Volume} * \text{Flux Time (min)}}$$

(Eq. 2.2)

The calculation of red cell volume and chondrocyte cell counting will be described in the next section.

2.3.2 Cell Counting

2.3.2.1 Erythrocytes

The volume of packed red cells per ml was measured by calculating the percentage haematocrit present in the solution from a haemoglobin determination. The was done by adding a 0.1ml aliquot of the red cell suspension to 5.0 ml of 1.25g/l Drabkin's Reagent (Sigma) which reacts with the haemoglobin to form cyanomethaemoglobin (Dacie and Lewis, 1975). The absorbance of cyanomethaemoglobin was then measured at 540 nm in a Perkin-Elmer Lambda 5UV/VIS spectrophotometer using unreacted Drabkin's Reagent as the blank. For human, lamprey, sheep red cells, the Drabkin's Conversion Factor is 247, 185, and 247 respectively (Ellory and Kirk, personal communication). The formula used for the conversion is set out below in equation 2.3.

$$\% \text{ Hct} = \frac{\text{Absorbance} * \text{Dilution Factor} * 100}{\text{Drabkin's Factor}}$$

(Eq 2.3)

where, absorbance is at 540nm and dilution factor is 51 in this case. This was repeated at least 3 times and the mean calculated. Multiplication of flux rate/ml by 1000 and division by the % haematocrit gives the rate of flux in terms of mmol (or μmol). 1 $cell^{-1}.min^{-1}$.

2.3.2.2 Chondrocytes

A 100µl aliquot of the isolated chondrocyte suspension in incubation medium was added to 200µl of 0.5% (w/v) of the vital dye Trypan Blue. A sample was placed on an improved Neubauer haemocytometer stage and examined under a light microscope (Olympus BH-2). The haemocytometer chamber consists of 25 large squares enclosed by triple lines, each of which is divided into 16 smaller squares. The total area of the 25 square chamber is 1mm and the depth beneath the coverslip is 0.1mm (Thonstone & Thorpe, 1988). Therefore, the total volume of cell suspension in the chamber is $0.1mm^3$. Conversion of the number of cells counted cells per square by 10,000 and by the dilution factor, this was repeated at least 4 times and the mean evaluated.

2.4 General Experimental Protocol

2.4.1 Zero-Trans Flux

Cells were depleted of intracellular amino acids by incubating at 37^0C for at least one and an half hours prior to the start of experiments. The majority of intracellular zinc is tightly bound to haemoglobin or enzymes. Negligible efflux was measured (Kalfakakou and Simons, 1990). A small fraction of ^{65}Zn counts measured was due to zinc binding to the cell surface, and accounted for less than 15% of total counts measured. The uptake rate for zinc was then calculated by subtraction of zinc surface binding from the total counts measured (see section 3.1 for details).

2.4.2 Time Course for Initial Uptake

When measuring the rate of uptake of any test molecule through a membrane, it is important to stop the flux before any backflux becomes significant. This is in order to maintain the amount of tracer in the cell in proportion to the molecules taken up during the flux period. In order to avoid any significant backflux it is necessary to determine the time course of the tracer accumulated by the cells and then to use a time point in subsequent experiments which is within the linear part of the curve.

Time course experiments were carried out by incubating the cells for the desired incubation period such that all the tubes could be removed from the waterbath simultaneously. Subsequent separation of cells from the extracellular medium was achieved by immediately using the rapid wash technique (see section 2.3.1).

2.4.3 Concentration-Dependent Uptake

After determining the time course for the initial uptake, it is usual to investigate the effect of substrate concentration on rates of uptake. If a plot of the initial uptake rate against substrate concentration gives a hyperbolic relationship, then this is good evidence for saturation kinetics with the data fitting the Michaelis-Menten equation (Eq 2.4).

$$v = \frac{V_{max} * [S]}{[S] + K_m} \quad \text{(Eq 2.4)}$$

where [S] is the concentration of substrate

V_{max} is the maximum rate of uptake

K_m is that substrate concentration which gives half the maximum rate

However, transport in biological situations rarely occurs by a single route but involves a variety of transport systems. The relationship between v and [S] in equation 2.4 under these circumstances would represent the sum of several components. A substrate may be transported by a saturable route and a component which has a linear relationship between rate and concentration which, in the simplest case, would represent passive diffusion (Eq 2.5).

$$v = \frac{V_{max} * [S]}{[S] + K_m} + k_d * [S] \qquad \text{(Eq 2.5)}$$

where [S] is the concentration of substrate

V_{max} is the maximum rate of uptake

K_m is that substrate concentration which gives half the maximum rate

k_d is the slope of the linear component (expressed as rate constant, sec^{-1}).

2.4.4 Estimation of Kinetic Parameter, K_m and V_{max}

The estimation of the kinetic parameters, K_mm and V_{max} has been done conventionally by rearranging equation 2.4 such that a plot of the data was linear; the Lineweaver-Burk (1/v vx. 1/[S]); Hanes ([S]/v vs. [S]); or Eadie-Hofstee (v vs. v/[S]). These were developed originally because it was difficult to draw a hyperbola through a set of experimental points.

Recently, software packages have become available which allow the fitting of data directly to the v against [S] hyperbola and the kinetic parameters computed from the best fit. "Enzymefitter" by Elsevier-Biosoft, Cambridge was used to fit the data in this thesis. This involved an iterative least squares nonlinear regression programme where estimates of K_m are repeatedly made until a minimum error occurs at which point the Vmax value and, if necessary, the k_d value are derived.

2.4.5 Other Characteristics

Four types of experiments were employed frequently in this thesis to characterize the transport system in question. They were performed to investigate Na-dependence, inhibitory effects, trans-stimulation and substrate stereospecificity of the transport system. A brief description for each type of experiment will be given below.

2.4.5.1 Na-Dependence

The difference in substrate uptake rates in Na-containing or Na-free media represents the Na-

dependent component. In such experiments, measurement of flux rate were carried out by incubating cells in control and Na-free medium. Na^+ was replaced by NMDG and the pH of the medium was adjusted to 7.4 by using concentrated HCl stock solution.

2.4.5.2 Inhibitor Experiments

Inhibitors (DIDS, SITS and competiting amino acids) were added to discriminate the transport routes. Uptake rate of substrate was measured within the period of the initial uptake in the presence and absence of inhibitors (10μM DIDS or SITS; 20 fold excess concentration of paradigm amino acids).

2.4.5.3 Trans-Stimulation

Trans-stimulation is an important property to distinguish a facilitated transport system from ion channels. Cells were preincubated with excess paradigm substrates or sucrose for at least 3 hours, washed 3 times and resuspended in the medium. Uptake of test substrate was then measured in control cells and in cells preloaded with substrate.

2.4.5.4 Substrate Stereospecificity

Uptake rates of test substrate were measured within a period of the initial uptake in the presence

of L-,or D-form competitive substrates in the medium. The difference in the two conditions represented the substrate stereospecificity of the transport system in question.

2.4.6 Estimation of Ionic Zinc, (Zinc-histidine) Complexes Concentration

Zinc can bind to histidine to form zinc-histidine complexes. The equilibrium can be expressed as:

Zn^{2+} + 1 histidine $\rightleftharpoons$ $(Zn\text{-histidine})^{+}$ (K_1)

Zn^{2+} + 2 histidine $\rightleftharpoons$ $(Zn\text{-histidine}_2)$ $(ß_2)$

$$K_1 = \frac{[(Zn\text{-histidine})^{+}]}{[Zn]_{free} * [histidine]_{free}} \qquad ß_2 = \frac{[Zn\text{-histidine}_2]}{[Zn]_{free} * [histidine]^2_{free}}$$

The two apparent stability constants were reported as $K_1=8* 10^4\ M^{-1}$and $ß_2= 2* 10^8\ M^{-2}$(Wensink, 1988) or $K_1=10^{6.7}\ M^{-1}$and $ß_2=10^{11.8}\ M^{-2}$ (Dawson *et al.*, 1986). A computer program in Fortran (shown on the facing page) was written to esitmate the concentration of Zn^{2+} and $(Zn\text{-histidine})^{+}$ using the former set of values for the stability constants. Table 2.1 lists the results of these estimated concentrations under the experimental conditions investigated in the present study.

2.5 Statistical Methods

Results presented in this thesis are the mean ± the standard error of the mean (sem) unless otherwise indicated where n is the number of independent determinations used to obtain the mean. Each observation of flux was the mean of duplicate or triplicate samples. TheStudent's t-test (paired or unpaired, as appropriate) was employed to test significance; differences were judged to be significant if the p value was less than 0.05. In experiments where results have been

normalised and expressed as a percentage of the control, the p value expressed was obtained from using the paired t-test on the raw data prior to normalisation.

Table 2.1 Estimations for Zn^{2+} & (Histidine-Zn)$^{+}$ Concentrations

$[His]_{total}$ (mM)	$[ZnCl_2]_{total}$ (μM)	$[Zn^{2+}]$ (μM)	$[(His\text{-}Zn)^{+}]$ (μM)
1	5	0.02	1.43
1	25	0.10	7.33
1	30	0.12	8.80
1	50	0.21	15.14
1	75	0.34	23.43
1	100	0.48	32.27
1	150	0.86	51.72

CHAPTER THREE

ZINC TRANSPORT IN HUMAN RED CELLS

Introduction

The band 3 anion exchanger has been reported recently to be the major route for the entry of free zinc into human erythrocytes, and different chemical formulae have been suggested for the species involved ($ZnCO_3Cl^-$, $Zn(HCO_3)Cl.OH^-$ and $Zn(HCO_3)_2Cl^-$ (Alda Torrubia and Garay, 1989; Kalfakakou and Simons, 1990). However, the experiments on which these conclusions were based were carried out with 2-100 μM $ZnCl_2$ in a medium containing no binding ligands. The concentration of free zinc in human plasma is negligible due to the fact that zinc binds to many ligands such as albumin, α_2-macroglobulin and amino acids (Cousins, 1985). The actual value of free zinc concentration in human plasma is not known, but a concentration of $2.1 * 10^{-10}$ M was measured in horse plasma (Magneson *et al.*, 1987). Therefore, the physiological relevance of the proposed pathway needs to be confirmed by studying zinc transport at near physiological conditions. It was reported recently that one of the zinc binding amino acids, histidine, stimulated zinc transport in rat erythrocytes (Aiken *et al.*, 1992). It was further suggested that the amino acid transport system might be involved in transporting the zinc-histidine complex; the precise mechanism remaining unclear (Aiken, 1992). In this chapter, these two mechanisms of zinc transport in human red cells have been studied in more detail. Firstly, the physiological significance of the band 3 pathway was assessed by adding albumin, the most abundant and important zinc-binding ligand in plasma, to the medium. Secondly, the effect of histidine on zinc

transport was investigated. The results showed that histidine did not only function as a zinc-binding ligand, but further that the zinc-histidine complex could actually be transported across red cell membranes via a carrier-mediated process. Finally, due to the poor understanding of histidine transport and the interest of histidine involvement in zinc movement, the transport of histidine in human red cells was studied aiming at identifying the principal pathways for the entry of histidine into cells.

Materials & Methods

Freshly drawn, heparinized human blood was centrifuged and the buffy coat removed. The red cells were washed 3 times with the medium in which the experiment was performed. After the final wash the cells were suspended at a haematocrit of 10% and incubated at 37 ^{0}C for at least two hours to deplete intracellular amino acids.

Tracer flux experiments were started by adding the zinc stock solution with radioactive ^{65}Zn into the cell suspensions at 37^{0}C. After the chosen incubation period, the cells were then immediately washed 5 times with ice cold media containing (mM): NaCl(150), HEPES(10), EDTA(10), pH 7.4 at room temperature to remove the extracelluar radioactivity. EDTA was added to remove most of the surface-bound zinc (Aiken *et al.*, 1990). Unless specified otherwise, the incubation medium contained (mM): NaCl(150), MOPS(10), D-glucose(5), KCl(5), buffered to pH 7.4 with 1M Tris at room temperature. Bicarbonate (5mM) was added to the medium as required. When DIDS or SITS were employed in the experiments to block the band 3 pathway, red cells were preincubated with the inhibitor at 37^{0}C for 30 minutes prior to the experiments; this period was found to be

efficient for DIDS and SITS to block the anion exchanger (data not shown). For each experiment stock solutions of DIDS or SITS (both 10mM in standard saline) were freshly prepared.

Results

3.1 Time Course for Zinc Uptake in Human Red Cells

Zinc uptake (5-250μM) into human red cells has a linear relationship with time for up to one hour after the isotope was added (Figure 3.1). Therefore a fixed incubation period of 15-30 minutes was chosen to measure the initial uptake rate of zinc in all following experiments. At the time zero, there was a small fraction of ^{65}Zn measured which could be due to ^{65}Zn binding to the cell surface. This value was subtracted when calculating the uptake rates.

3.2 Effect of DIDS (or SITS) & Bicarbonate

Uptake of zinc (30μM) was greatly inhibited by the presence of anion exchanger inhibitors DIDS or SITS (Figure 3.2); treatment with 10μM DIDS (or SITS) abolished over 90% of the zinc influx. In this study there was found to be no difference between the inhibitory effect of DIDS and SITS on zinc transport via the band 3 transporter (data not shown). Zinc uptake (30μM) was stimulated greatly by the presence of 5mM bicarbonate in the medium (Figure 3.3). The uptake rate of zinc (30μM) was increased by more than 5 fold from 2.6 ± 0.4 μmol.l $cell^{-1}.min^{-1}$ (±sem, n=3) in bicarbonate-free medium to 15.5 ± 1.1 μmol.l $cell^{-1}.\ min^{-1}$ (±sem,n=12) in bicarbonate-containing

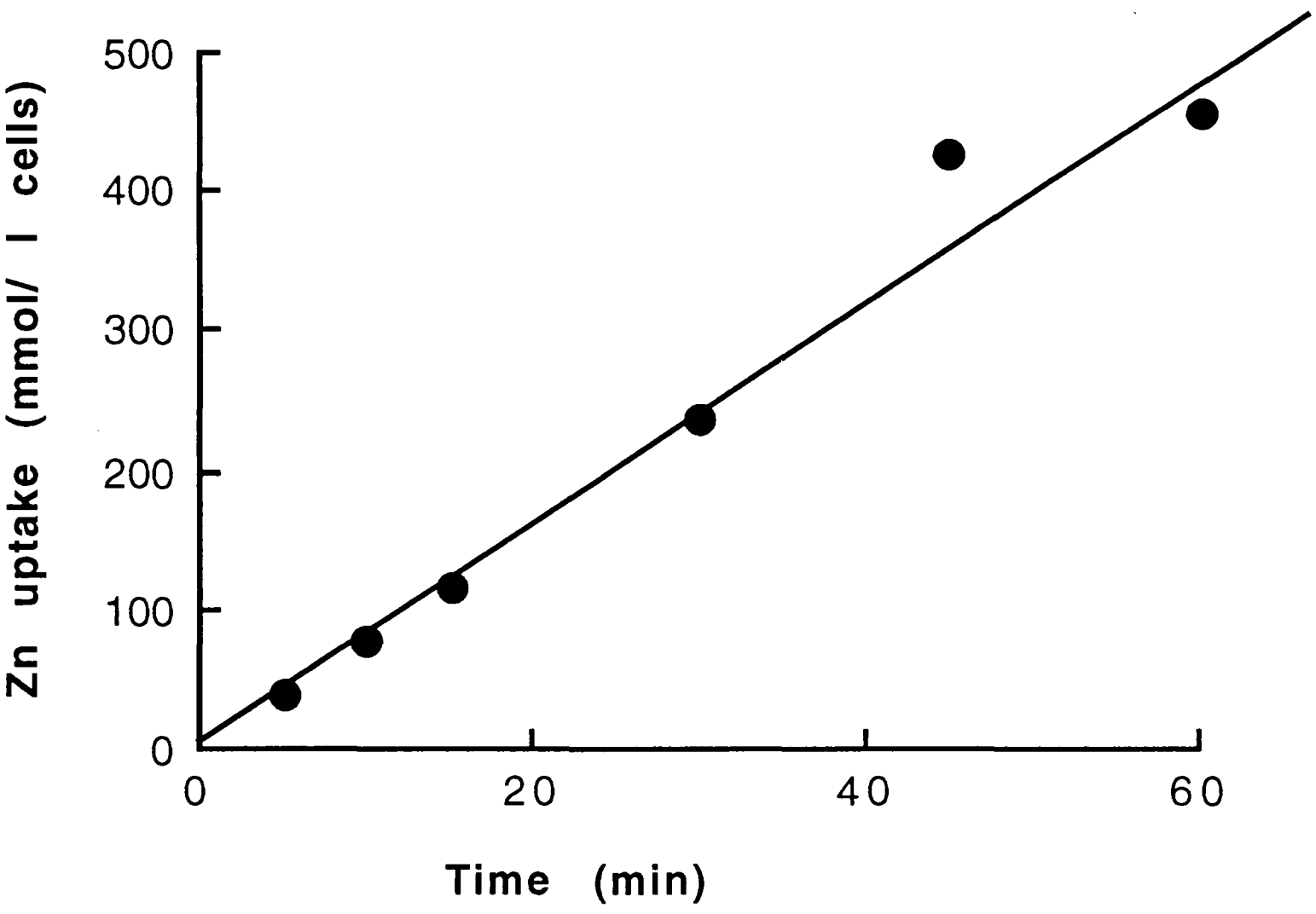


Figure 3.1

Time course for zinc influx into human red cells. The external Zn concentration was 0.25mM. The incubation medium contained(mM): NaCl(145), HEPES(10), glucose(5), $KHCO_3$(5). The uptake rate was 7.63 µmol.l $cell^{-1}.min^{-1}$ (r=0.999).

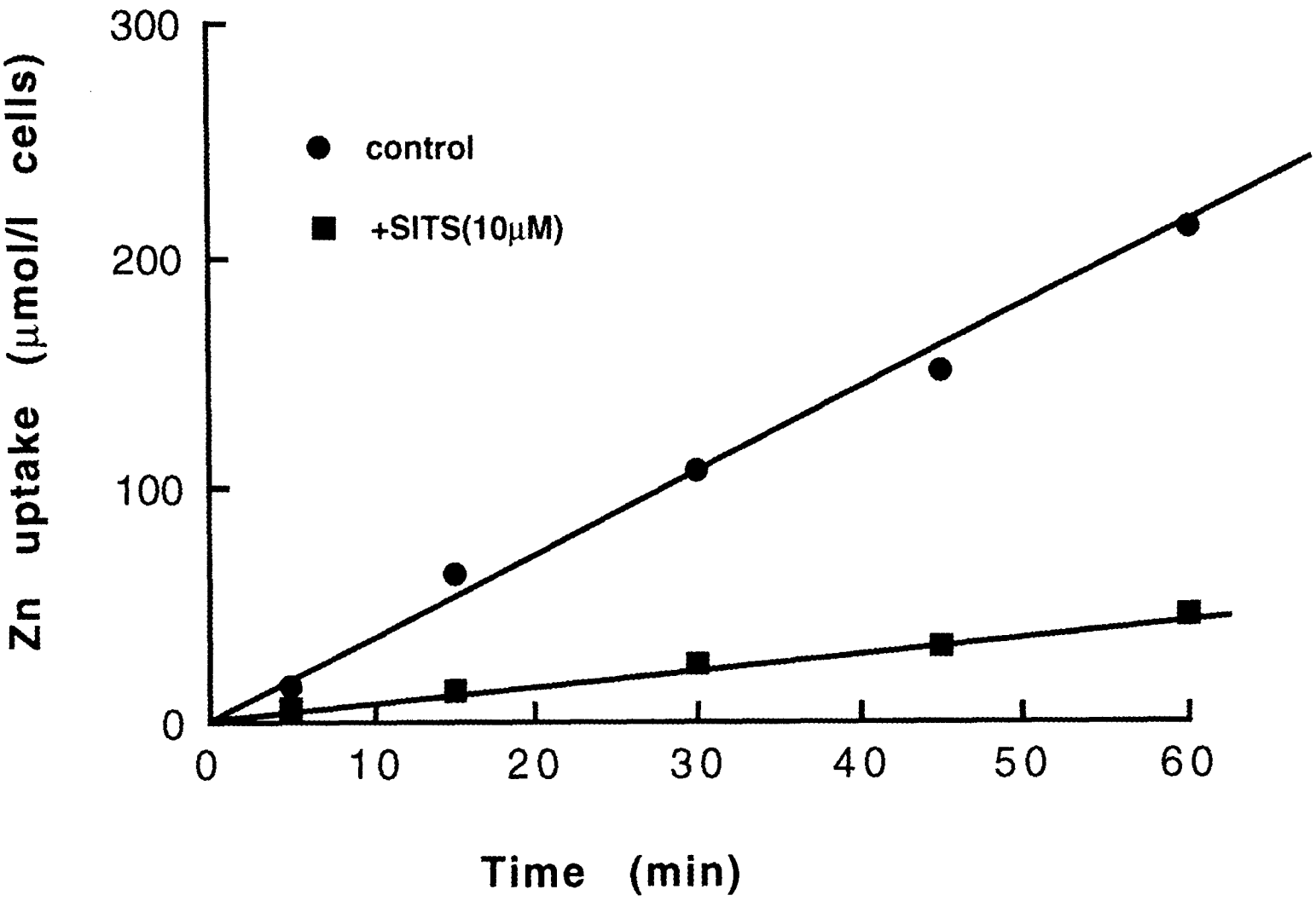


Figure 3.2

An example of the effect of SITS on Zn uptake. The external Zn concentration was 30μM. Addition of 10μM SITS inhibited over 90% of the flux. The uptake rate was 3.44 μmol.l cell^{-1}.min^{-1} (r=0.997) in the control group and 0.72 μmol.l cell^{-1}.min^{-1} (r=0.995) in the SITS treated group.

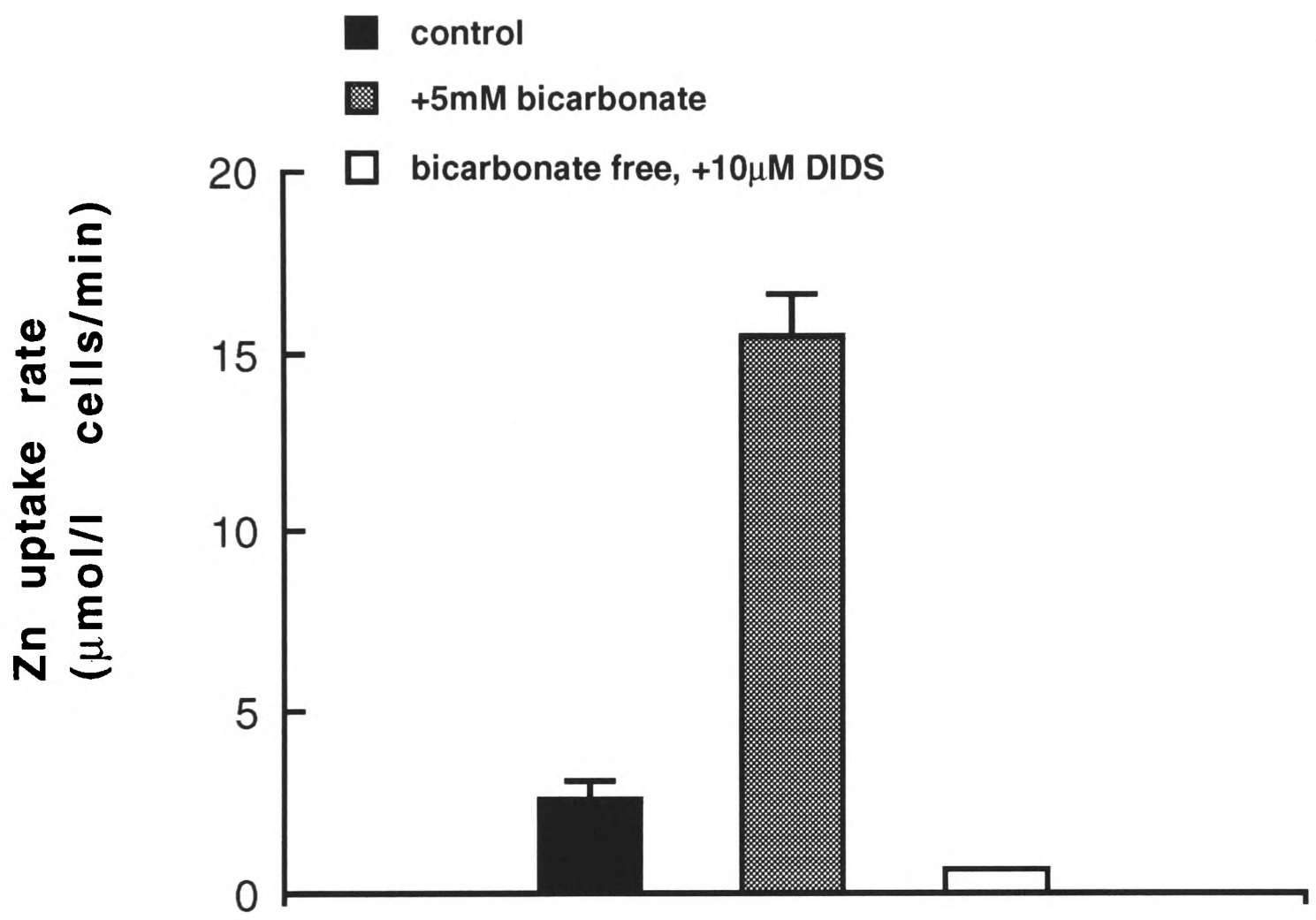


Figure 3.3

Zinc uptake (30μM) in human red cells. The measurements were conducted over 15 minutes. The uptake rates of zinc were 2.61 ± 0.43 (n=3); 15.54 ± 0.14 (n=12); 0.62 ± 0.07 (n=4) (±sem) for the control, bicarbonate-containing, and DIDS-treated groups respectively.

media.

3.3 Effect of Albumin

Though the band 3 pathway appears to be the dominant route for free zinc uptake, it should be emphasised that the vast bulk of plasma zinc is not free but is bound to a variety of ligands (see section 1.2). The most important of these is albumin which is present in human plasma of a concentration of 2-3.5 % (g/dl) (Dittmer, 1961). Zinc uptake (30μM) was measured in the presence of 1% bovine serum albumin (BSA). Figure 3.4 shows that the presence of 1% BSA in the medium abolished nearly all the uptake of zinc (10-150μM) (over 98%). Notice that these experiments were conducted in the medium containing 5mM bicarbonate which allowed the anion exchanger route for zinc transport to be activated. Thus in the presence of albumin, zinc transport via band 3 pathway was greatly reduced if not completely prevented.

From the result above, it seemed that the band 3 pathway was unable to transport zinc when in the albumin-bound form. This form of Zn complex accounts for the biggest exchangeable pool of zinc in the systemic circulation (Cousins, 1985). Thus, the band 3 pathway might not be a significant route for zinc transport under physiological conditions. In the rest of the chapter, alternative pathways for the transport of zinc across the erythrocyte membrane were investigated. To ensure that the zinc flux via the band 3 component did not contribute to the fluxes measured, in the subsequent experiments bicarbonate-free media were used and red cells were pretreated with DIDS or SITS unless otherwise stated.

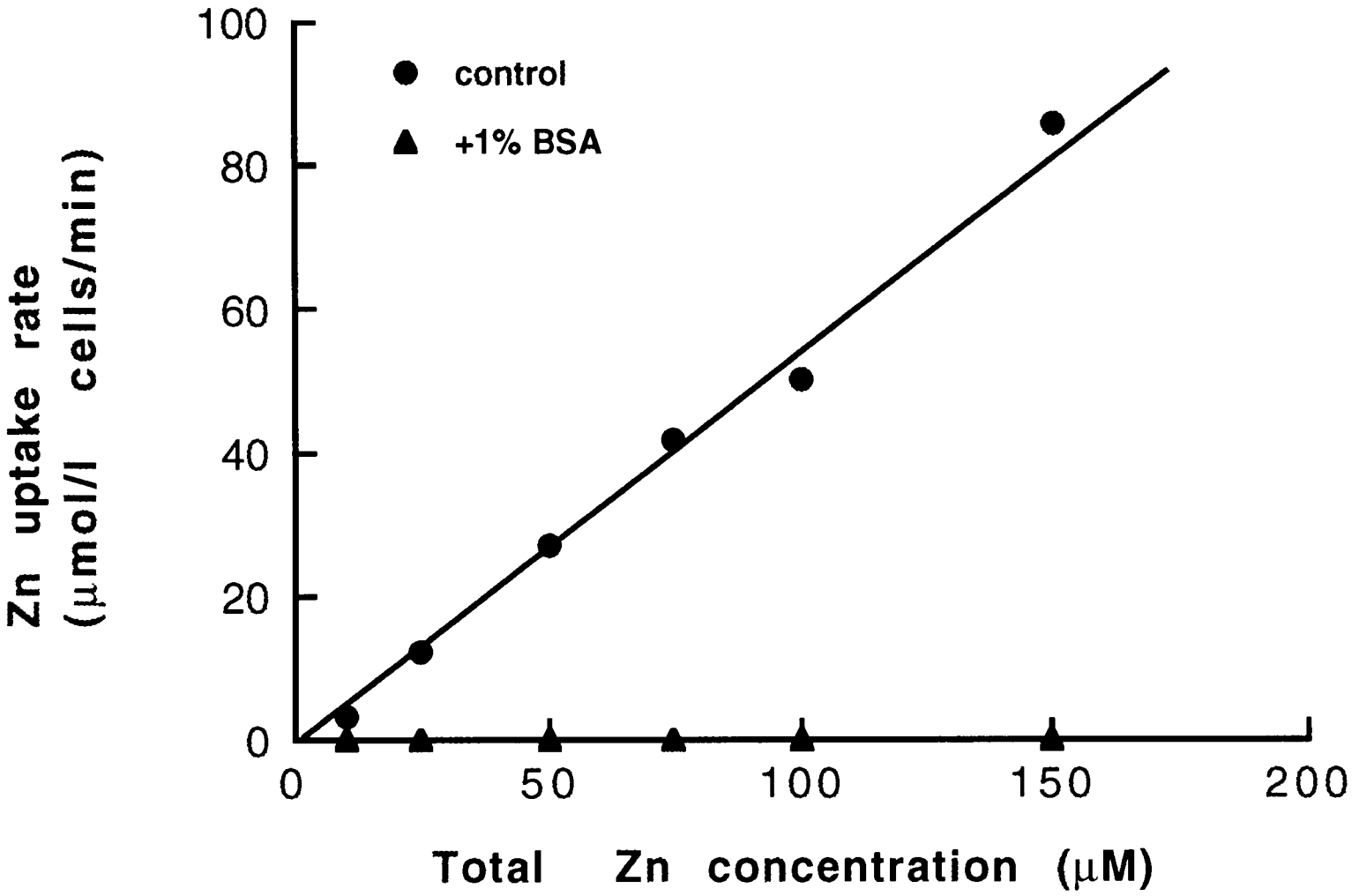


Figure 3.4

Effect of 1% bovine serum albumin on zinc uptake over a range of 10-150 μM. The medium contained 5mM bicarbonate.

3.4 Effect of Histidine

1mM L-, or D-histidine was added to the medium to study the effect on zinc (30μM) uptake. The results are shown in Figure 3.5. As shown in Figure 3.5 (a), the presence of histidine (either L- or D- form) caused a decrease in zinc uptake when compared to the uptake rate of total zinc concentration of 30μM. The decrease in zinc uptake rates caused by histidine was expected as we observed the similar chelating effect in the presence of albumin (see section 3.3). The concentration of free zinc (ionic zinc) was greatly reduced because of the formation of zinc-ligand complexes when the ligands were present in the medium. In the case of albumin, since the (albumin-zinc) complex was not able to enter the cells, the uptake of zinc was mostly prevented because there was almost no ionic zinc available in the medium to cross the cell membranes. In the case of histidine, the free ionic zinc concentration was estimated to be about 0.12 μM (see section 2.4.6). At this concentration the rate of zinc uptake (assuming that only the free zinc but not zinc-histidine complexes were transported) was about 0.002 μmol.l $cell^{-1}.min^{-1}$. However, in the presence of L-histidine, the rate was 2.1± 0.1 μmol.l $cell^{-1}.min^{-1}$ (±sem, n=3) and in the presence of D-histidine the rate was 0.07± 0.004 μmol.l $cell^{-1}.min^{-1}$ (±sem,n=3). These values are respectively 100- and 35-fold higher than that expected if only the free zinc is transported (Figure 3.5(b)).

3.4.1 Concentration-Dependent Uptake

The extracellular concentrations of both zinc and histidine were changed to study the relation between the concentration of the (zinc-histidine) complex and zinc uptake in human red cells. In

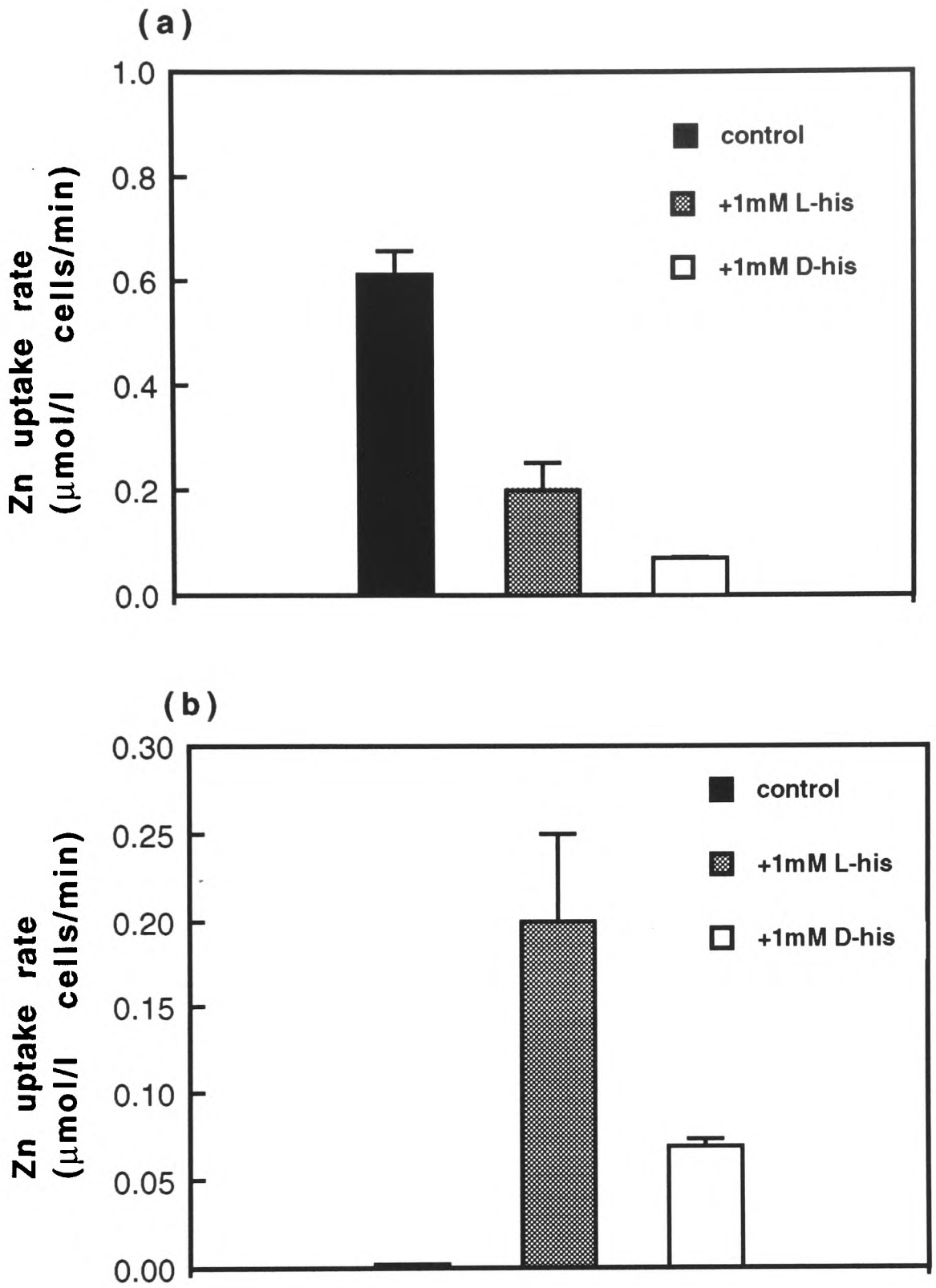


Figure 3.5

Effect of 1mM histidine on Zn uptake. (a) The uptake rate of zinc (in a total concentration of 30μM) was 0.62 ± 0.07 μmol.l cell^{-1}.min^{-1} (±sem, n=3). (b) The uptake rate of zinc (in a free zinc concentration of 0.01μM) was 0.002 μmol.l cell^{-1}.min^{-1}. Zinc uptake rate in L-histidine-containing medium was 0.2 ± 0.1 μmol.l cell^{-1}.min^{-1} (±sem, n=5) and 0.07 ± 0.004 μmol.l cell^{-1}.min^{-1} (±sem, n=4) in D-histidine containing medium.

all these experiments, the entry via the band 3 pathway was prevented by pretreating red cells with DIDS and using bicarbonate-free saline. First, the uptake rates of ^{65}Zn were measured in the medium which contained 5-150μM $ZnCl_2$ and 1mM L-, or D-histidine. The concentrations of zinc-histidine complex were estimated according to the method described in section 2.4.6. The uptake of zinc-histidine was found to be saturable according to the external (Zn-histidine) concentration, which gave a good fit to the Michaelis-Menten equation (Figure 3.6). The kinetic parameters were estimated by the method described in section 2.4.4 and are listed in Table 3.1. Secondly, the uptake rates of ^{65}Zn were measured in the medium which contained 1% BSA, 30μM $ZnCl_2$, and L-,or D-histidine (0-1mM). The results are shown in Figure 3.7. The uptake rates of ^{65}Zn measured were increased with the increase of external L-histidine concentration while the increase of D-histidine concentration caused a gradual decrease in ^{65}Zn uptake rates.

3.4.2 Effect of Albumin

In a previous section (section 3.3), albumin was used to test the physiological significance of the band 3 pathway in its ability to exchange with albumin-bound zinc. In the same way, the importance of the entry of zinc in a form of zinc-histidine complex needs to be tested by studying the effect of albumin on this mechanism of zinc transport. Alanine which is a non-zinc-binding amino acid, was chosen to compare with histidine. The uptake of zinc (30μM) was measured in the presence of 1% BSA and 1mM L-,D-histidine or alanine. The result of Figure 3.8 shows that when the medium contained BSA, the uptake of zinc was greatly reduced to a very small magnitude and only L-histidine was found to be able to increase significantly zinc uptake rate ($p=0.036$, $n=6$) whereas other amino acids (D-histidine, L-,D-alanine) failed to have any effect.

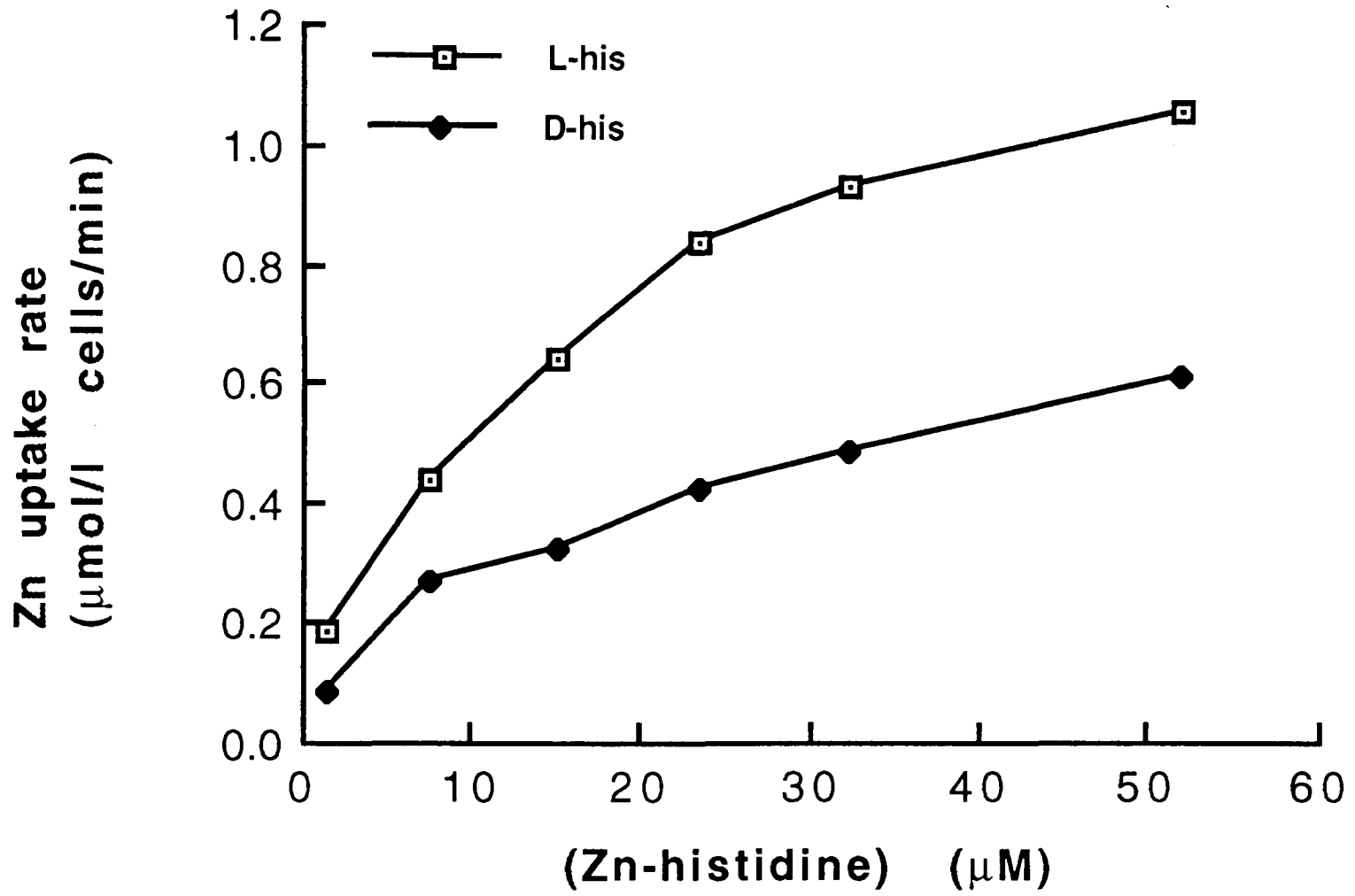


Figure 3.6

Concentration-dependent histidine linked-zinc uptake in human red cells. The red cells had been pretreated with 10 μM DIDS to inhibit the anion exchanger.

Table 3.1 Estimation of Kinetic Parameters for (Zinc-histidine) Uptake

	Kinetic parameters (mean ± sem, n=3)
(L-histidine-zinc)	V_{max}=0.83 ±0.25 μmol. l cell^{-1}. min^{-1}
	K_m= 10.22 ± 3.96 μM (L-histidine-zinc)
	k_d= 0.006 ± 0.002 sec^{-1}
(D-histidine-zinc)	V_{max}=0.57 ± 0.37 μmol. l cells^{-1}. min^{-1}
	K_m= 9.80 ± 6.40 μM (D-histidine-zinc)
	k_d= 0.003 ± 0.002 sec^{-1}

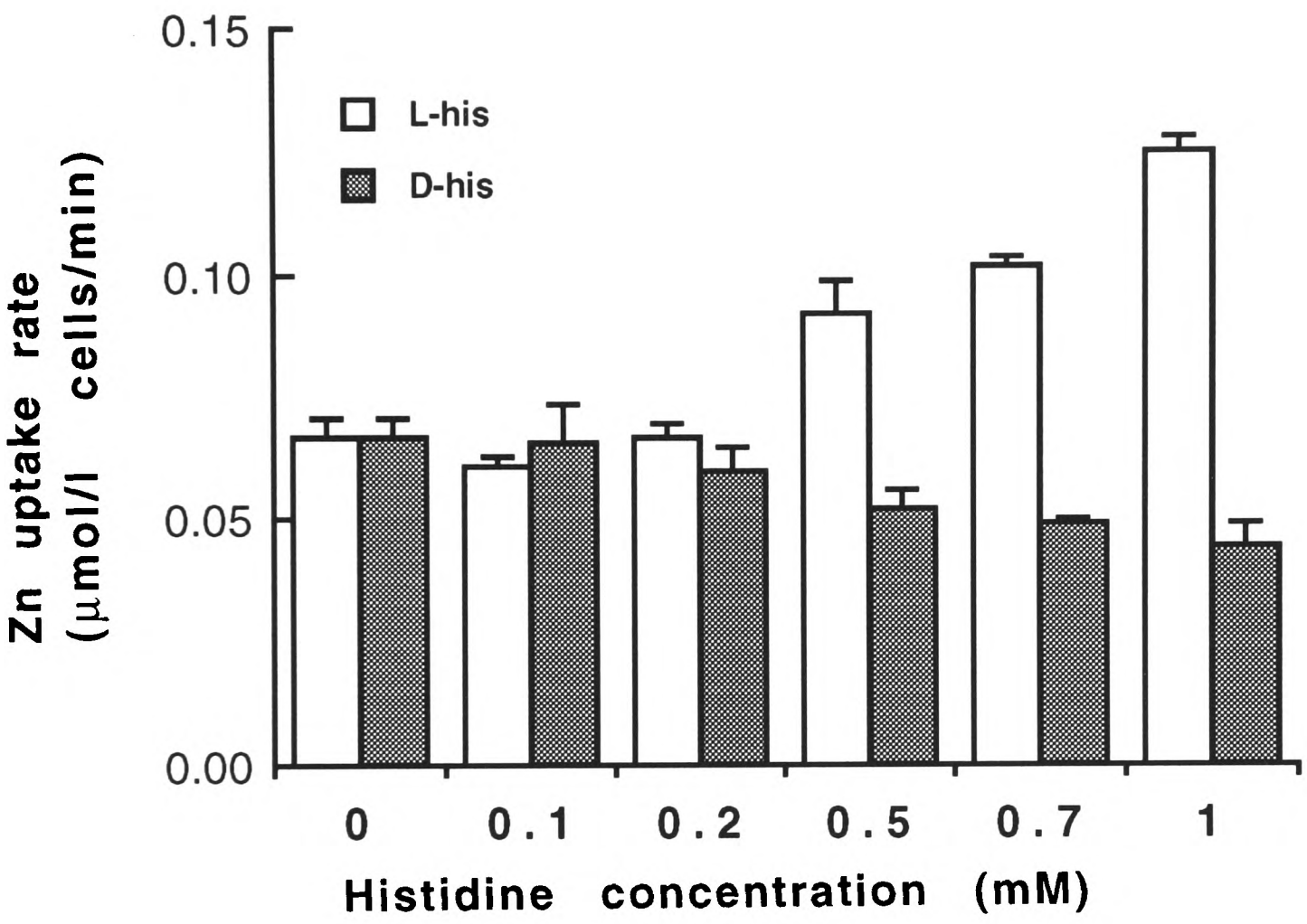


Figure 3.7

Effect of L-,D-histidine (0-1mM) on zinc uptake. The medium contained 1% BSA. Red cells were treated with DIDS and bicarbonate-free medium was used.

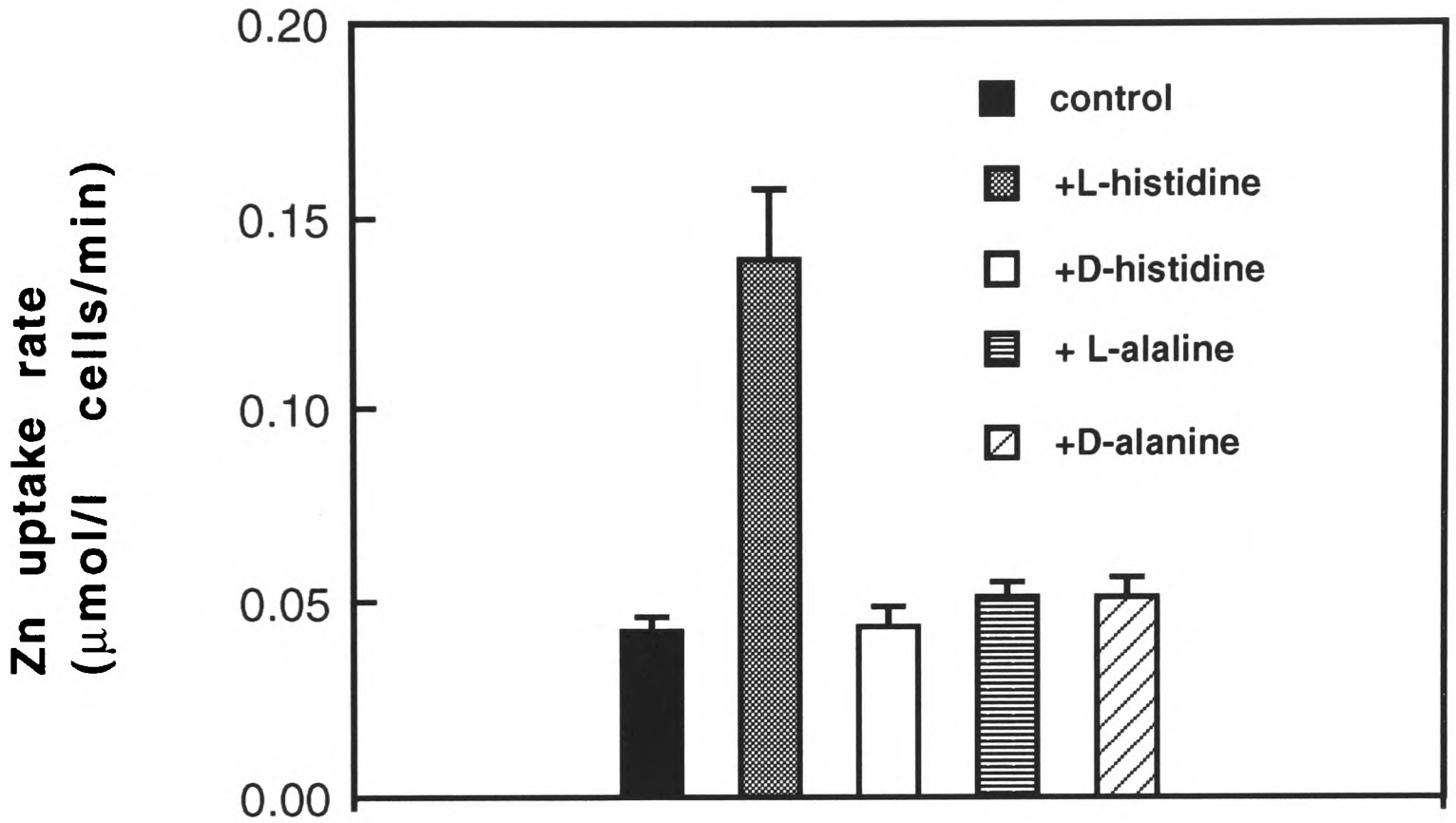


Figure 3.8

Effect of 1% BSA on histidine-linked zinc uptake. Zinc uptake (30μM) was measured in the presence of 1mM L,D-histidine and alanine. The bicarbonate-free medium contained 1% of BSA. The uptake rates of zinc were 0.04 ± 0.004; 0.14 ± 0.02; 0.04 ± 0.01; 0.05± 0.003; 0.05 ± 0.01 μmol.l $cell^{-1}.min^{-1}$ (± sem, n=3 except for the L-histidine-containing group, n=6) respectively.

Thus, it was clear that L-histidine was able to exchange with albumin-bound zinc and facilitate zinc transport across the red cell membranes in a form of (zinc-histidine) complex.

3.4.3 Temperature Effects

It might be thought that the effect of histidine on zinc uptake could be caused by the increase of zinc binding to cell surface rather than an actual increase of uptake into cells. It is normally agreed that the transport process is more sensitive to the change of temperature than the binding. By studying the temperature-sensitivity of this histidine-facilitated zinc uptake, it is hoped to differentiate the passive binding and Zn uptake into cells. It should be noted that the band 3 pathway was inhibited by using bicarbonate-free media and DIDS-treatment in these experiments. The uptake of zinc (10-150μM) was measured at 37 ^{0}C and 4^{0}C in the medium which contained 1% BSA and 1mM L-histidine. As it is clear from Figure 3.9, the uptake at this condition was greatly affected by the change of temperature. At 4^{0}C, over 75% of measured ^{65}Zn uptake was abolished.

3.4.4 Na^+-Dependence

The facilitated uptake of zinc by histidine was further characterized by studying its dependence on the extracellular Na^+ concentration. In the presence of 1mM L-histidine, zinc uptake (over 5-150μM) was measured with or without Na^+ in the medium. The result of Figure 3.10, over this concentration range, indicates that histidine-facilitated zinc uptake had little (less than 5%) Na^+-dependence.

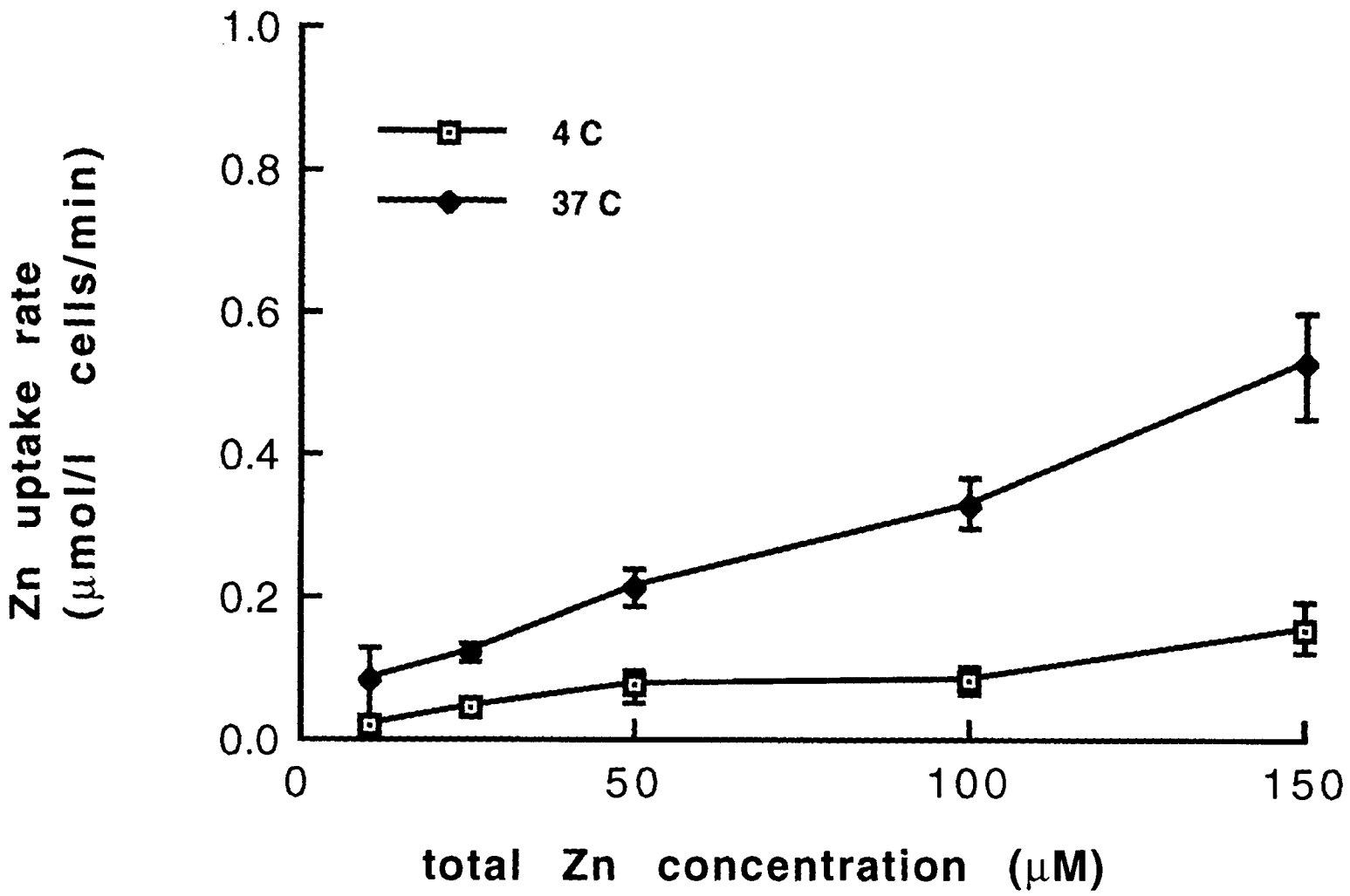


Figure 3.9

Effect of temperature on (zinc-histidine) uptake. Zinc uptake (30μM) was measured at 37 and 4 ^{0}C with the presence of 1mM L-histidine and 1% BSA.

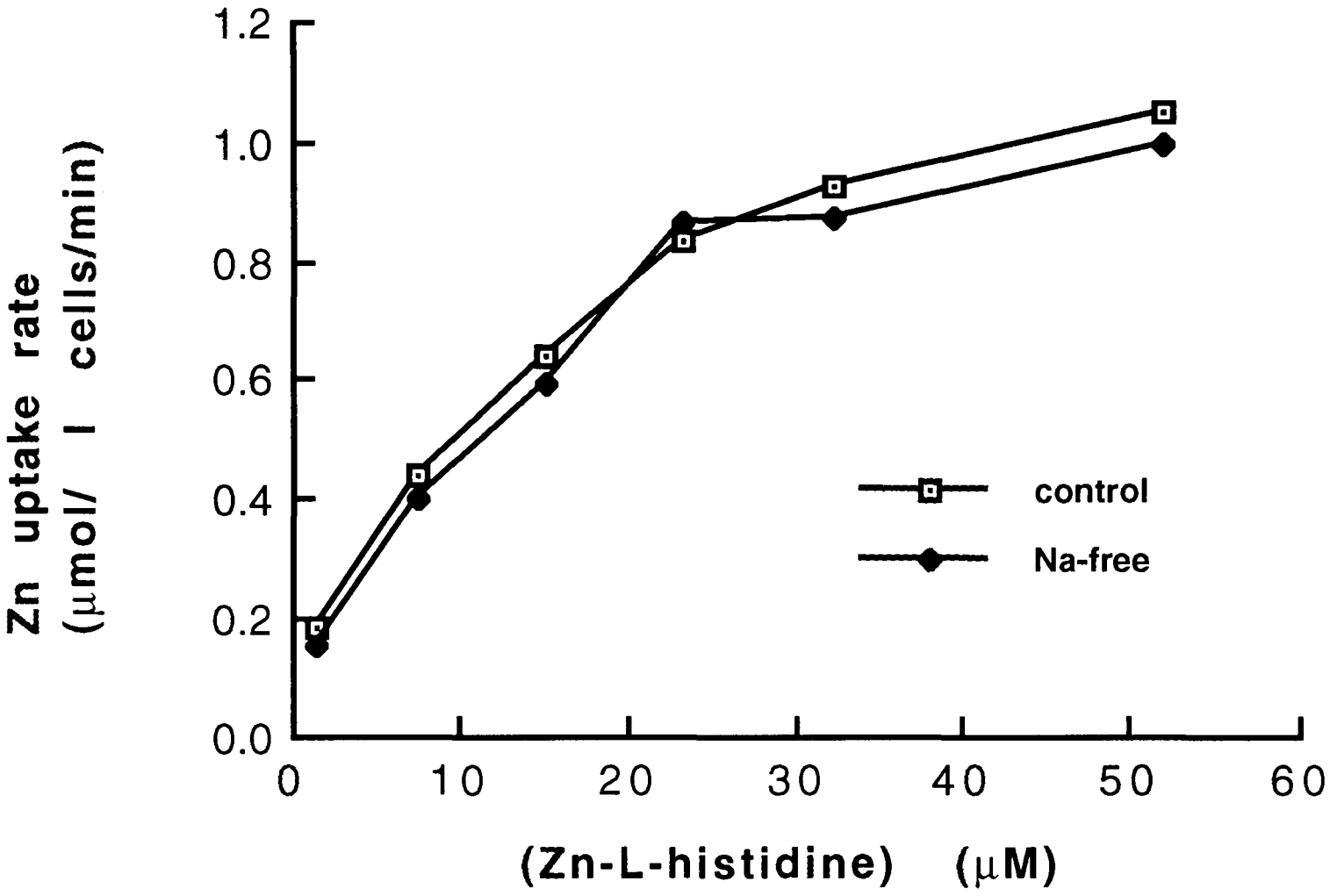


Figure 3.10

Na-dependence of (zinc-histidine) uptake (total Zn of 5-150 μM; L-histidine 1mM) in human red cells. DIDS was added to inhibit the anion exchanger. Zinc uptake rates were measured in Na^+- containing and Na^+ free saline. Red cells were pretreated with 10μM DIDS.

3.4.5 Cis-, & Trans-Effect of L-leucine and L-lysine

From the evidence presented so far, it was apparent that zinc-histidine was transported across red cell membranes via a carrier-mediated process which had a stereoselectivity in favour of (zinc-L-histidine) over (zinc-D-histidine). Hence, it was reasonable to propose the possibility of a histidine transport system being involved in the transport of such a zinc-histidine complex. Histidine transport in human red cells is mainly via two Na^+-independent amino acid transport systems, system L and y^+ which have leucine and lysine as the most preferred substrate respectively (see section 3.5). In the following experiments, the cis- and trans-effect of L-leucine and L-lysine on the uptake of zinc-histidine was studied in order to investigate if zinc-histidine shared the same routes as leucine or lysine. As before, red cells were pretreated with DIDS with media containing no bicarbonate to prevent the contribution of the band 3 pathway to zinc uptake. The uptake of ^{65}Zn (30μM final concentration) was measured in a medium which contained 1% BSA, 1mM L-histidine and 5mM L-leucine or L-lysine. Under the same conditions, the effect of 5mM external L-leucine or L-lysine on ^{14}C-L-histidine uptake was also investigated. Figure 3.11 (a) shows that the presence of L-histidine increased the measured ^{65}Zn uptake rate but this effect was not affected by the presence of external L-leucine and L-lysine. Figure 3.11 (b) shows that at the same condition, the uptake of histidine was not inhibited by 5mM L-leucine or L-lysine. On the other hand, in the experiments in which red cells were preloaded with excess L-leucine and L-lysine, internal L-leucine and lysine had a trans-stimulating effect on histidine facilitated zinc uptake as shown in Figure 3.12. Internal L-leucine induced a significant (over 80%) increase in zinc-histidine uptake as did L-lysine (over 40%).

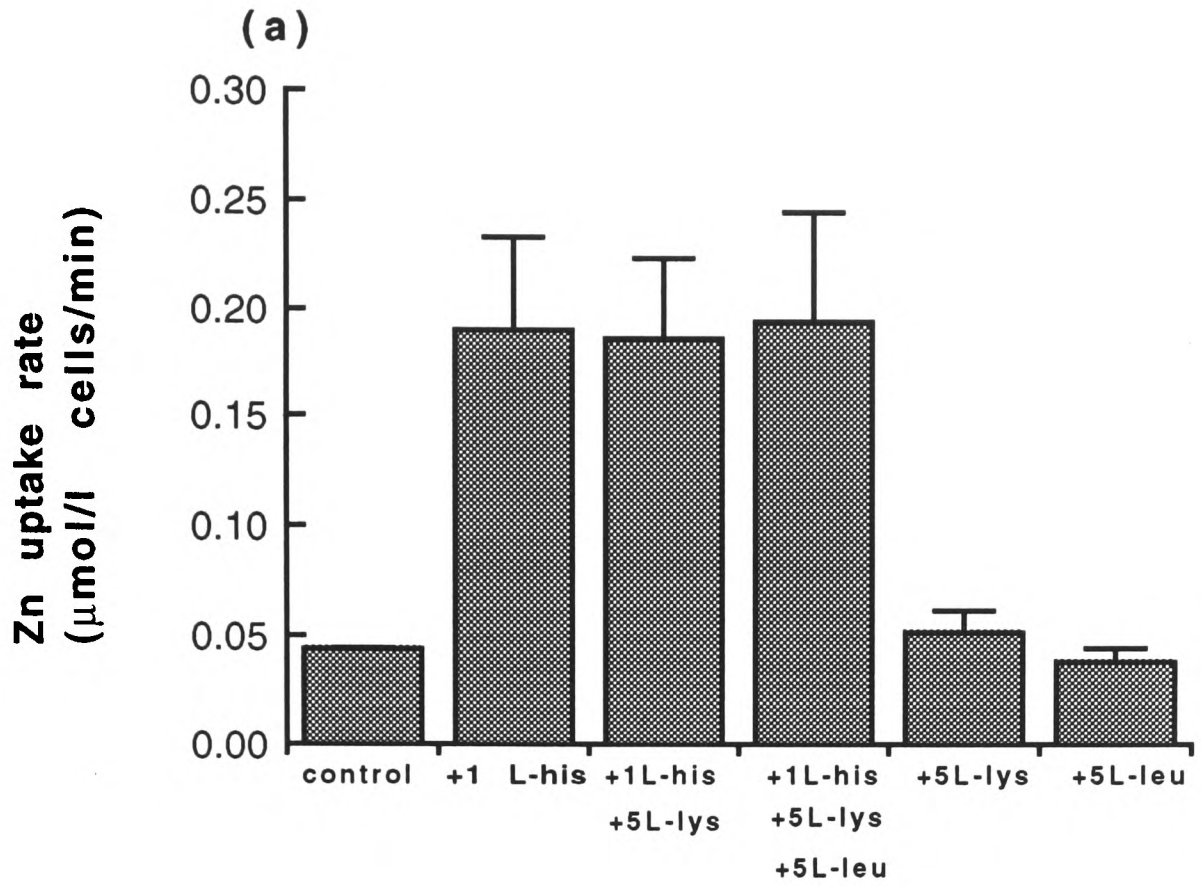


Figure 3.11

Effect of cis L-leucine and L-lysine (5mM) on L-histidine linked zinc uptake (total zinc 30μM; L-histidine 1mM; 1% BSA) in human red cells. Cell were treated with 10μM SITS and the media contained no bicarbonate. The incubation time course was 5 min. (a) The measured ^{65}Zn uptake rates were 0.04±0.001; 0.19±0.04; 0.19±0.04; 0.19±0.05; 0.05±0.01; 0.04±0.01 (±sem, n=3) μmol.l cell^{-1}.min^{-1}respectively. (b) The measured L-histidine uptake rate were 0.017±0.001; 0.015±0.001; 0.0015±0.001 μmol.l cell^{-1}.min^{-1} (±sem, n=3) respectively.

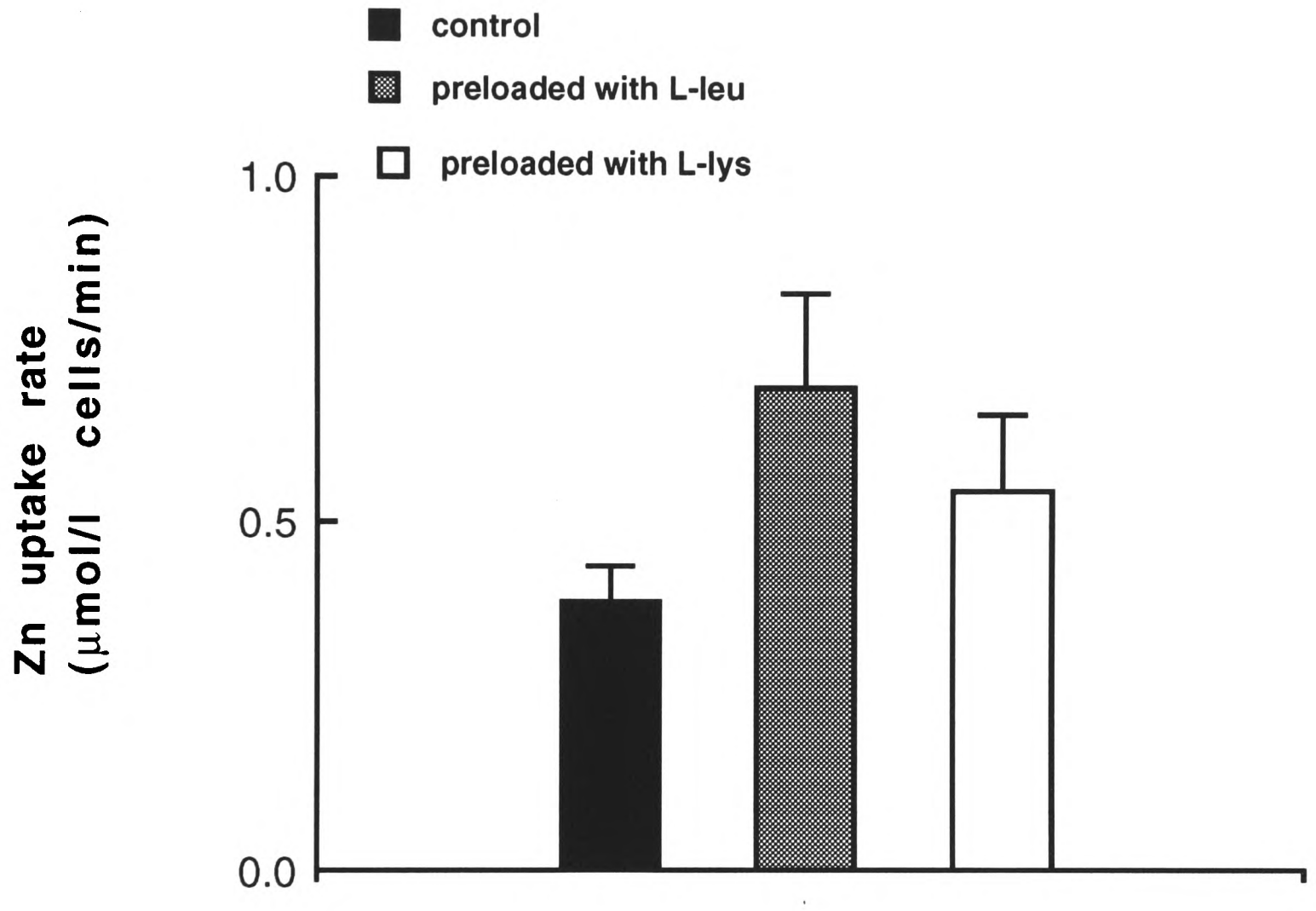


Figure 3.12

Trans-effect of internal L-leucine and L-lysine on L-histidine linked zinc uptake. Cells were preloaded with 10mM L-leucine or L-lysine for 3 hours and treated with 10µM SITS. The media contained no bicarbonate. The incubation time course was measured over 5 min. The Zn uptake rates were 0.39±0.05 µmol.l cell^{-1}.min^{-1} (±sem, n=5) in the control group and 0.76±0.13 (±sem, n=3); 0.55±0.11 (±sem, n=4) µmol.l cell^{-1}.min^{-1} in cells preloaded with L-leucine and L-lysine respectively.

3.5 Histidine Transport in Human Red Cells

Little is known about histidine transport in human red cells. In Ehrilch cells, histidine was reported to be transported by both the Na^+-independent L system and the Na^+-dependent A system (Christensen, 1968). It was reported that in rat hepatocytes histidine might be a good substrate for Na^+-dependent system N (Kilberg *et al.*, 1980).

3.5.1 Time Course

The uptake of histidine (up to 10mM) was linear with time for at least 10 minutes (Figure 3.13). The fixed incubation period of 5 min was chosen to measure initial uptake rates in the following experiments.

3.5.2 Concentration-Dependent Histidine Uptake

The uptake of histidine (0.1-10mM) was measured in standard bicarbonate-free saline (Figure 3.14). The uptake of histidine at this concentration range was found to be saturable, which was well-fitted to the Michaelis-Menten equation with apparent kinetic parameters K_m= 11.1± 4.7 mM, V_{max}= 2.2± 1.4 mmol. l $cell^{-1}$. min^{-1} and k_d = 0.002± 0.002 (±sem, n=4) sec^{-1}.

3.5.3 Na^+-Dependence

The Na^+-dependence of histidine uptake was studied by measuring 0.1mM L-histidine uptake in standard saline and Na^+-free medium (replaced with NMDG). Figure 3.15 shows that the

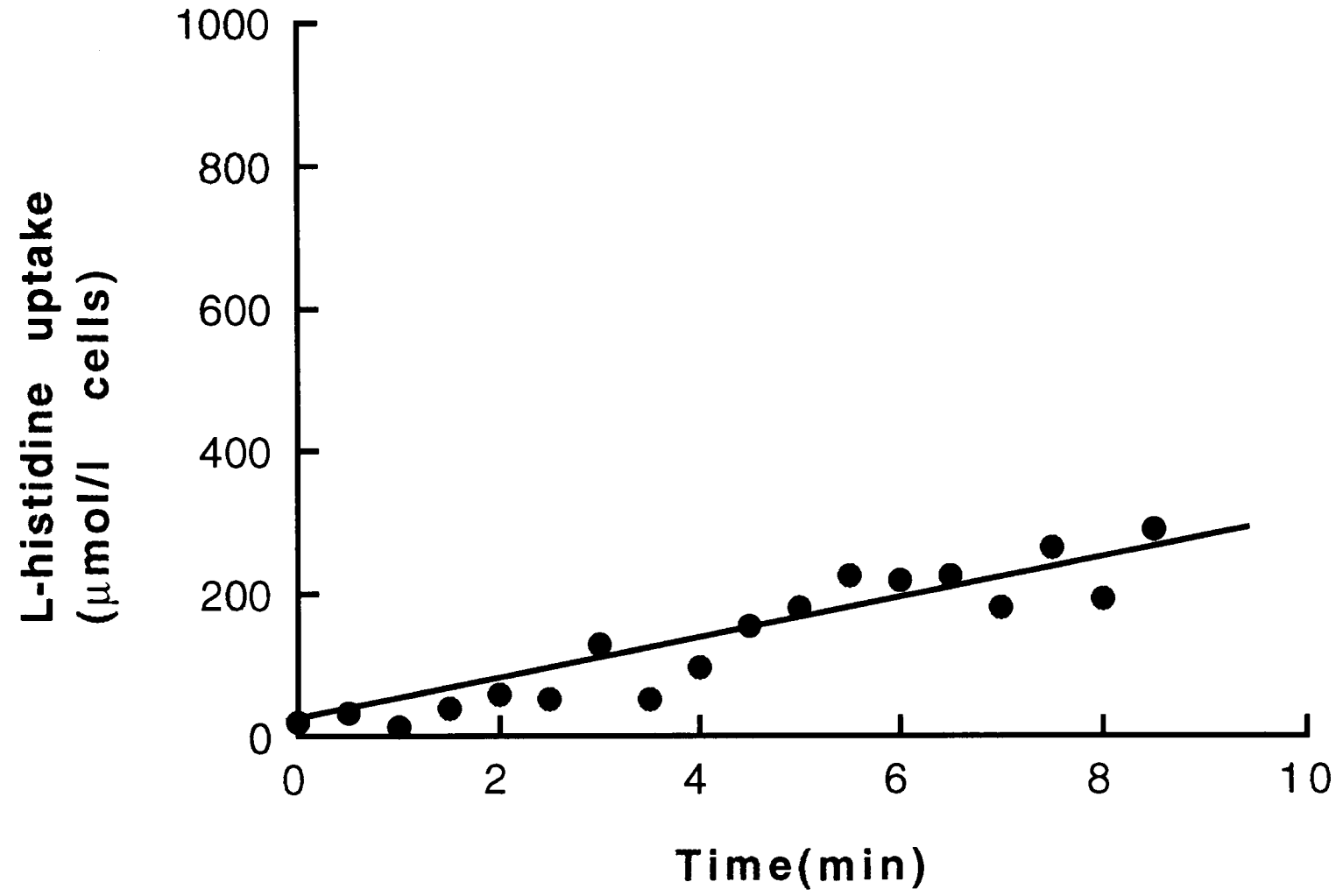


Figure 3.13

Time course for histidine influx into human red cells. The external histidine concentration was 1mM. The data points fitted a linear regression with a slope of 32 μmol. l cell^{-1}.min^{-1} (r=0.867).

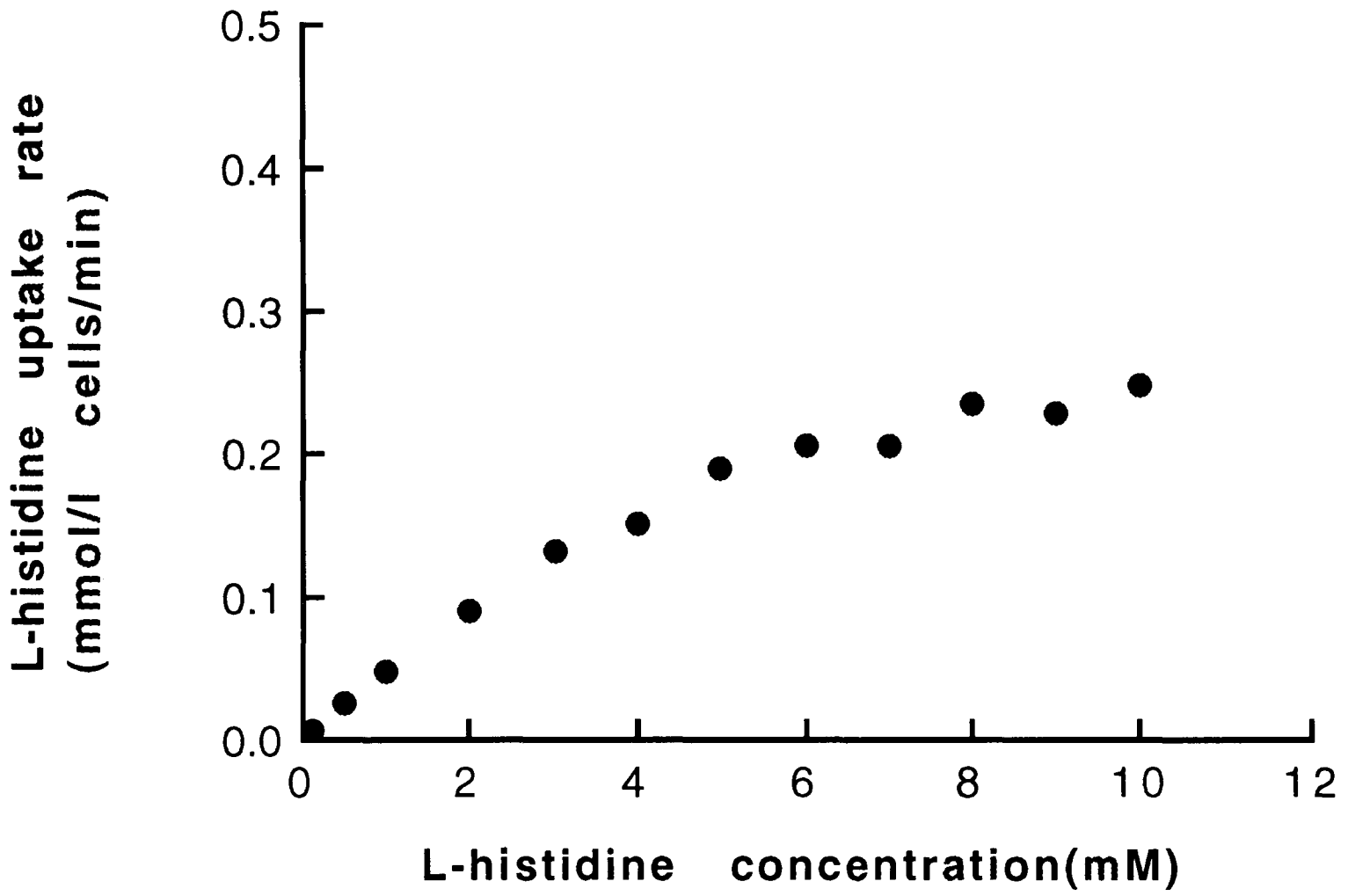


Figure 3.14

An example of concentration-dependent histidine uptake in human red cells.

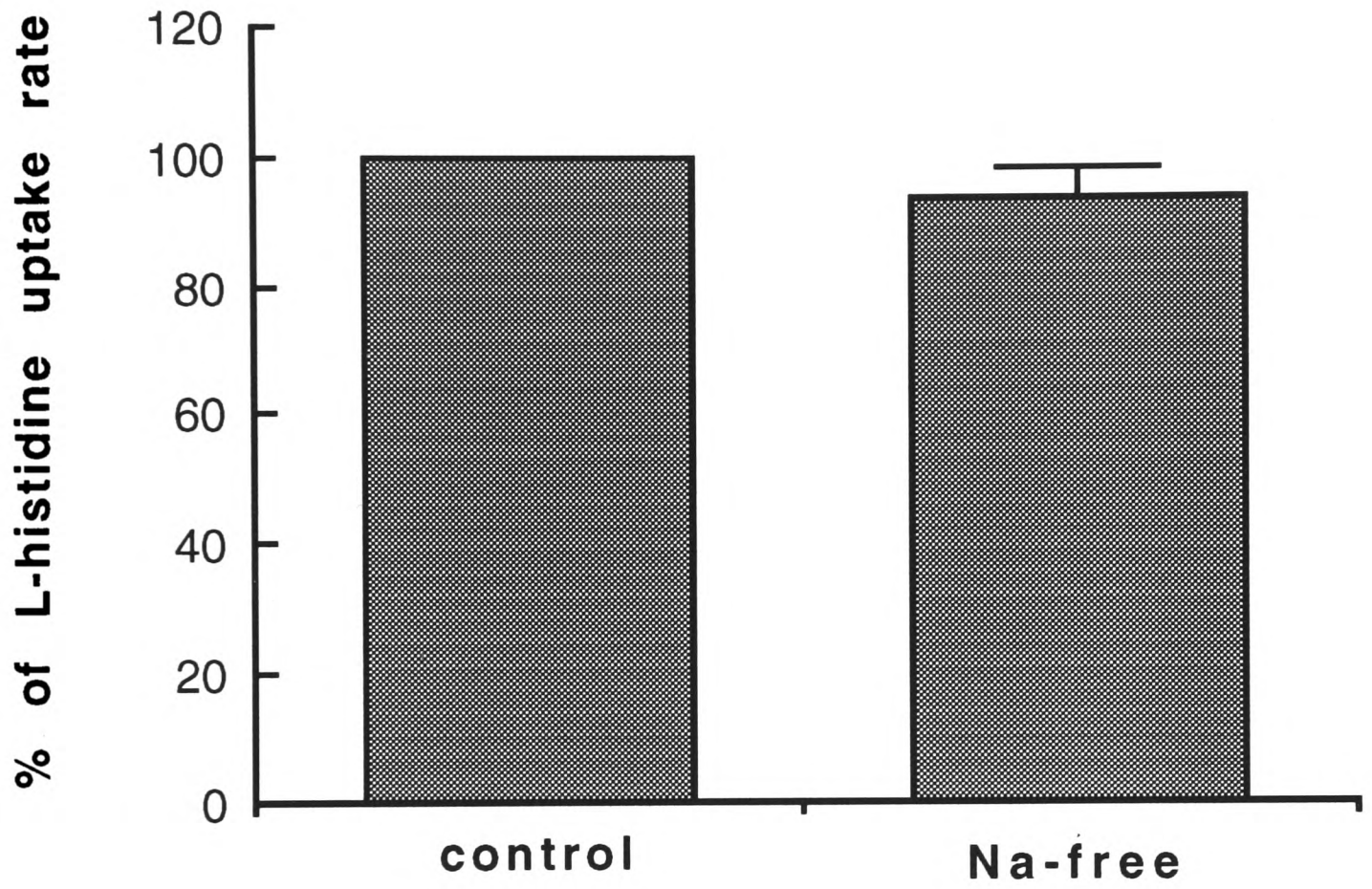


Figure 3.15

Effect of Na^+-replacement on histidine uptake in human red cells.

The replacement of Na^+ caused a 6.3% ± 4.4% (±sem, n=3) decrease in histidine uptake.

replacement of Na^+ had a small effect on the uptake of histidine (0.1mM) which caused a less than 7% (6.3± 4.4 %, ±sem, n=3) decrease in uptake rate.

3.5.4 Investigation of Histidine Pathways

In these experiments, excess concentration of paradigm substrates of known amino acid transport systems were used as competitive inhibitors to identify histidine transport pathways. Serine, glycine and DIDS which are the model substrate and inhibitor for the ASC, gly and the anion exchanger respectively had no effect on histidine uptake (data not shown). The result of the effects of L-lysine, L-glutamine, L-leucine and L-tryptophan (as preferred substrates for y^+, N, L and T transport systems respectively) on histidine transport (0.1mM) is shown in Figure 3.16. The result indicates that systems y^+, and L, contributes about 30% of total histidine uptake each, and an as yet unresolved Na^+-independent component (possibly passive diffusion) is also involved in histidine transport in human red cells.

Discussion

The results of this study confirm that the entry of zinc via the band 3 anion exchanger is the major route for zinc uptake when the medium contains no binding ligands (Alda Torrubia and Garay, 1989; Kalfakakou and Simons, 1990). This DIDS-sensitive, bicarbonate-stimulated zinc uptake accounted for over 90% of total zinc uptake (Figure 3.3). However, it may not represent a physiological route for zinc uptake into cells because this anionic mechanism was not able to mediate transport of Zn bound by albumin (Figure 3.4) which suggests that the formation of the anionic complexes of zinc which are the substrates for the anion exchanger did not take place

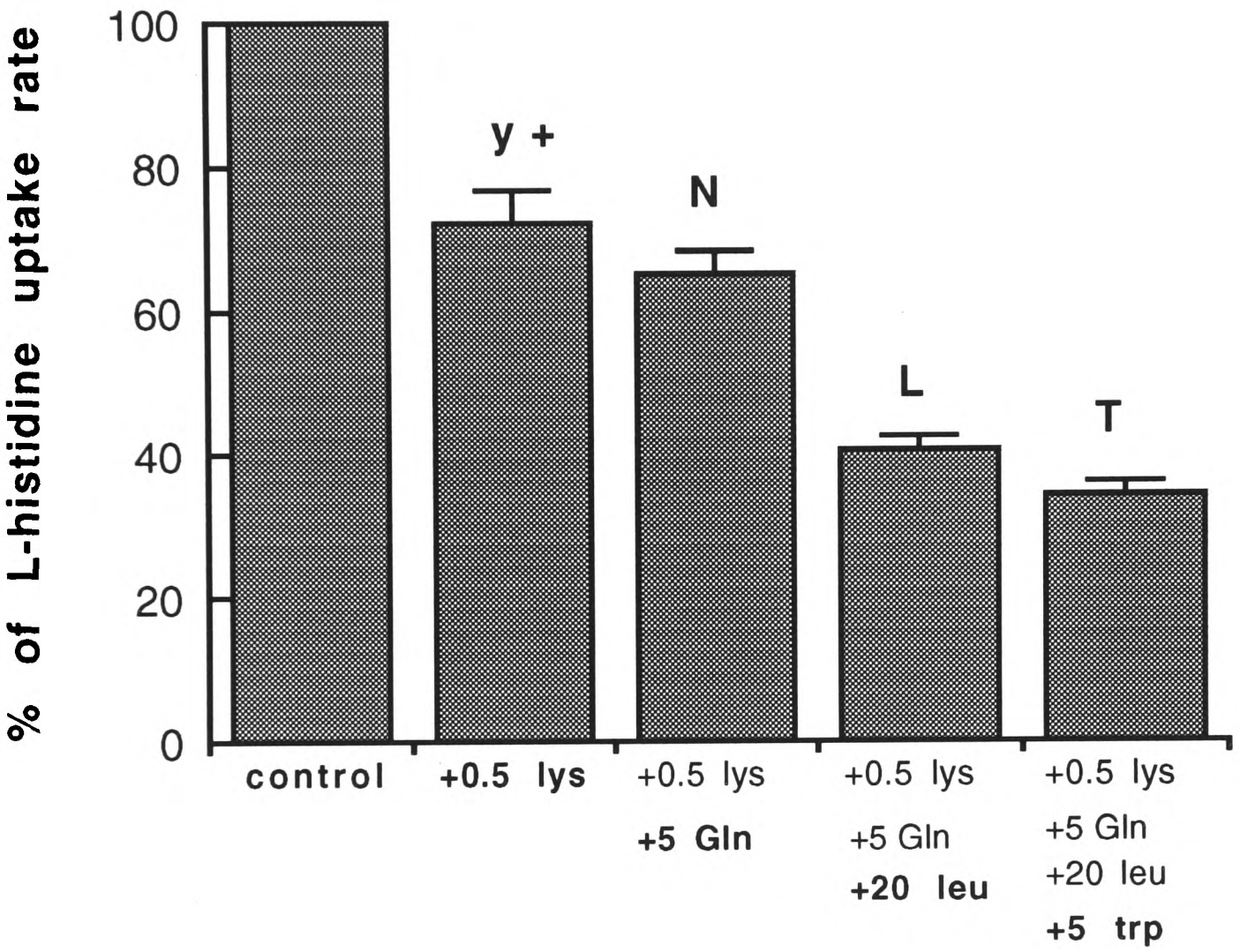


Figure 3.16

Possible pathways involved in L-histidine transport.

when albumin was present in the medium. It is known that under physiological condition the majority of plasma zinc was albumin-bound (Cousins, 1985).

The presence of histidine in the medium was found to have two different effects on zinc transport. First, by a general chelating effect as all other binding ligands, the presence of histidine reduced the ionic zinc in the medium available for transport and therefore caused a decrease in the measured uptake rates (Figure 3.5 (a)). However, if taking into account the chelating effect and estimating the available ionic zinc for transport, the presence of histidine had a great facilitating effect on zinc uptake (Figure 3.5 (b)). The histidine effect was stereospecific (Figure 3.5 (b)), saturable according to the external zinc-histidine concentration (Figure 3.6), functional even in the presence of BSA (Figure 3.8), temperature-sensitive (Figure 3.9), Na^+-independent, and trans-stimulated by internal L-leucine and L-lysine (Figure 3.12). All the evidence strongly suggests a mechanism of zinc-histidine cotransport via a carrier-mediated process. In this study, the major pathways for histidine transport in human red cells have been identified as two Na^+-independent system, system L and y^+. The result of the cis-effect of L-leucine and L-lysine (Figure 3.11 (a) & (b)) was surprising that in the presence of albumin the addition of 5mM external L-leucine and L-lysine failed to inhibit the uptake of both zinc & histidine. Perhaps it could be explained by albumin reducing the concentration of available zinc and histidine for transport by a very large amount, such that the measured uptake represented a very small flux of zinc-histidine entry via system L or y^+. In this case, it might be difficult to observe the competitive inhibition caused by L-leucine and L-lysine. The result of Figure 3.12 is better evidence to support system L and y^+ participating in the transport of the zinc-histidine complex. Since both of these two Na^+-independent systems are known to be strong in their exchange mode (Ellory, 1982), the fact that internal L-leucine and L-lysine could stimulate the flux of zinc-histidine suggests that they might

share the same transport pathway.

It is difficult to obtain direct evidence of zinc-histidine cotransport via the amino acid transport system. Nevertheless, all the results seemed to suggest the amino acid transport systems are the most plausible candidate for this transport process. A possibility is that there is an unknown transport system specific for the zinc-histidine complex, which is stereospecific for its substrates. Another possibility is that the presence of histidine can assist to present zinc to the the cell membrane in such a form that zinc uptake is enhanced, but it is difficult to imagine such a mechanism should be stereoselective between L-, and D-histidine. A report in human fibroblasts suggested that the binding ligand histidine did not have such a specific effect but only free zinc was transported across cell membrane (Ackland and Mcardle, 1990). This cannot be the case with the present results in human red cells.

The results show that in human red cells a mechanism of zinc-histidine cotransport rather than an ionic mechanism is the likely physiological route for zinc uptake into cells. With a knowledge of such mechanism it might be proposed to use histidine to increase the zinc absorption or transport into tissues and cells since zinc deficiency is found in many pathological conditions and the application of zinc supplementation grows increasingly popular (Endre *et al.*, 1990). However, such a proposal is unlikely to be useful in practice because even though at the cellular level histidine can bind zinc and facilitate its entry into cells, the chelating effect is actually greater than its facilitating effect. This means on the whole the increase of histidine in the plasma would not increase the intake of zinc into tissues or cells but rather reduce it. Nevertheless, the role of histidine and other zinc-binding amino acids is significant in not only acting as a binding ligand but providing a route for the entry into cells.

CHAPTER FOUR

ZINC TRANSPORT IN LAMPREY RED CELLS

Introduction

The lampreys belong to a subclass of the Cyclostomes (the only remaining class of Agnathans or jawless fish) and are among the most primitive vertebrates known. Lamprey red cells have been reported to have a minimum activity of band 3 anion exchanger, which is the main route for Cl^-, HCO_3^- (and several other anions) in red cells of all higher vertebrates (Ohnishi and Asai, 1985; Nikinmaa *et al.*, 1986). This characteristic of lamprey red cells makes this cell type a model system to clarify the role of the band 3 anion exchanger in zinc transport across cell membrane. In human red cells, there are at least two mechanisms for zinc uptake (Chapter 3). Firstly, zinc forms anionic complexes and enter the cells via the band 3 anion exchanger. Secondly, zinc may be cotransported with binding to the amino acid histidine via amino acid transport systems. Nothing is known about zinc and amino acid transport in lamprey red cells. In this chapter, both mechanisms of zinc transport (anion-dependent and histidine-linked) are examined in lamprey red cells.

Materials and Methods

Blood. River lampreys (*Lampetra fluviatalis*; 25-80g) were a generous gift from Dr K.D. Kirk. After being captured in the Simojoki River in Northern Finland, lampreys were transferred to Oxford and kept in well-aerated, dechlorinated tap water (maintained at a temperature of 4^0C) for several weeks before experimentation. They were anaesthetized with MS222 (2g/l) and then bled from the tail. Blood was suspended into an ice-cold , heparinised saline containing(mM): NaCl(130), HEPES(10), EGTA(0.1), pH 7.6. The cell suspension was then filtered through a polymer wool column and washed immediately three times with lamprey Ringer solution which contained (in mM): NaCl(114), HEPES(10), $MgCl_2$(1), $CaCl_2$ (1), glucose(5), KCl (4), and pH was adjusted to the pH 7.6 with 1M Tris.

Flux measurements. The measurement of ^{65}Zn and ^{14}C-histidine transport was carried out by employing the rapid wash technique and scintillation counting as described previously. Cell suspension (50µl; with a haematocrit of 7-10%) was mixed with 50µl of the appropriate stock solution containing radioactive tracer to start uptake experiments. Unless otherwise stated, both cell suspensions and stock solutions were already in a 20 ^{0}C water bath for at least 5-10min before starting the influx incubation in order to ensure the temperature-equilibration (Figure 4.1). After a chosen incubation period, 1ml ice-cold washing solution (150mM $MgCl_2$, 10mM EDTA, 10mM HEPES, pH 7.4) was quickly added to the cell suspension to stop the fluxes and then immediately followed by 5 washes. The cell pellet was lysed with 0.5ml 0.5% (v/v) Triton and deproteinized by 0.5ml 0.5% (w/v) TCA followed by centrifugation (10,000g, 10min). The intracellular radioactivity was then measured by scintillation counting.

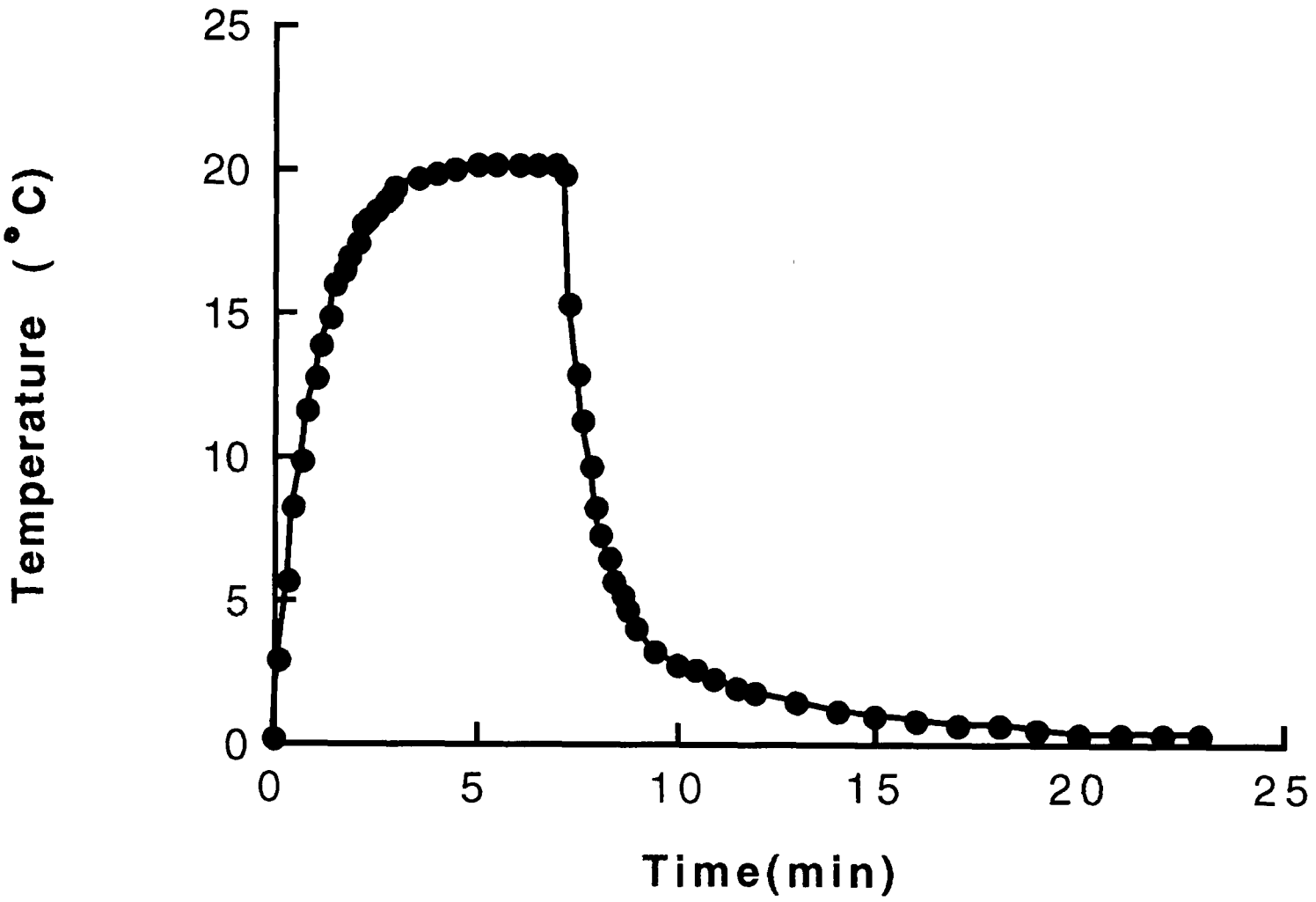


Figure 4.1

Temperature change within a Epperdorf tube. 0.1ml lamprey standard saline was placed in an Epperdorf tube. At time zero the tube was placed in a 20^{0}C water-bath and the temperature of the saline was meaured at a chosen time period with a microthermometer. The change of temperature reached its equilibration within 5 minutes.

Results

4.1 Zinc Uptake in Lamprey Red Cells

4.1.1 Time Course

The concentration of total Zn in lamprey plasma is not known. A concentration of 30μM was chosen here because of the convenience in comparing the results with red cells of other species investigated in this thesis. The measurement of Zn flux was performed in standard bicarbonate-free lamprey saline. The uptake of zinc (30μM) showed a linear relationship with time for at least 30 min with a uptake rate of 6.3 ± 2.1 μmol.l cell^{-1}. min^{-1} (±sem, n=6) (Figure 4.2). Therefore, an incubation period of 10-15min was used whenever the measurements of initial uptake rates of zinc were performed.

4.1.2 Effect of DIDS & Bicarbonate

In the following experiments, the effects of DIDS and bicarbonate on zinc transport were studied. DIDS (final concentration of 10μM) was added to the cell suspension (haematocrit of 5-10%) at 20^{0}C 30 minutes before the flux measurement commenced. In both control and DIDS-treated cells, the saline in which red cells were suspended, contained no bicarbonate. After a 30-minute incubation, red cells were then quickly washed twice with ice-cold washing solution to remove extracellular i.e. unbound DIDS. Next, the ^{65}Zn stock solution was added to start the flux experiments. Figure 4.3 shows the result of these experiments. The uptake of zinc in lamprey red

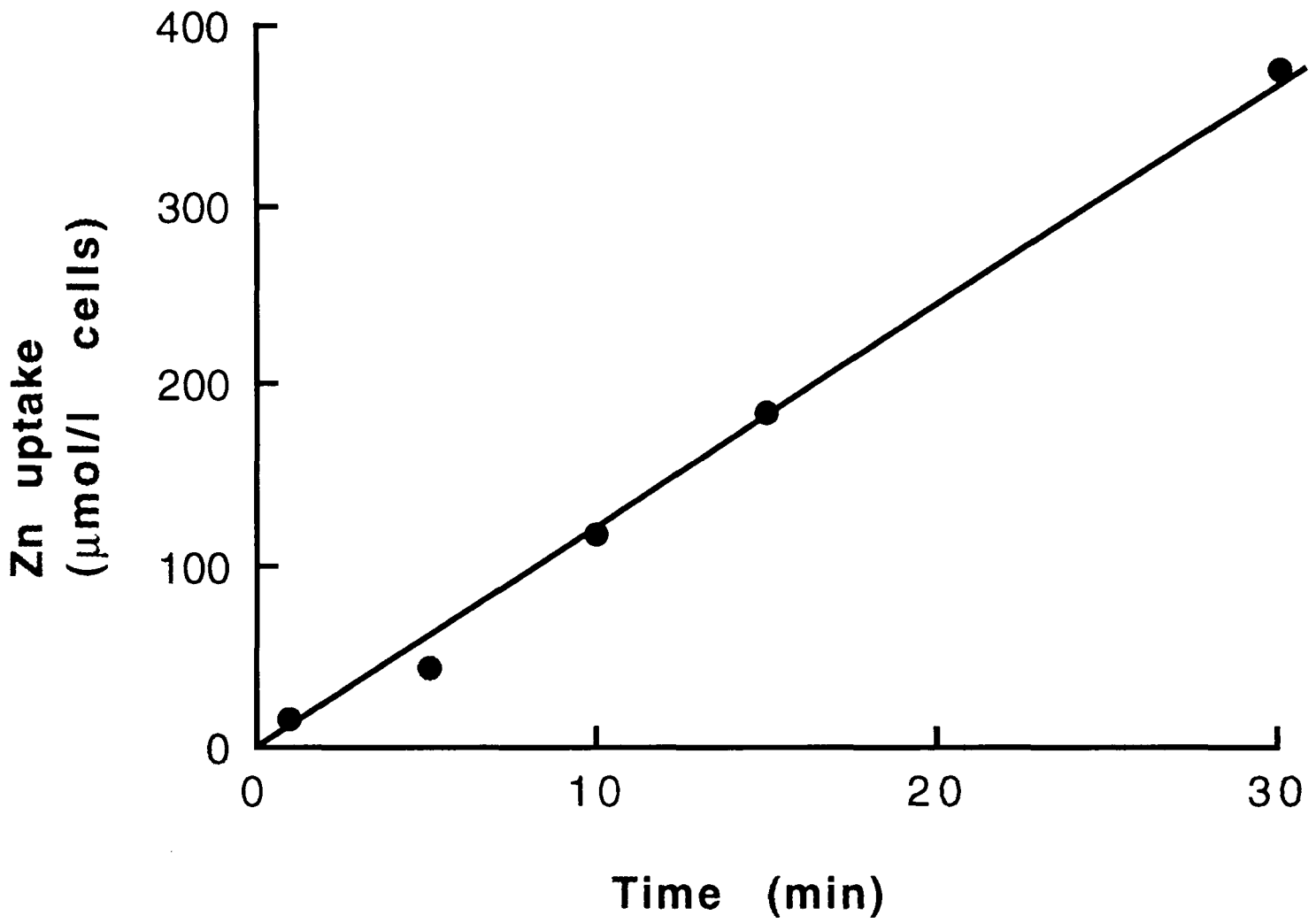


Figure 4.2

Time course for zinc uptake in lamprey red cells. The extracellular Zn concentration was 30μM. The data points fitted a linear regression with a slope of 12.6 μmol.l cell^{-1}.min^{-1}. (r=0.993).

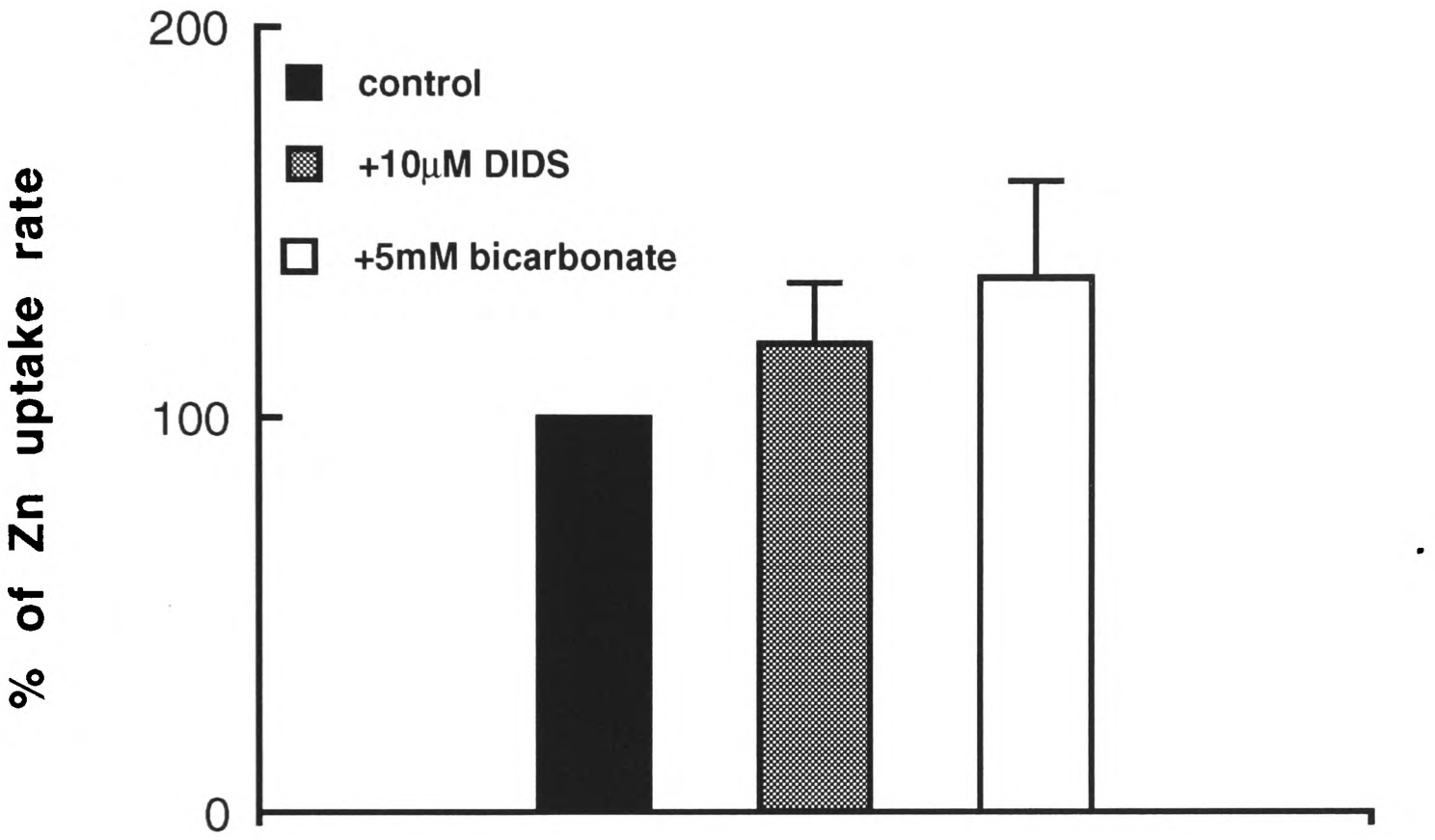


Figure 4.3

Effect of DIDS treatment(10μM) and the addition of bicarbonate(5mM) on zinc(30μM) uptake in lamprey red cells. Data have been normalized and expressed as a percentage of the control value (n=3). Both DIDS treatment and the addition of bicarbonate had no significant effect on zinc uptake, p=0.319 and 0.255 respectively (n=3).

cells was not affected by the treatment with 10μM DIDS (p=0.319, n=3) or the presence of 5mM bicarbonate in the medium (p=0.225, n=3). This result indicated that in lamprey red cells zinc uptake was not via an anion-dependent pathway as was found in human red cells.

4.1.3 Concentration-Dependent Zinc Uptake

Zinc uptake at various concentrations (5-200μM) was measured to see if the uptake of zinc showed a concentration-dependent saturation. Up to the concentration of 200μM, the uptake of zinc was linear with respect to time for at least 15 minutes (data not shown). Thus a period of 10 minutes was used to measure the initial uptake rate of zinc at this concentration range. Figure 4.4 showed an example of such experiments. Over the range of 5-200μM, the uptake of zinc in lamprey red cells demonstrated a linear relationship with the external total zinc concentration. The uptake rate was 0.17 ± 0.04 μmol.l $cell^{-1}.min^{-1}.[\mu M^{-1}]$ external Zn (±sem, n=3).

4.2 Effect of Histidine on Zinc Uptake in Lamprey Red Cells

The effect of histidine in lamprey red cells was investigated in the experiments described below to see if there existed a similar mechanism to that found in human red cells ie that histidine could stimulate zinc uptake despite its chelating effect. L, or D-histidine (final concentration 0.1mM) was added into the medium in which lamprey red cells were suspended. The uptake of zinc (30μM) was then measured in standard bicarbonate-free saline. The result shows that the presence of both L, and D-histidine in the medium caused a small increase in zinc uptake rate (Figure 4.5).

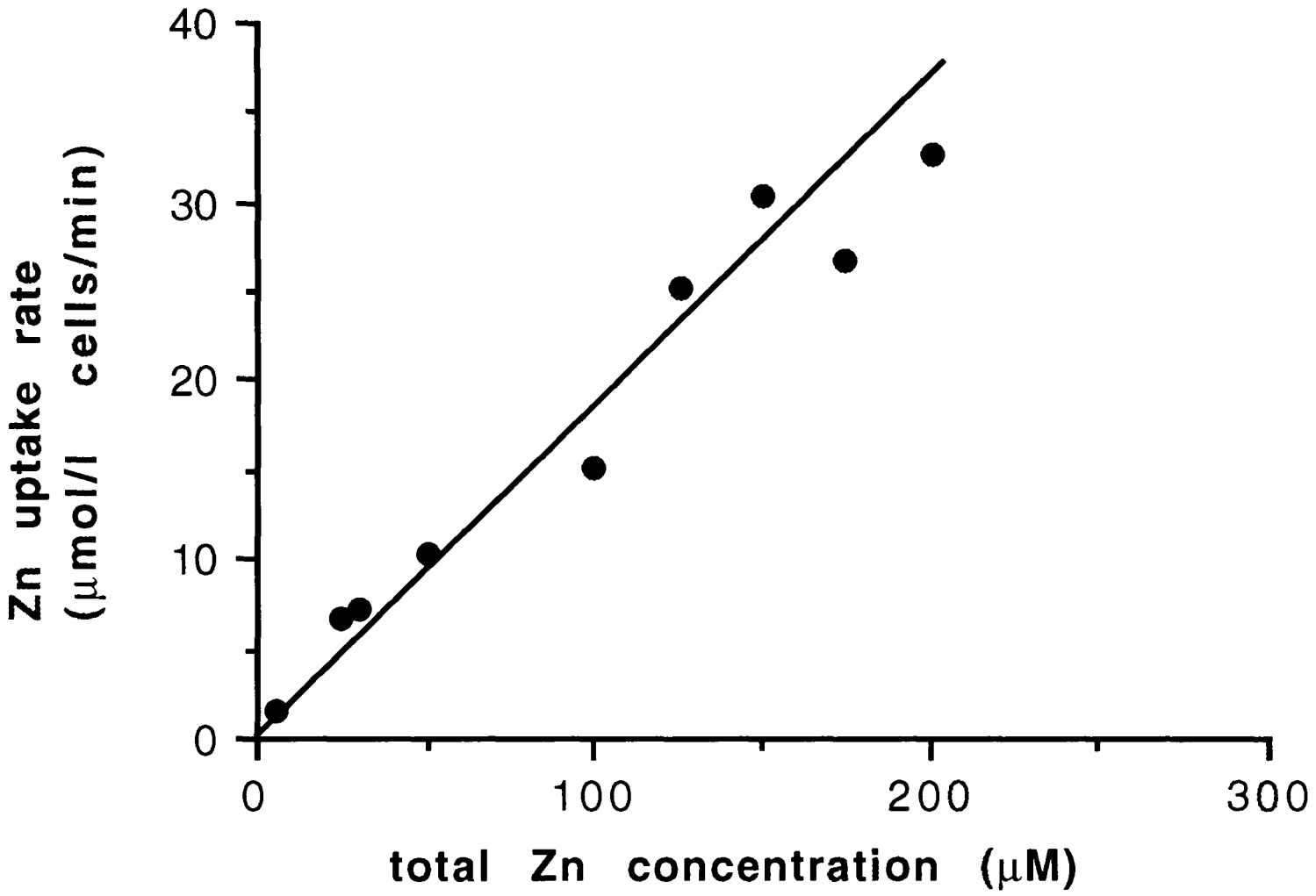


Figure 4.4

Concentration-dependent zinc uptake in lamprey red cells. Zinc uptake over a range of 10-200μM was measured in standard lamprey saline. The data points of this typical experiment fitted a linear regression with a slope of 0.159 μmol.l cells^{-1}.min^{-1}.[μM] external Zn (r=0.973). The mean of three independent experiments is 0.17±0.04 μmol.l cells^{-1}.min^{-1}.[μM] external Zn (±sem, n=3).

The free zinc concentration was estimated to be 3.95 μM at this condition (see section 2.4.6 for calculation method). Accordingly, the uptake rate of zinc at this concentration was estimated to be 0.67 μmol.l cell^{-1}.min^{-1}. The uptake rate of zinc was increased to 4.2 ± 2.3 μmol.l cell^{-1}.min^{-1} in the presence of L-histidine and 5.8 ± 2.9 μmol.l cell^{-1}.min^{-1} in D-histidine containing medium. There was no difference between the effects of L, and D-histidine (p=0.123,n=3).

Nothing is known about the transport of histidine in lamprey red cells. In order to explain this stimulating effect by L-, and D-histidine on zinc uptake, histidine transport was studied and the results presented in the following sections.

4.3 L-histidine Uptake in Lamprey Red Cells

4.3.1 Time Course

The uptake of L-histidine (0.1mM) in lamprey red cells appeared to be linear with time for at least first three min with an initial uptake rate of 65.4 ± 9.6 μmol.l cell^{-1}.min^{-1} (±sem,n=4)(see Figure 4.6). Therefore, most of the following flux measurements were performed with an incubation time of 30 seconds unless otherwise stated.

4.3.2 Investigation of L-histidine Transport Pathways

This section describes a series of experiments which studied the effect of excess extracellular paradigm amino acids on histidine transport to identify the transport systems for L-histidine entry

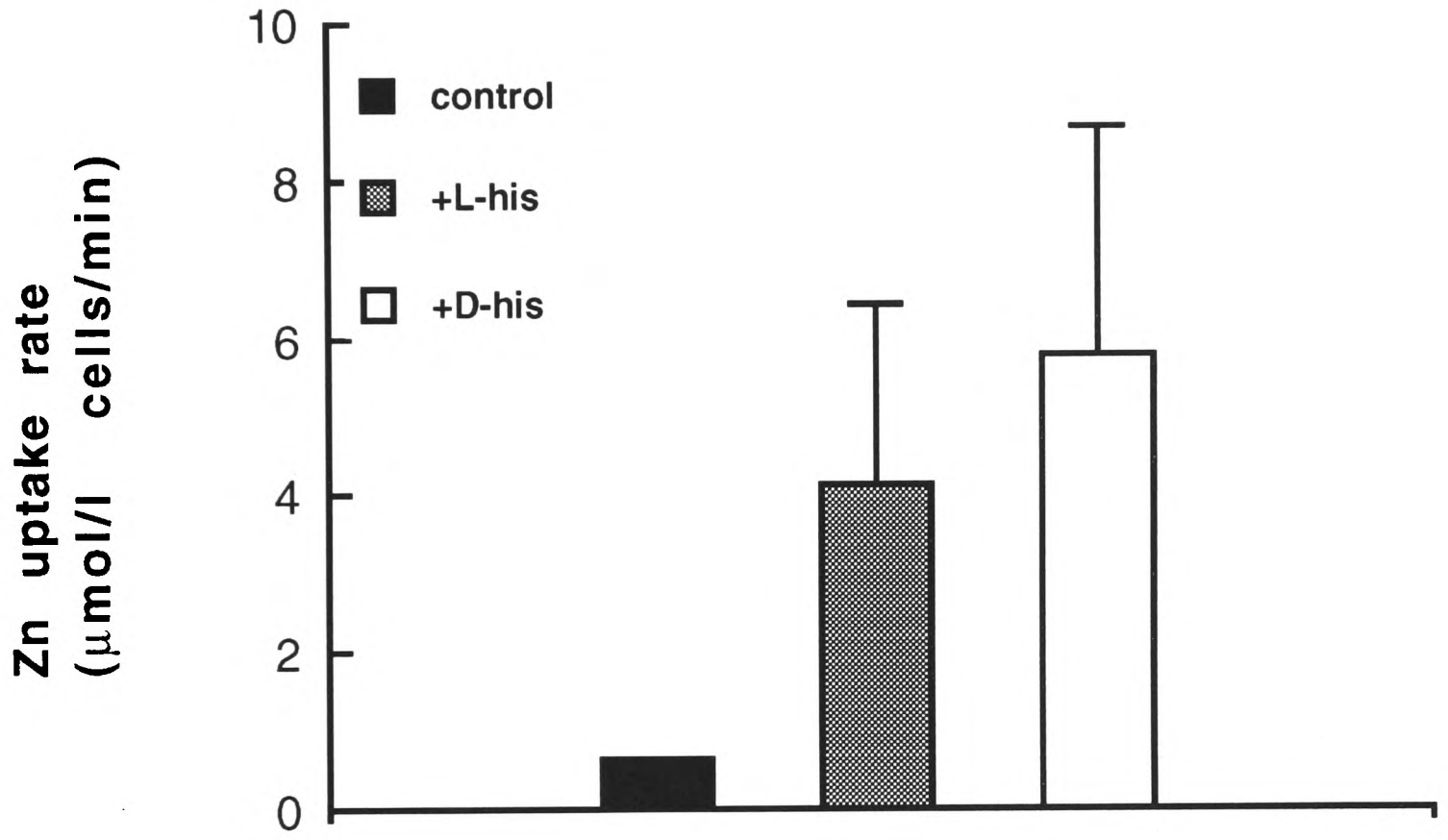


Figure 4.5

Effect of 0.1mM L- & D-histidine on zinc uptake (30µM) in lamprey red cells. The uptake rate of free zinc was estimated to be 0.67 µmol.l cells.min, whereas the uptake rates of zinc in the presence of 0.1mM L,D-histidine were 4.16± 2.29 and 5.79± 2.91 µmol.l cells^{-1}.min^{-1} respectively (±sem, n=3).

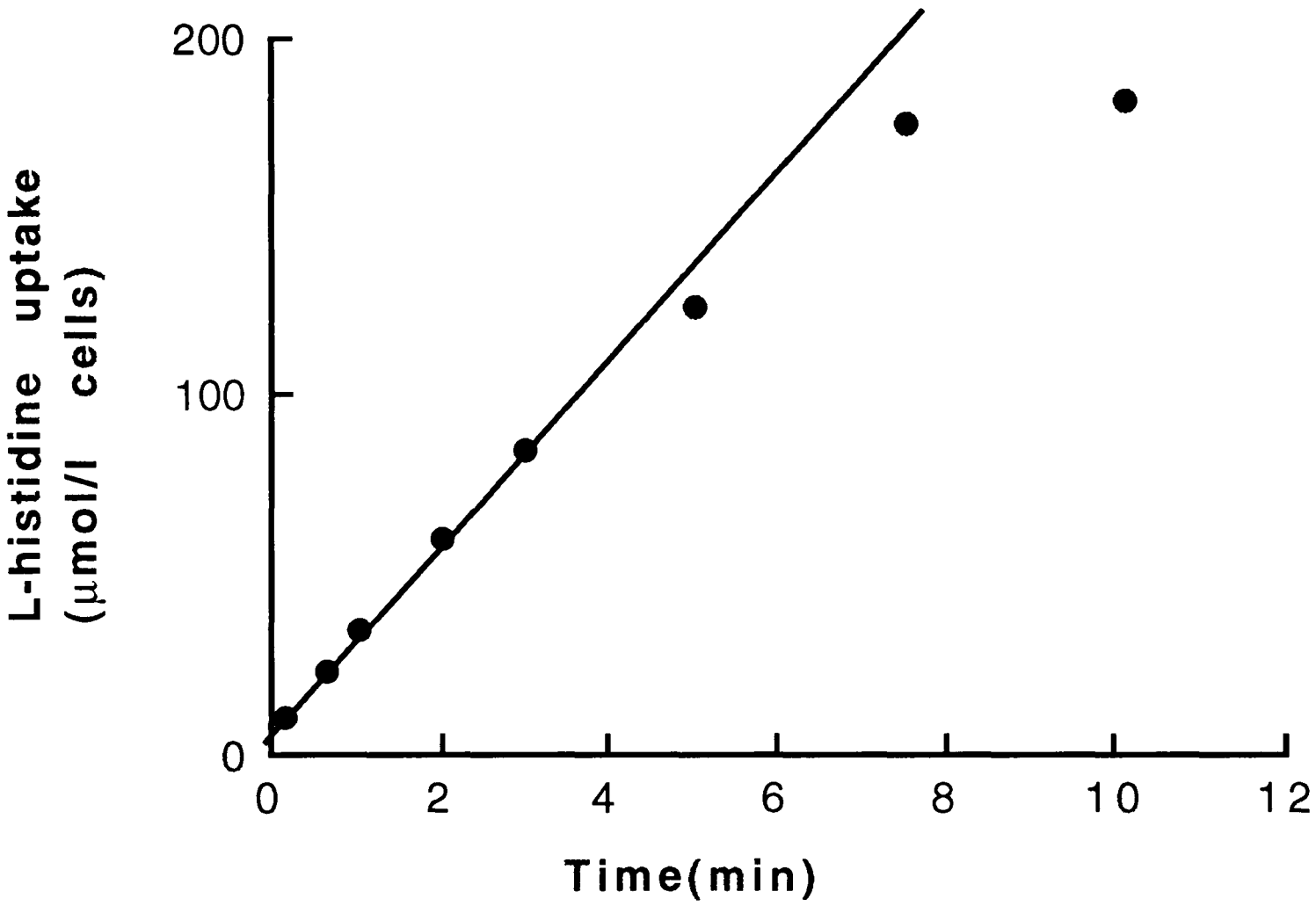


Figure 4.6

Time course for L-histidine (0.1mM) uptake in lamprey red cells. The data of this typical example fitted a linear regression with a slope of uptake rate 12.7 μmol.l cells^{-1}.min^{-1} (r=0.976).

into lamprey red cells. L-proline (5mM), glycine (5mM), L-leucine (15mM), L-alanine (5mM) and L-lysine (5mM) were used to inhibit the transport systems, pro, gly, L, asc, y^+, if they were present in lamprey red cells as has been shown in hagfish red cells (Fincham, 1990 and discussion). L-histidine uptake (0.1mM) was then measured within a period of 30 seconds with the presence of extracellular paradigm amino acids (Figure 4.7). The uptake of L-histidine in lamprey red cells was not affected by the presence of glycine, proline, alanine and glutamine. Only L-leucine and L-lysine were found to have a significant effect on L-histidine uptake which causing a decrease of over 15% & 75% respectively in the uptake rate of L-histidine (0.1mM). This result suggests that L-histidine transport in lamprey red cells might share the same routes with L-leucine and L-lysine.

4.3.3 Substrate Stereospecificity of L-histidine Transport Systems

The histidine effect in lamprey red cells was not found to be stereospecific (section 4.2). However if this histidine effect was due to the cotransport of zinc-histidine via histidine transporters in lamprey red cells, one would expect to find that histidine transporters were not stereoselective for their substrates. These experiments described below were designed to investigate the substrate stereospecificity of histidine transporters in lamprey red cells. L, or D-lysine and L, or D-leucine (final concentration of 20mM) were used to inhibit the uptake of 0.1mM L-histidine. Lamprey red cells were incubated in the standard lamprey saline containing 0.1mM L-histidine in the presence of 20mM L and D-lysine and L and D-leucine. Figure 4.8 illustrates the result of the inhibition caused by lysine which was significantly different ($p=0.009$, $n=3$) between L, and D-lysine of which L-lysine inhibited over 90% (91±1%, ±sem, n=3), of total histidine uptake and

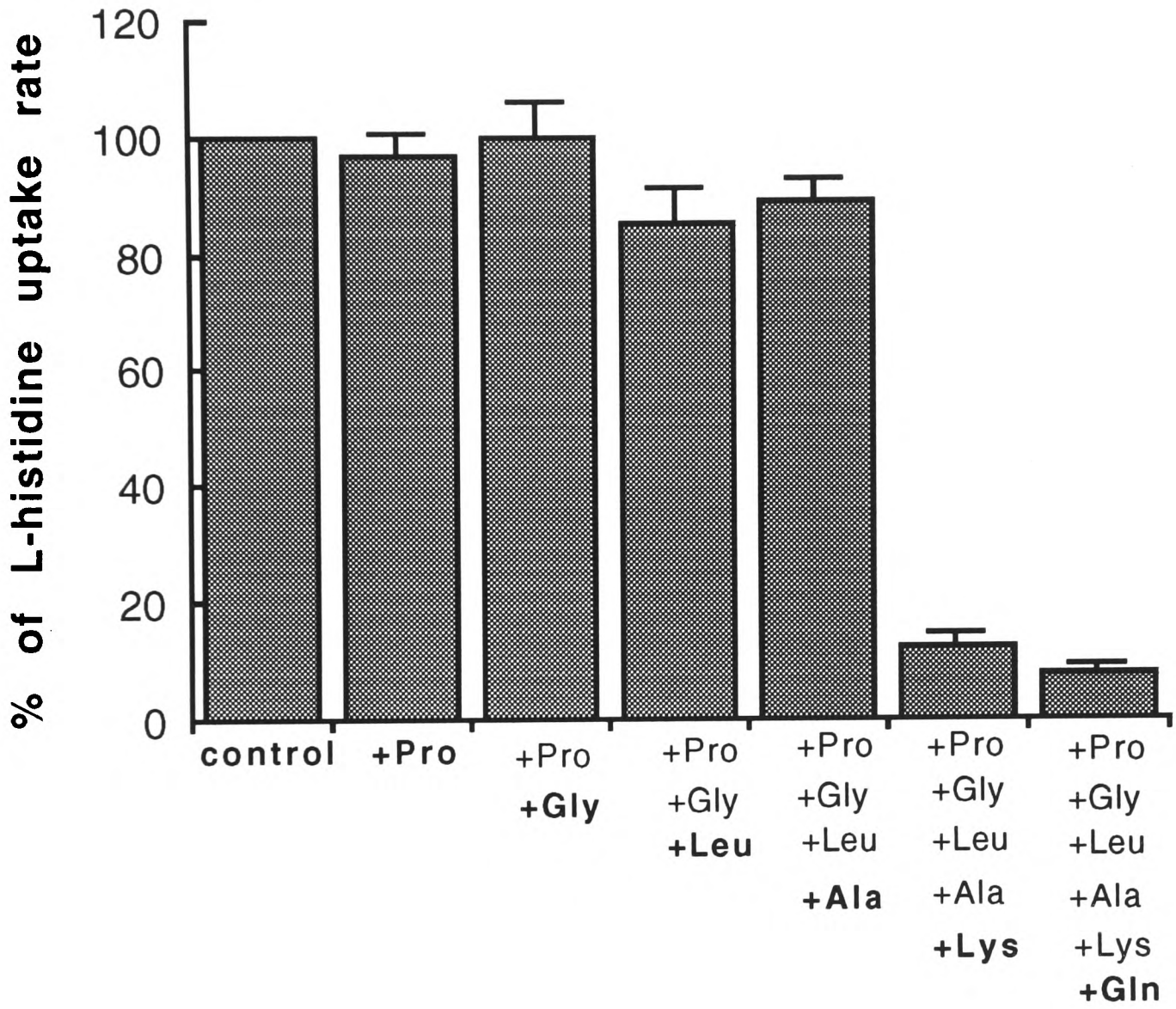


Figure 4.7

Possible pathways for histidine uptake in lamprey red cells. The uptake of 0.1mM L-histidine was measured with the excess concentration of paradigm substrates (in mM); proline (5), glycine (5) , L-leucine (15), L-alanine (5), L-lysine (5) and L-glutamine. The incubation period for influx was 30 sec.

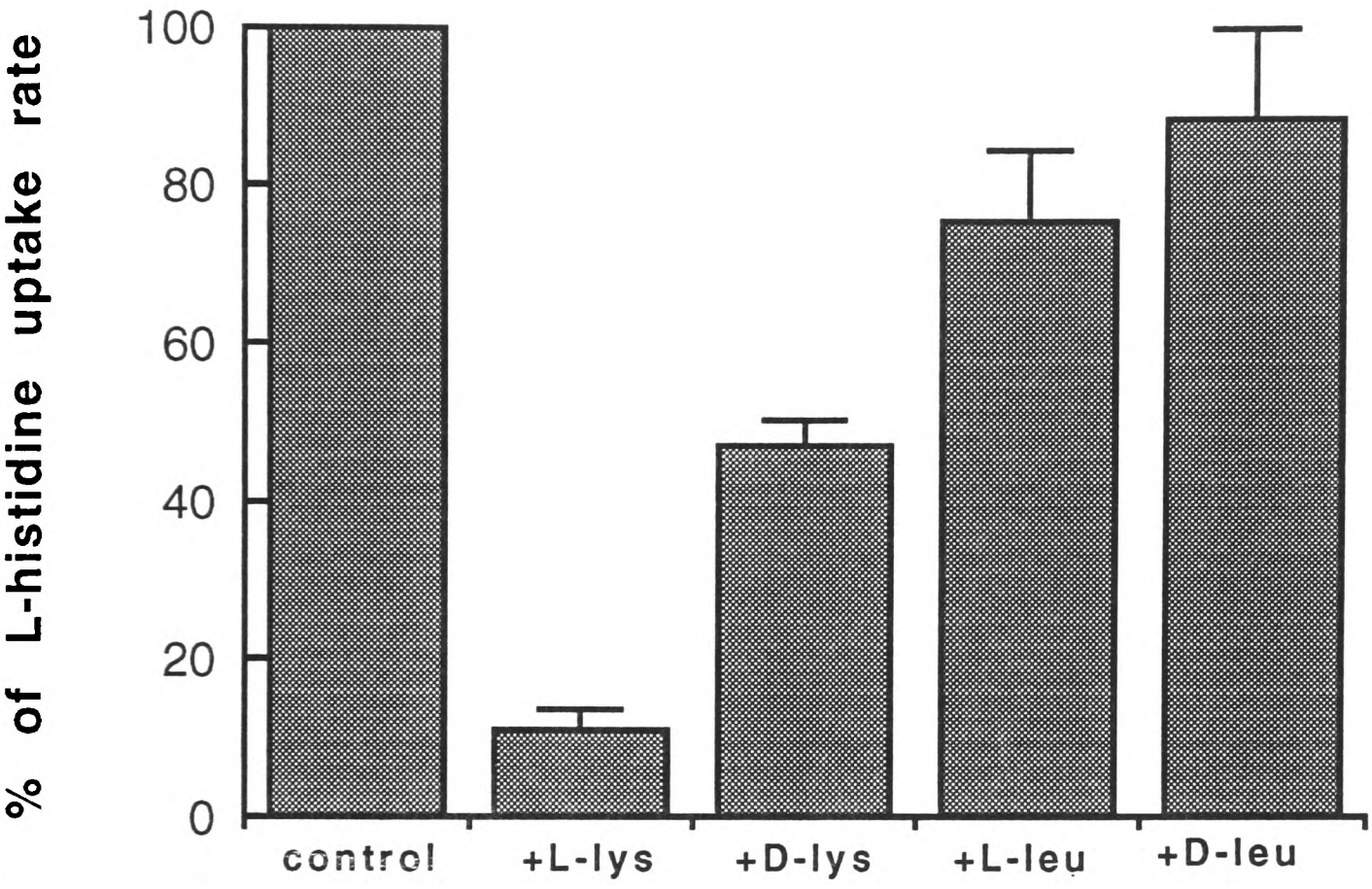


Figure 4.8

Substrate stereospecificity of the lysine and leucine transport system in lamprey red cells. The uptake of L-histidine (0.1mM) was measured with the presence of 20mM L- & D-lysine and L- & D-leucine in the medium. Data have been normalized and expressed in % of total L-histidine uptake rate. The percentages of inhibition caused by L,D-lysine and L,D-leucine were 91.1±0.7%, 55.4±3.5%, 27.4±12.6%, and 14.2±6.3% respectively (±sem, n=3). The inhibitory effect of amino acids is significantly different between L- and D-lysine (p=0.001) but not between L- and D-leucine (p=0.485).

D-lysine caused an over 50% (53±4%, ±sem, n=3) decrease. However, there was no significant difference in the inhibitory effect of L & D-leucine (p=0.45, n=3). L-leucine inhibited L-histidine uptake by 27±13 % (±sem, n=3) and D-leucine caused a decrease of over 10 % (14±6 % , ±sem, n=3). From this result, it is clear that there are at least two distinct transport pathways for histidine transport in lamprey red cells. One is stereoselective in favour of the L-form substrate such as L-lysine whereas the other one did not have a clear preference for L-, over D-leucine. These two transporters may represent a y^+-like, and L-like system in lamprey red cells.

4.4 *Trans*-effect of Leucine and Lysine on (Zinc-histidine) Uptake

If the zinc-histidine complex enters the lamprey red cells via the non-stereospecific L-like system, direct evidence for this cotransport would be the determination that internal leucine activates the exchange mode of the transporter and consequently results in an increased uptake of zinc-histidine via trans-stimulation. In other words, the preloading of leucine has a trans-stimulating effect on zinc-histidine influx. The experiments described below were designed to test this hypothesis. Lysine was compared with the effect of leucine to distinguish the role of two histidine transporters; an L-like and y^+-like system. Lamprey red cells were preloaded with 50mM L-leucine and L-lysine for an hour. Cells were then washed twice quickly with ice-cold EDTA washing solution to remove the extra extracellular leucine and lysine. Following the washes, red cells were quickly resuspended in standard saline, and the ^{65}Zn stock solution which contained total 30μM Zn and 0.1mM L-histidine was added to start the flux. Figure 4.9 shows the results. The uptake of zinc-histidine complex was significantly increased (over 400%) in cells which were preloaded with L-leucine but not in cells preloaded with L-lysine. Thus, the result strongly support the

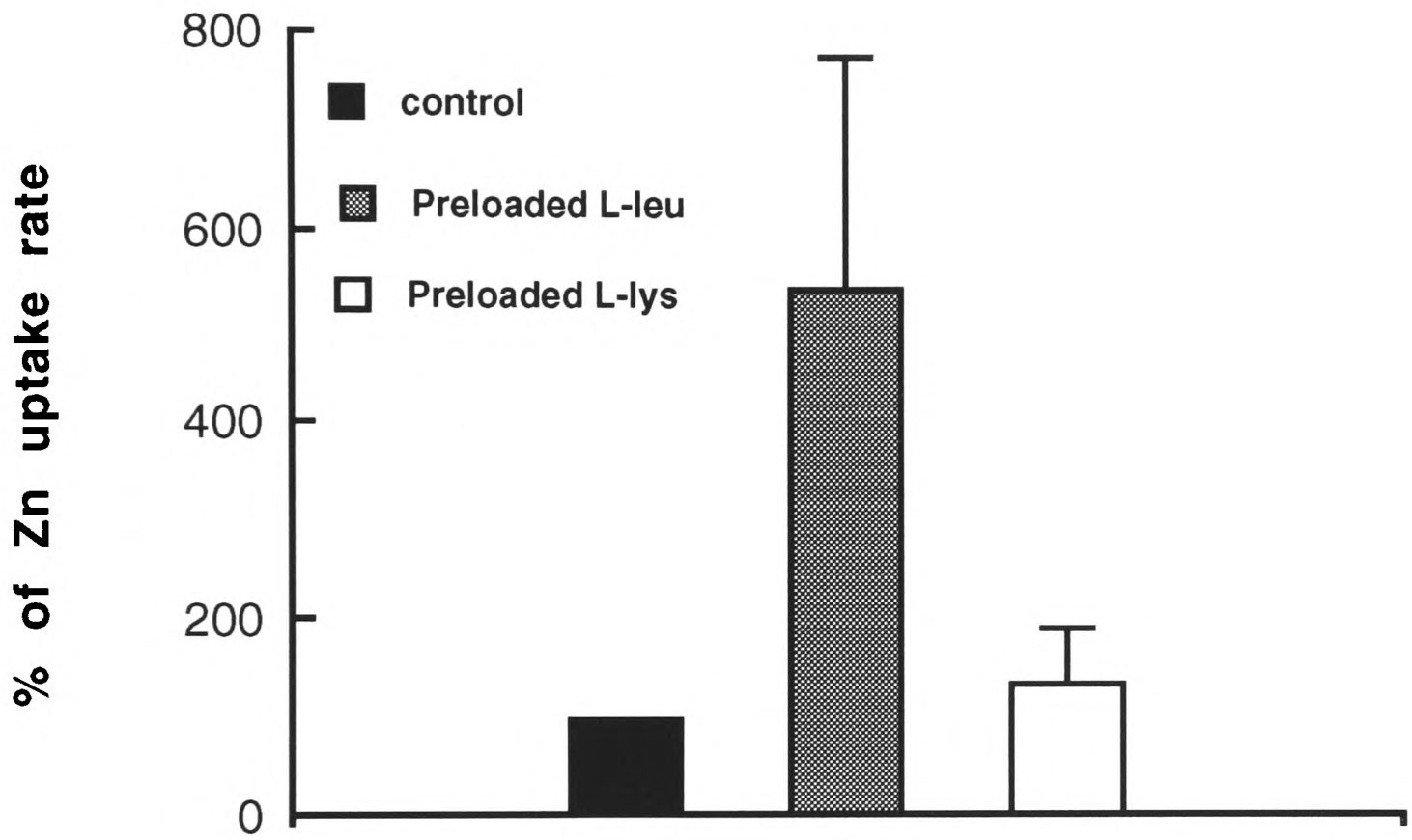


Figure 4.9

Effect of internal L-leucine and L-lysine on (zinc-histidine) uptake (30μM $ZnCl_2$; 0.1mM L-histidine). Data have been normalized and expressed as a percentage of the control value. In the group with cells preloaded with L-leucine, the uptake rate of zinc (in percentage) was 536.3± 237.6% (±sem, n=3) and 136.4± 56.4% (±sem,n=3) when cells were preloaded with L-lysine.

hypothesis that in lamprey red cells zinc-histidine was transported via a non-stereospecific L-like system.

4.5 Na^+-Dependence

The uptake of zinc, histidine, & the zinc-histidine complex in lamprey red cells was further characterized by studying its Na^+-dependence. Flux measurements were carried out in standard lamprey saline or Na^+-free saline (Na^+ replaced with NMDG). Figure 4.10 shows that the uptakes under all three conditions had little (< 10%) Na^+-dependence.

Discussion

The data presented here indicate that free zinc uptake in lamprey red cells was not affected by DIDS treatment or the presence of bicarbonate (Figure 4.3). This suggests that in lamprey red cells zinc uptake was not via an anion-dependent route as found in human red cells (Kalfakakou & Simons, 1990; Alda Torrubia & Garay, 1989). This result is consistent with the earlier reports that lamprey red cells have no band 3 anion exchanger (Ohnishi and Asai, 1985; Nikinmaa *et al.*, 1987).

A recent study on amino acid transport in red cells of the Pacific hagfish (Fincham *et al.*, 1990) can be compared directly with the results obtained here. Hagfish are similar to lampreys in many respects; since they are among the most primitive vertebrates alive today. Red cells from hagfish were also reported to have minimum activity of band 3 anion exchanger (Ellory, 1987). In their

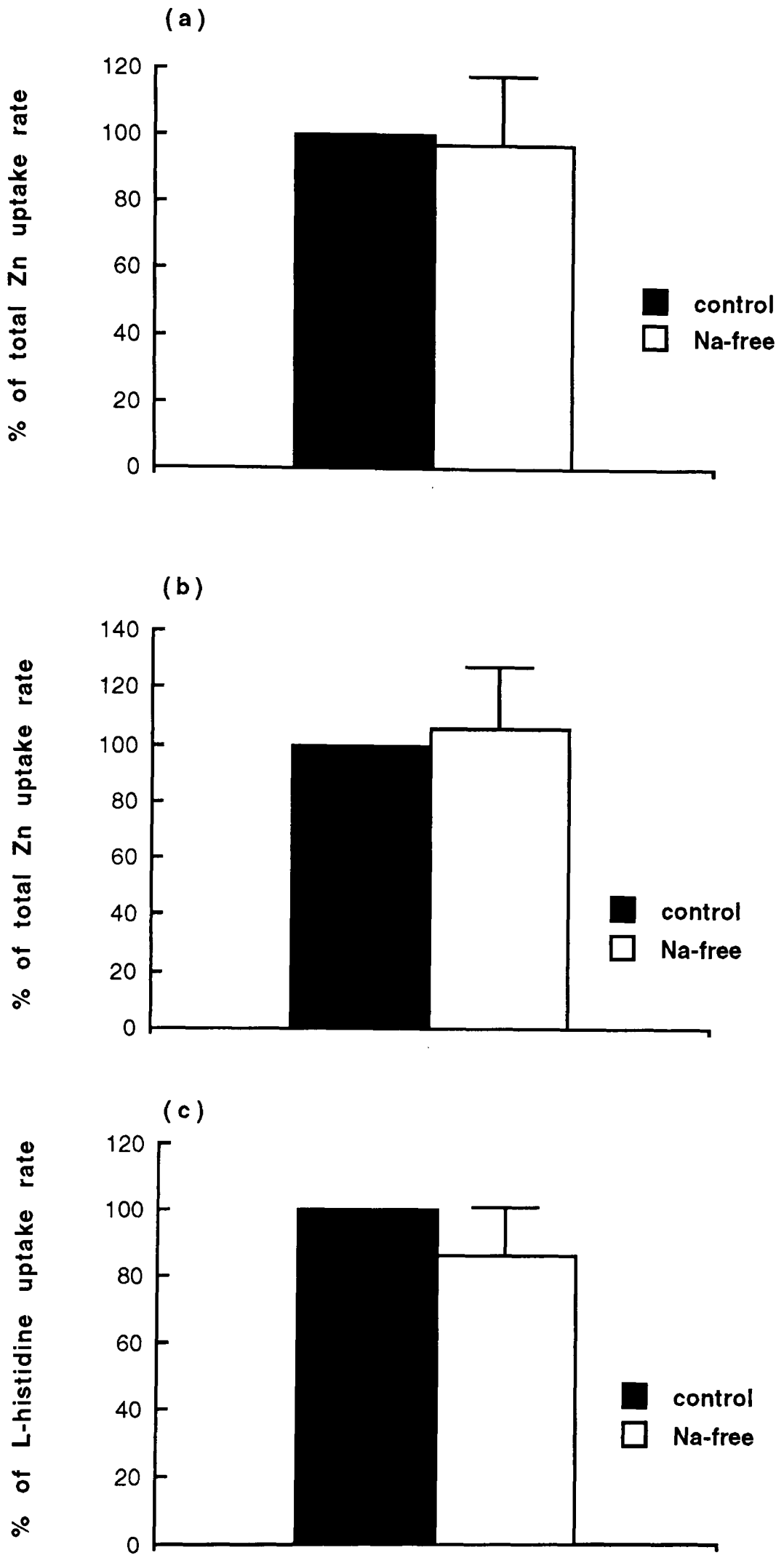


Figure 4.10 (a), (b) & (c)

Na^+-dependence of Zn, Zn-histidine, and L-histidine uptake in lamprey red cells. The uptake of 30 μM Zn (a), 30μM Zn and 0.1mM L-histidine (b), and 0.1mM L-histidine was measured in standard lamprey saline and Na^+-free saline (replaced with NMDG). Data have been normalized and expressed as a percentage of the control value.

study, Fincham *et al.* (1990) indicated that hagfish red cells had very potent amino acid transport and high intracellular concentrations of amino acids. For example, system asc in hagfish red cells was suggested to have a 10^4 fold greater activity than a similar ASC system found in human red cells (Eavenson and Christensen, 1967; Young, 1967; Fincham, 1987). Uptake rates for L-leucine and L-histidine were even higher (Fincham *et al.*, 1990). There were at least four distinct systems for amino acids which had been identified in hagfish red cells (Fincham, 1990); two Na-dependent systems specific for proline and glycine and two Na-independent systems which had high affinity for neutral amino acids and mainly operated in exchange mode, system asc and L-type system.

No studies have been carried out on amino acid transport in lamprey red cells. Therefore, nothing is known about the characteristics of lamprey amino acid transport systems such as their substrate specificity, exchange properties or substrate stereospecificity. In order to perform typical paradigm substrate experiments, we made the reasonable assumption that lamprey red cells might resemble hagfish red cells in their amino acid transport systems. Therefore, proline, glycine, L-leucine, L-alanine, and L-lysine were chosen to be the competitive inhibitors to identify L-histidine transport pathways. The result of Figure 4.6 shows that lamprey red cells are similar to hagfish red cells since they showed a very rapid L-histidine transport rate with the greatest initial uptake rate among all the cell types which had been examined in this thesis (see Table 4.1). Figure 4.7 illustrates that L-leucine and L-lysine were able to inhibit L-histidine uptake suggesting that they might share the same pathways. However, it needs to be emphasized that the percentage of inhibition caused by these amino acids should not be interpreted literally as the percentage of contributions by different transport pathways. In view of the fact that the amino acid transport systems in the lamprey are of exceptionally high activity and possibly operating in the exchange mode, it is not impossible that paradigm amino acids can quickly enter the cells during the

incubation period and then activate transporters from trans-side. Thus trans-stimulation might have occurred when we expected a cis-inhibition effect by excess paradigm substrates. This might be the reason for the slight increase we observed on the addition of L-alanine (Figure 4.7). L-alanine might be transported via asc and L-type systems into lamprey red cells during the 30 second incubation and then activate both L and asc system from trans-side. Nevertheless, the result of Figure 4.7 does show that the presence of L-leucine and L-lysine inhibited L-histidine uptake into lamprey red cells, suggesting that L-histidine might share the same routes with L-leucine and L-lysine, even though the percentages of inhibition may not have represented the real contributions of these transporters.

The result of Figure 4.8 shows clearly that there are at least two distinct transporters for L-histidine in lamprey red cells which are different in their substrate stereospecificity. The lysine-inhibitable pathway showed stereospecific preference for L-lysine. On the contrary, the leucine-inhibitable transporter did not show a significant stereoselectivity for its substrate. Unlike in human red cells, the effect of histidine on zinc uptake in lamprey red cells was not different between L-, and D-histidine (Figure 4.5). If such an effect was operating on the same mechanism of the cotransport of zinc and histidine via the histidine transport system, the non-stereospecific L-type system seemed to be the possible route for zinc-histidine entry into lamprey red cells. The finding that the uptake of zinc-histidine was able to be trans-stimulated by internal leucine but not lysine strongly supports the hypothesis that the zinc-histidine complex entered the cells by the L-type amino acid transport system (Figure 4.9).

Little is known about the composition of lamprey plasma. Nevertheless, if taking into account the fact that lamprey red cells have highly potent amino acid transport and possibly also have a

greater concentration ratio of amino acids over free zinc ions in plasma as observed in many other vertebrates, it may be justified to propose that amino acid transport systems account for the principal route for zinc uptake across lamprey red cell membranes in a form of zinc-amino acid complex. In the case of the zinc-histidine complex, an L-type system is the most potent pathway.

CHAPTER FIVE

ZINC TRANSPORT IN SHEEP RED CELLS

Introduction

Erythrocytes from Finnish Landrace sheep show a genetically controlled difference in their amino acid transport properties. This phenomenon has been extensively studied and reported as the absence of a single dominant amino acid transport system (system C) (Young *et al.*, 1976, 1977, 1980; Ellory,1987), which results in many manifestations, including decreased permeability for several neutral amino acids, low intracellular GSH, raised internal amino acids (particularly lysine and ornithine), altered Na^+ and K^+ levels and reduced life span of the red cell (Young and Ellory, 1977). Nothing is known about zinc and histidine transport in sheep red cells.

The purpose of this chapter is to study zinc transport in sheep red cells by examining the role of the two mechanisms (anion-dependent and histidine linked) suggested in previous chapters. By comparing the results from red cells from both normal and system C deficient sheep, it was hoped to clarify the role of amino acid transport systems in the zinc transport process.

Material and Methods

Sheep blood was a generous gift from Dr E.M. Tucker of A.F.R.C. Institute of Animal Physiology, Babraham, Cambridge. Whole blood from Finnish Landrace sheep was collected by jugular venepuncture into heparinized vacutainers. The cell suspension was filtered through a

polymer wool column to remove white blood cells; the erythrocytes were then washed three times in a medium containing (mM); NaCl(145), MOPS(10), D-Glucose(5), KCl(5), pH 7.4. The buffy coat was discarded. After the final wash erythrocytes were suspended at a haematocrit of 5% and incubated at 37^{0}C for at least one and half hours (the incubation medium being changed every half hour) to deplete intracellular amino acids. Uptake of zinc was measured by mixing 0.45 ml 10% cell suspension with 0.45 ml of ice cold incubation medium containing ^{65}Zn. 5mM bicarbonate was added as appropriate. Experiments were started by mixing the cell suspension with the stock solution at room temperature and then transferring the samples to a 37 ^{0}C water bath. At chosen time points during the incubation, samples were taken and put onto ice for 2-3 min before washing the cells 5 times by centrifugation with ice-cold medium containing (mM): NaCl(150), HEPES(10), EDTA(10), pH 7.4. The packed cells were lysed in 0.5 ml of 0.5% Triton X-100 and 0.5ml 5% of TCA (trichloroacetic acid) was added. The precipitate was removed by centrifugation (10,000 g, 5min), and the radioactivity in the supernatant was measured by scintillation counting.

Results

5.1 Time course

Red cells from normal and system C deficient sheep were incubated in buffered saline medium containing 30μM $ZnCl_2$ (including ^{65}Zn) for various time periods between five and sixty minutes. The results of a typical experiment are shown in Figure 5.1. The results fitted a simple linear

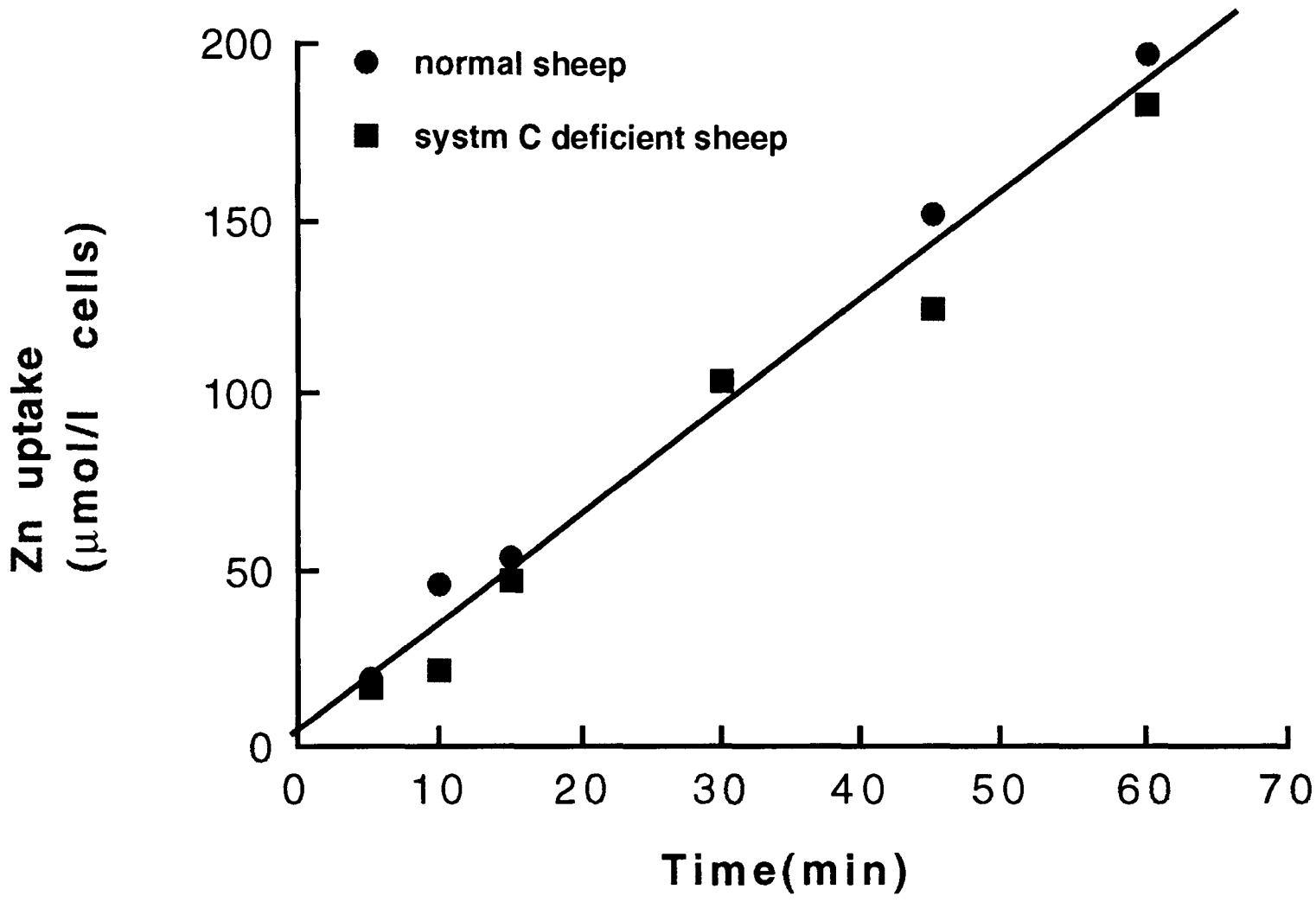


Figure 5.1

Time course for zinc uptake in sheep red cells. The extracellular zinc concentration was 30 μM. The data points fitted a linear regression with a slope of 3.18 (r=0.997) μmol. l cell^{-1}. min^{-1} for normal sheep red cells and 3.08 (r=0.982) μmol. l cells^{-1}. min^{-1} for system C-deficient sheep red cells.

regression. Zinc uptake (30μM) in red cells from both normal and system C deficient sheep appeared linear with time for up to an hour. Zinc uptake did not differ in either cell type. Therefore, all subsequent flux measurements were performed with an incubation period of 10-15 min at 37 ^{0}C to measure the initial uptake rates.

5.2 Effect of SITS & Bicarbonate

The effects of 10μM SITS and 5mM bicarbonate on zinc (30μM) uptake were studied in these experiments to see if sheep red cells possess an anion-dependent, SITS-inhibitable mechanism for zinc entry as was found in human red cells. The results are shown in Figure 5.2. The presence of 5mM bicarbonate in the medium significantly ($p<0.05$) stimulated zinc uptake by 4 fold in both types of sheep red cells. The stimulus of bicarbonate was inhibitable by 10μM SITS. In the bicarbonate-free medium, SITS caused a further slight decrease (over 40%) in zinc uptake rates. The uptake rates of zinc (30μM) in these experimental conditions did not differ between red cells of normal or system C deficient sheep. The result indicated that entry via an anion-dependent, SITS inhibitable pathway which accounted for over 80% of zinc uptake at 30 μM in sheep red cells under these conditions. As in human red cells, the band 3 anion exchanger might account for the major route for zinc uptake in sheep red cells when the medium contains no binding ligands. From the data here, it could also be concluded that the transport activity of the band 3 anion exchanger was identical in both types of sheep red cells.

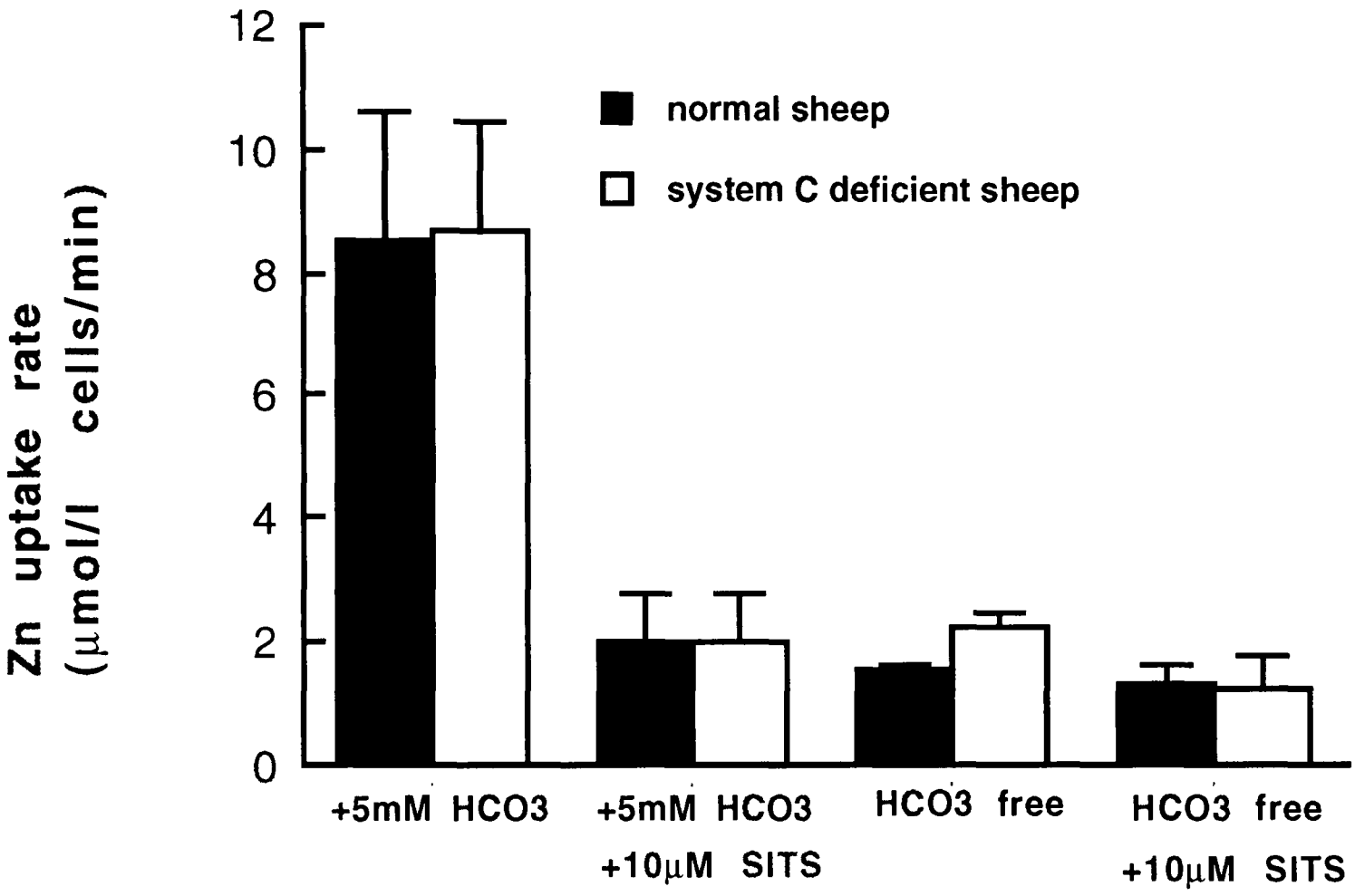


Figure 5.2

Effect of SITS treatment (10μM) and the addition of bicarbonate (5mM) on zinc (30μM) uptake in sheep red cells. The uptake rates in these conditions are 8.5±2.1 (n=4), 8.7±1.8 (n=4), 2.0±0.8 (n=4), 2.0±0.7 (n=4), 1.5±0.1 (n=6), 2.2±2.3 (n=6), 1.3±0.3 (n=3), 1.2±0.6 (n=3) μmol. l cell^{-1}. min^{-1} (±sem) respectively.

5.3 Effect of L,D-histidine on SITS-insensitive Component

Having established that sheep red cells share a similar mechanism of anion-dependent zinc uptake to that found in human red cells, the following experiments were aimed at investigating if zinc uptake in sheep red cells was also affected by the presence of histidine. Uptake of zinc (total extracelluar concentration 30μM) was measured in red cells from normal and system C deficient sheep. All the red cells were pretreated with SITS to inhibit the band 3 anion exchanger. The flux measurement was carried out in bicarbonate-free medium containing 1mM L-, or D-histidine. Under these conditions, the concentration of [Zn] was estimated to be 0.12μM. Thus, the uptake rate of Zn at this concentration could be estimated to be expected to be about 0.005 μmol.l cells^{-1}.min^{-1}. from the results in Figure 5.1 if the transport is passive and linear. The results in Figure 5.3 show that in both types of sheep red cell the uptake rates of zinc were significantly ($p< 0.05$) greater (over 60 fold) than the estimated value when the medium contained 1mM L, or D-histidine. In normal sheep red cells, there was no significant difference between the effects caused by L, or D-histidine ($p=0.444$). In system C deficient sheep red cells, the zinc uptake rate in the presence of D-histidine was slightly lower than that in the L-histidine containing medium. However, the difference was not significant ($p=0.173$). The uptake of zinc at this experimental condition did not show any significant difference between the two types of sheep red cells either.

5.4 L-histidine Uptake

In human & lamprey red cells, it was suggested that the effect of histidine on zinc uptake could be due to transport of a zinc-histidine complex via an amino acid transport system(s). In order

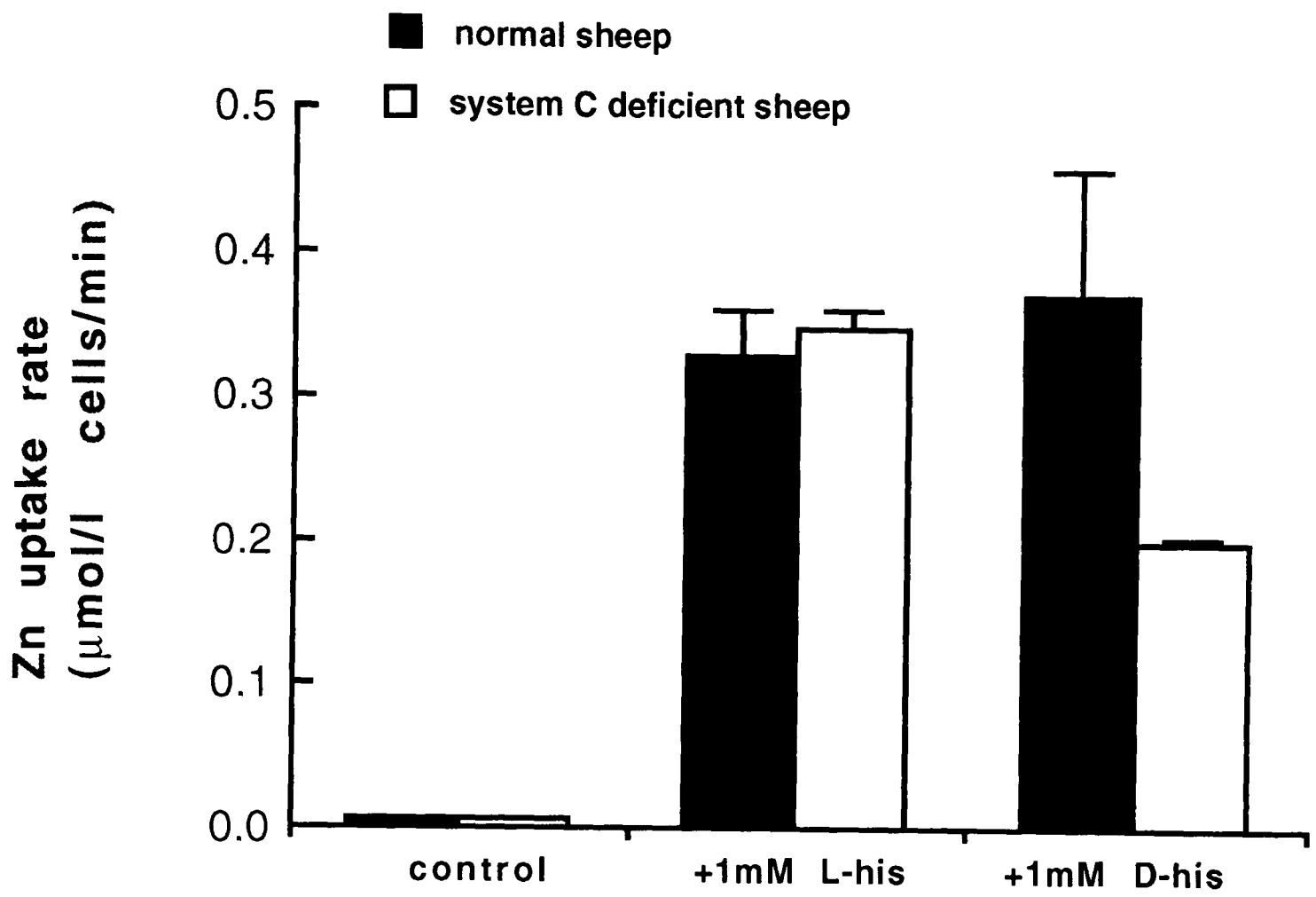


Figure 5.3

Effect of 1mM L-, or D-histidine on zinc (30μM) uptake in sheep red cells. The estimated uptake rate for the control group in the presence of 1mM histidine is 0.005 μmol. l cell^{-1}. min^{-1}. The uptake rates for L-histidine and D-histidine groups in normal and system C-deficient sheep red cells are 0.3±0.03, 0.35±0.01, 0.37±0.09, and 0.2±0.00 μmol. l cell^{-1}. min^{-1} (±sem, n=3) respectively.

to explain this non-stereospecific histidine effect observed in sheep red cells, a series of experiments have been carried out to study histidine transport using red cells from normal and system C deficient sheep.

5.4.1 Time Course

Red cells of both types of sheep were washed and preincubated for one and half hours at 37 ^{0}C to deplete intracellular amino acids. Red cells were then suspended (haematocrit of 10-15%) in buffered bicarbonate-free saline medium containing 1 & 10mM L-histidine for various time intervals between 5-60 minutes. Figure 5.4 shows the result of a typical experiment carried out in normal sheep red cells. Uptake of L-histidine over a concentration range up to 10mM showed a linear relationship with time for at least 60 minutes in red cells from both normal and system C-deficient sheep. Therefore, all the following histidine experiments were carried out with an incubation period of 10-15 minutes to measure the initial uptake rates.

5.4.2 Concentration-Dependent Uptake

Though it was known that red cells of system C deficient sheep had a markedly diminished permeability to several amino acids (including L,D-alanine, α-amino-n-butyrate, valine, cysteine, serine, threonine, asparagine, lysine and ornithine (Young, 1976)), it was not clear if histidine uptake did differ between the two types of sheep red cell. The uptake of histidine at three different concentrations (0.1, 1 and 10mM) was measured in both types of sheep red cells and the results are shown in Table 5.1. At lower concentrations of 0.1 & 1 mM, there was no difference in L-histidine uptake between the two types of sheep red cells ($p=0.623$, & 0.285 at the

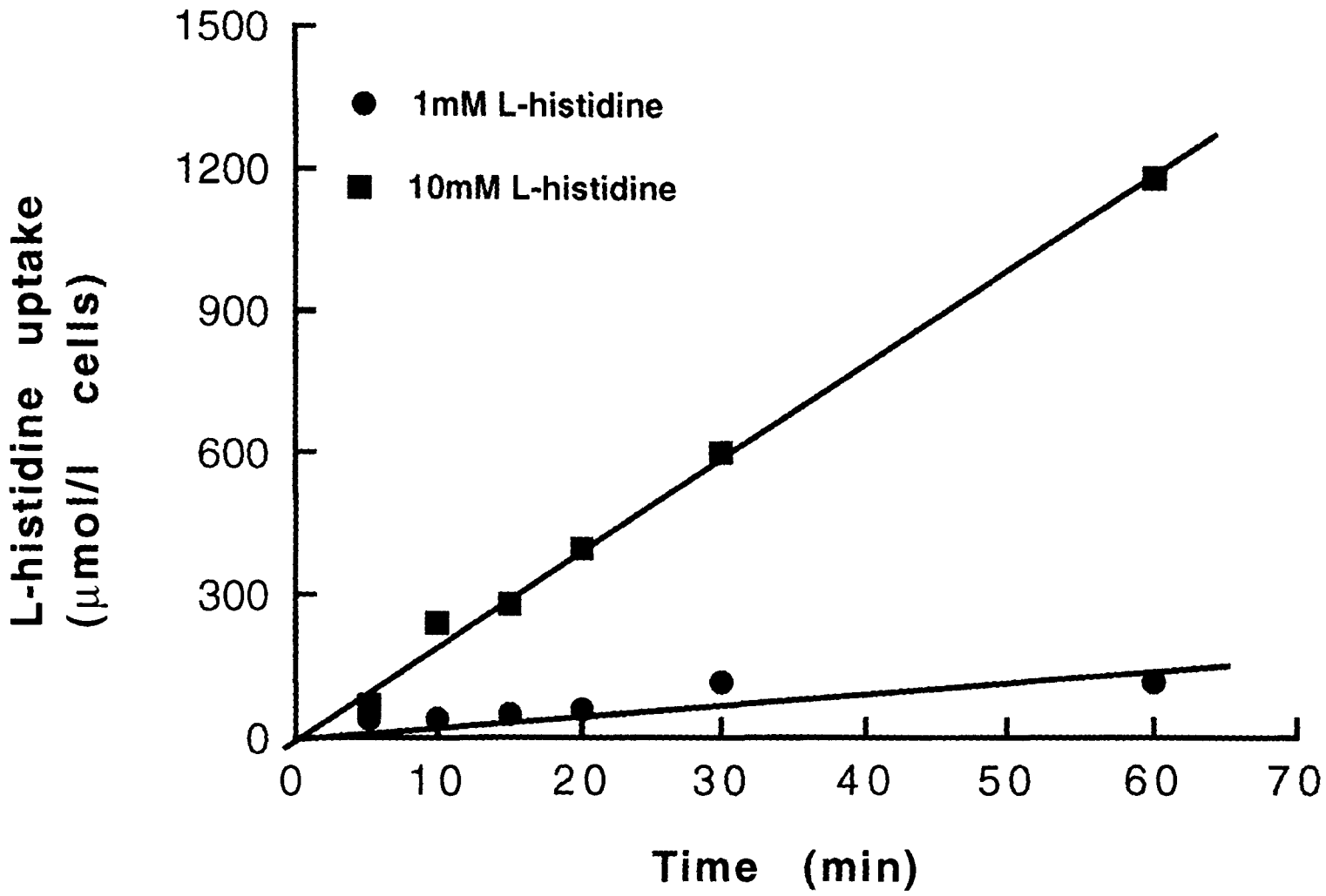


Figure 5.4

Time course for L-histidine (1 and 10mM) uptake in normal sheep red cells. The data of this typical example fitted a linear regression with a slope of uptake rate 19.8 µmol.l cells^{-1}.min^{-1} (r=0.998) for 10mM L-histidine uptake and 1.6 µmol. l cells^{-1}. min^{-1} (r=0.859) for 1mM L-histidine.

Table 5.1 L-histidine Uptake in Sheep Red Cells

Concentration(mM)		Uptake rate (μmol. l cell^{-1}. min^{-1})	
	(p value)	Normal sheep (±sem, n=3)	System C$^-$ sheep (±sem,n=4)
0.1	(p=0.6)	0.20 ± 0.04	0.19 ± 0.04
1	(p=0.3)	1.64 ± 0.06	1.28 ± 0.28
10	(p<0.05)	18.00 ± 2.60	9.29 ± 2.5

concentration of 0.1, & 1mM). However, at an extracelluar L-histidine concentration of 10 mM, L-histidine uptake in normal sheep red cells was significantly greater than in system C-deficient sheep red cells ($p<0.05$).

5.4.3 Effect of SITS on L-histidine Uptake

The experiments described below were designed to test if the uptake of histidine in either type of sheep red cell is inhibitable by SITS. Red cells from both types of sheep were preincubated with 10μM SITS for half an hour in standard bicarbonate-free saline. After the incubation, red cells were quickly washed 3 times to be rid of extracellular SITS and resuspended in saline to start the flux measurements. As the result in Figure 5.5 shows, treatment by SITS had no effect on L-histidine (1mM) uptake in red cells of both normal and C-deficient sheep ($p=0.217$ in normal sheep red cells; $p=0.711$ in system C-deficient sheep red cells).

Discussion

Entry via the Band 3 Anion Exchanger

By examining the effect of SITS and bicarbonate on zinc uptake (Figure 5.1 & 5.2), it was clear that in both types of sheep red cell zinc uptake was via a SITS-inhibitable, bicarbonate-stimulated pathway when the medium contained no binding ligands, similar to that found in human red cells. The result also clearly indicates that this anion-dependent route (which represented the entry via the anion exchanger) was of identical activity in both types of sheep red cell.

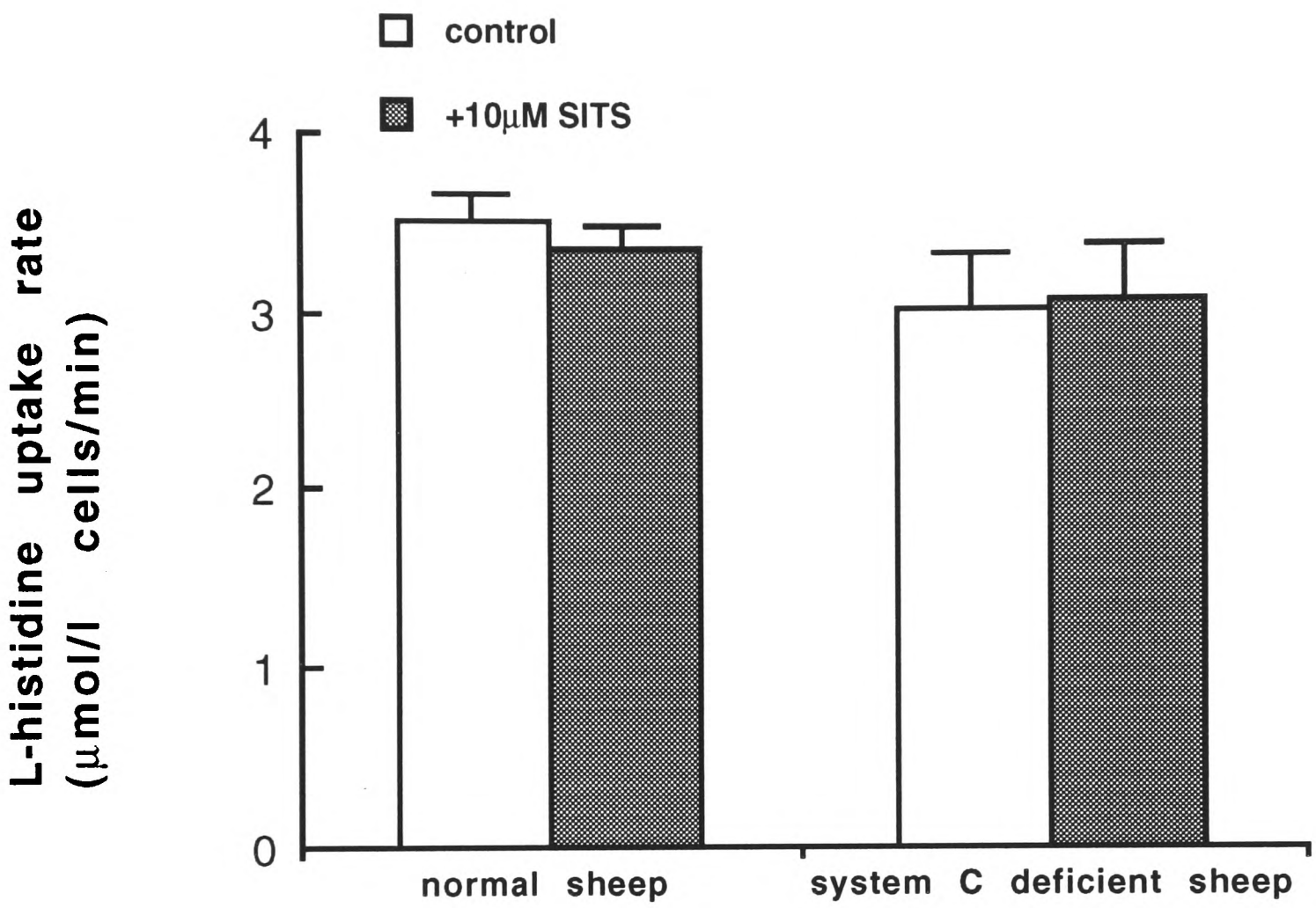


Figure 5.5

Effect of SITS (10μM) treatment on histidine (0.1mM) uptake in sheep red cells. The uptake rates of histidine in these condition are 3.5±0.2, 3.3±0.1, 3.0±0.3, and 3.1±0.3 μmol. l cell^{-1}. min^{-1} (±sem, n=5) respectively.

Histidine Effect on Zinc Uptake

System C was reported to be the dominant amino acid transport system for neutral and dibasic amino acids in sheep red cells (Young *et al.*, 1976) with several characteristics of being substrate-specific, highly stereospecific, Na-independent, and most important of all, completely absent in red cells from system C deficient sheep (Young *et al.*, 1976; 1977; 1979; 1983). This genetic variant has provided an excellent model for examining the role of the amino acid transport system in zinc transport across cell membranes. The results of Figure 5.3 demonstrate that the presence of histidine in the medium was able to increase the estimated zinc uptake rate by 60 fold. However, it was surprising to observe that this histidine effect was not stereospecific in favour of the L-isomer and did not show any significant difference in either type of sheep red cell. Evidence from human and lamprey red cells has strongly suggested that the histidine effect was due to facilitated zinc-histidine transport across cell membrane via an amino acid transport system (see Chapter 3 & 4). If this was also the case with sheep red cells, one would expect a different result from that obtained in Figure 5.3. Firstly, it would be expected that zinc uptake in system C deficient sheep red cells would be significantly lower than in normal sheep red cells because of the absence of system C. Secondly, one would expect that in normal red cells the effect of histidine would be stereospecific since system C is highly stereospecific for its substrates. For instance, red cells from normal red cells showed a 30-fold stereoselectivity for L-alanine as against D-alanine, while red cells from system C deficient sheep gave identical slow uptakes for both isomers (Young *et al.*, 1976). Further experiments on histidine transport were made to resolve this controversy.

Histidine Transport in Sheep Red Cells

The results of Table 5.1 reveal the important fact that though system C is very substrate specific, histidine is a poor substrate for this system. At the concentrations of 0.1 & 1mM, histidine uptake did not show any difference between two cell types. Histidine uptake in sheep red cells is much slower than in human & lamprey red cells (Table 5.2). It is likely that system C has a very low affinity for histidine so that histidine transport at a low concentrations occurs mainly via a passive diffusion process or by another transporter. At a concentration of 10mM, the involvement of system C results in a significant difference in histidine uptake between the two types of sheep red cell.

Possible Explanations for Histidine Effect

There may be several reasons for the non-stereospecific effect of histidine on zinc uptake. Firstly, it is possible that system C facilitates zinc-histidine across cell membranes. The lack of a difference between the two cell types (Figure 5.3) could be explained by histidine having a low affinity for system C (see Table 5.1). Secondly, it is possible that in both types of sheep red cells histidine was transported via some unknown amino acid transporter which might contribute to the transport of zinc-histidine complex. Finally, the possibility of a transport system specific for the zinc-histidine complex should be considered. The possibility that the band 3 anion exchanger might transport zinc-histidine complex and contribute to this non-stereospecific histidine effect in both cell types was ruled out by the facts that the histidine effect was obtained in red cells in which the anion exchanger was fully inhibited by SITS. Further evidence was demonstrated in

Table 5.2 Histidine Uptake in Human, Lamprey & Sheep Red Cells

	Histidine Uptake Rate (μmol. l cell^{-1}. min^{-1})
Human (1mM; 37^{0}C)	32
Lamprey (0.1mM; 20^{0}C)	6.5
Sheep (1mM; 37^{0}C)	1.6 (normal sheep) 1.3 (system C$^-$ sheep)

Figure 5.5 that histidine uptake in both types of sheep red cell was not via the anion exchanger at all.

Little is known about sheep plasma composition. Nevertheless, it is fair to presume a low free zinc concentration in sheep plasma due to the binding to plasma proteins as observed in many other vertebrates. Thus, an amino acid linked zinc uptake may represent the major mechanism for zinc entry into sheep red cells under physiological conditions as we have proposed in previous chapters. In the case of sheep red cells, the identity of such a transport system remains to be established.

CHAPTER SIX

ZINC TRANSPORT IN HUMAN SICKLE CELLS

Introduction

Sickle cell anaemia is associated with a modified haemoglobin molecule (Hb-Hb-SS), in which a single base pair in the ß-globin gene has been mutated from adenine to thymine, resulting in a change in the sixth amino acid of the protein from glutamic acid to valine (Luzzatto, 1989). Under conditions of low oxygen tension, the abnormal haemoglobin tends to polymerize, causing a deformation in red blood cell shape termed sickling. The sickled red blood cells lead to occlusion of small blood vessels and hence difficulty in oxygen transport, which can result in 'crisis', a term that describes a wide spectrum of events, from relatively brief painful episodes to massive thrombosis or infarction in bone, lung or brain (Rifkind, 1990). Over the past 15 years, many studies have shown that sickle cell anaemia patients suffer from zinc deficiency as a result of continued haemolysis and hyperzincuria (Reed *et al.*, 1987; Endre *et al.*, 1990). Markedly decreased levels of zinc in plasma, erythrocytes, and hair were commonly found in sickle cell anaemia patients (Reed *et al.*, 1987; Endre *et al.*, 1990). The clinical features of this disease include abnormal growth and sexual development, defective functioning of the immune system, impaired healing of chronic leg ulcers and adverse haematologic effects (Reed *et al.*, 1987). All such symptoms can be reduced or even eliminated by oral zinc supplementation (Reed *et al.*, 1987). At the cellular level, zinc was reported to have beneficial effects in the treatment of sickle

red cells. Firstly, the adminstration of zinc in vitro reduced the number of irreversibly sickled cells from 28% to 18% (Rifkind, 1990). Secondly, zinc was able to increase the oxygen affinity of erythrocytes by binding to haemoglobin (Rifkind, 1990). It was reported that the loading of zinc into sickle cells, *in vitro*, caused a 3-4 fold increase in the oxygen affinity of HbSS and furthermore improved the filterability of sickle cells at intermediate partial pressures of oxygen (Taylor, 1991).

However, despite the delineation of these important *in vitro* effects, little is known about the transport mechanism for zinc uptake across the sickle cell membrane. The aim of this chapter is to investigate zinc transport in sickle red cells and to compare the results with those obtained in normal human red cells.

Material and Methods

Blood samples were a generous gift from Prof. J. Stuart, Dept of Haematology, Birmingham University. Blood was taken from healthy controls and out-patients with homozygous sickle cell disease who were in the asymptomatic steady-state. The preparation of the red cells was carried out by Miss Susan Ody with a method described previously (Ellory, 1991). Sickle red cells were then washed 3 times and suspended in the standard saline which experiments were carried out. Normally saline contained 145mM NaCl, 10mM MOPS, 5mM KCl, 5mM glucose, pH 7.4. 5mM bicarbonate was added as required.

Results

6.1 Time Course

Zinc uptake (30μM) was measured in sickle cells at different time intervals. Figure 6.1 shows a typical example of three similar experiments. The uptake of zinc showed a linear relationship with time for at least 15 minutes. Therefore, a period of 5-10 min was chosen to measure the initial uptake rate in the following experiments.

6.2 Effect of DIDS & Bicarbonate

The effects of DIDS and bicarbonate on zinc uptake in sickle red cells were investigated to see if sickle red cells possess a similar anion-dependent mechanism for zinc uptake. Similar to our findings in normal red cells, zinc uptake in sickle red cells was inhibitable by DIDS treatment and stimulated by the presence of 5mM bicarbonate (Figure 6.2). The treatment of DIDS (10μM) inhibited over 52% of the total zinc uptake in sickle red cells. The presence of bicarbonate in the medium stimulated the zinc uptake by about 2 fold. The results in Figure 6.2 also shows that the uptake of zinc in these three experimental conditions was not significantly different between normal and sickle red cells.

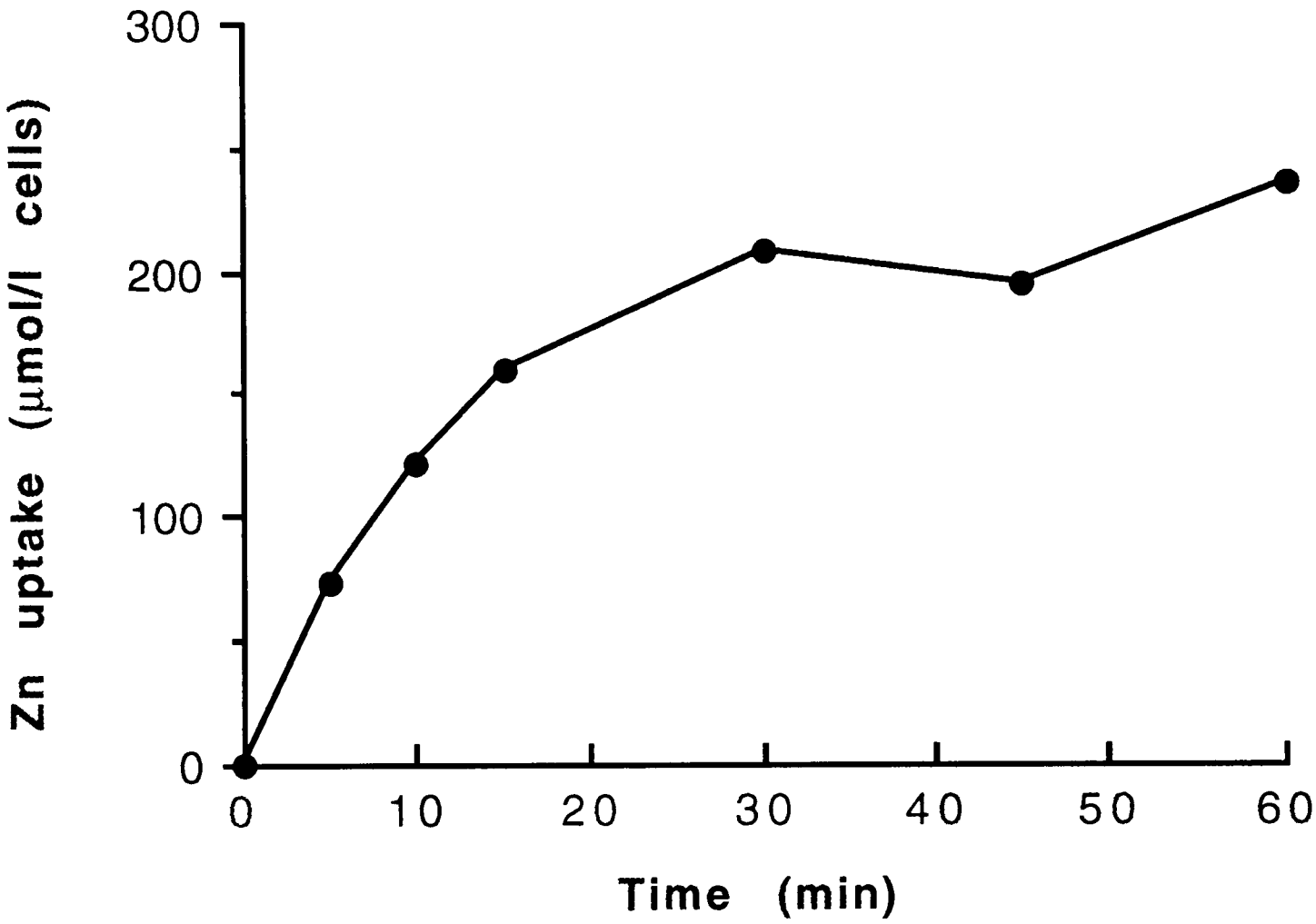


Figure 6.1

Time course for zinc influx into sickle red cells. The external Zn concentration was 30 µM. The incubation medium contained (mM): NaCl (145), HEPES (10), glucose (5), $KHCO_3$. The data points of this typical experiment fitted a linear regression with a slope of 8.97 (r=0.997) for the first 15 minutes. The mean of seven independent experiments is 19.8 ± 6.5 µmol.l $cell^{-1}.min^{-1}$ (±sem).

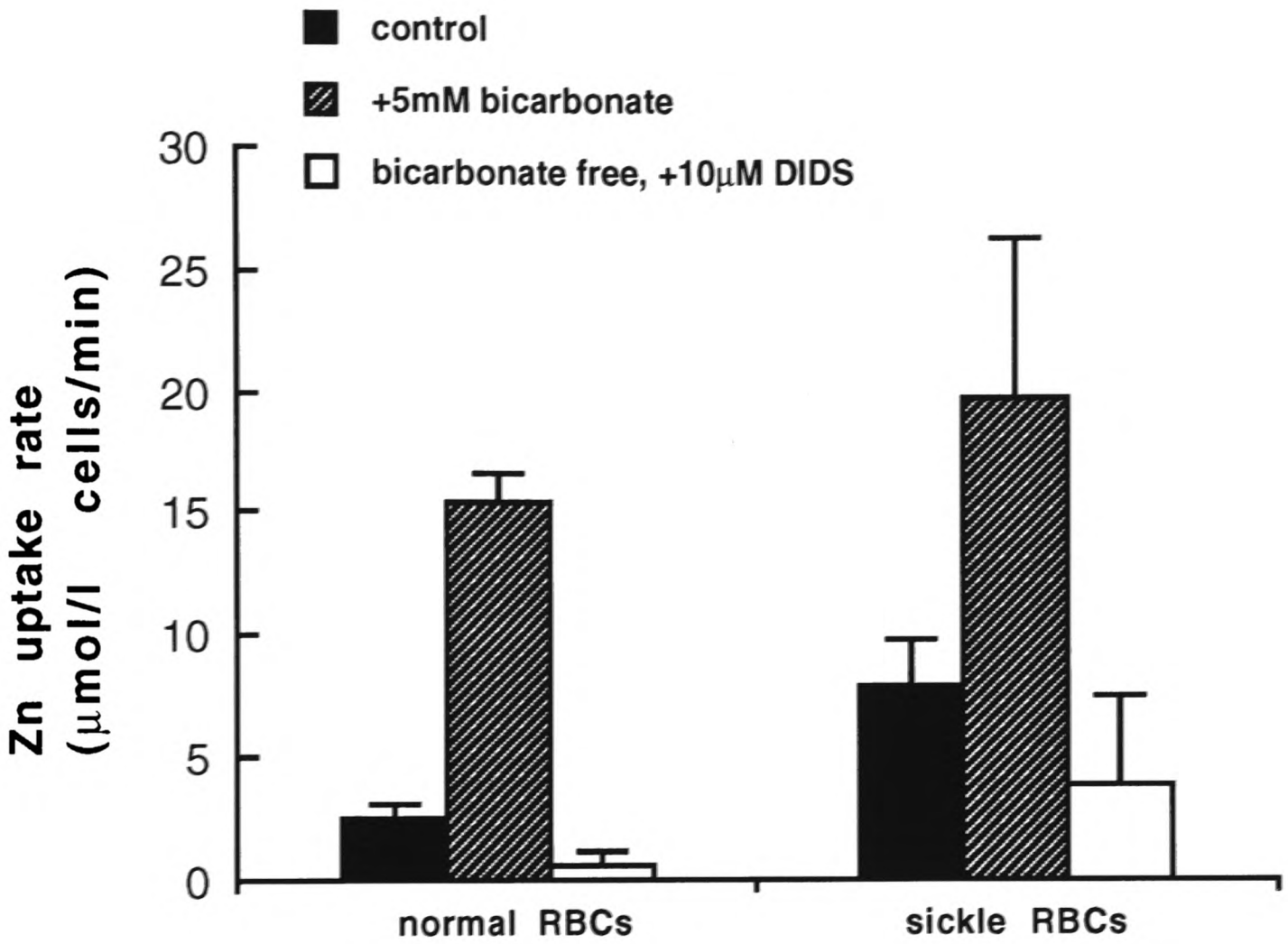


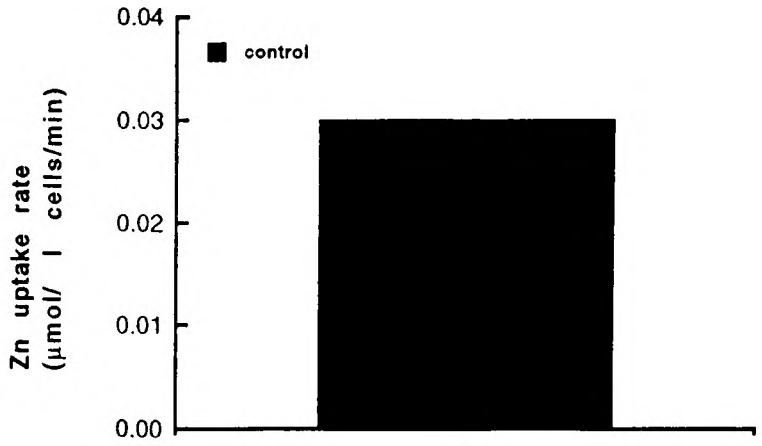
Figure 6.2

The uptake of Zn (30μM) in normal and sickle red cells. The uptake rates of Zn in these conditions were 2.6±0.4 (n=3), 15.5±1.06 (n=12), and 0.6±0.07 (n=3) (±sem) μmol.l cell^{-1}.min^{-1} in normal red cells and 7.9±2.0 (n=3), 19.8±6.5 (n=7), 3.8±1.7 (n=3) (±sem) μmol.l cell^{-1}.min^{-1} in sickle red cells.

6.3. Effect of Histidine via Non-band 3 Pathways

Having investigated the anion-dependent mechanism for zinc uptake, it was interesting to see if sickle cells operate a similar mechanism of amino acid linked zinc entry as we found in human, lamprey and sheep red cells. In the following experiments, the effect of histidine on zinc uptake in sickle cells was investigated (Figure 6.3). Red cells have been pretreated with 10μM DIDS to inhibit the anion exchanger.

1mM L, or D-histidine was added to standard bicarbonate-free saline and the uptake of zinc(30μM) was measured in sickle red cells. Again, as previously described, in the presence of histidine (1mM), the concentration of ionic zinc could be estimated to be 0.01 μM (using the calculation method described in section 2.4.6). Since the uptake rate of Zn was linear with the external concentration at this concentration (data not shown), we were able to estimate the zinc uptake rate from the previous result of Figure 6.2. The uptake rate of Zn at this concentration of 0.01 μM was about 0.03 μmol.l $cell^{-1}.min^{-1}$. The result in Figure 6.3 used this value as the uptake rate of the control group. Thus, when the medium contained histidine (either L, or D-isomer) the uptake rate of zinc measured was significantly greater than the estimated value of the control group. Furthermore the effect of histidine was strongly stereospecific in favour of the L-isomer (the significance of the difference between the effects of L, and D-histidine is ($p=0.01$)). The presence of L-histidine increased the zinc uptake rate by over 600 fold whereas D-histidine caused just over a 90 fold increase.



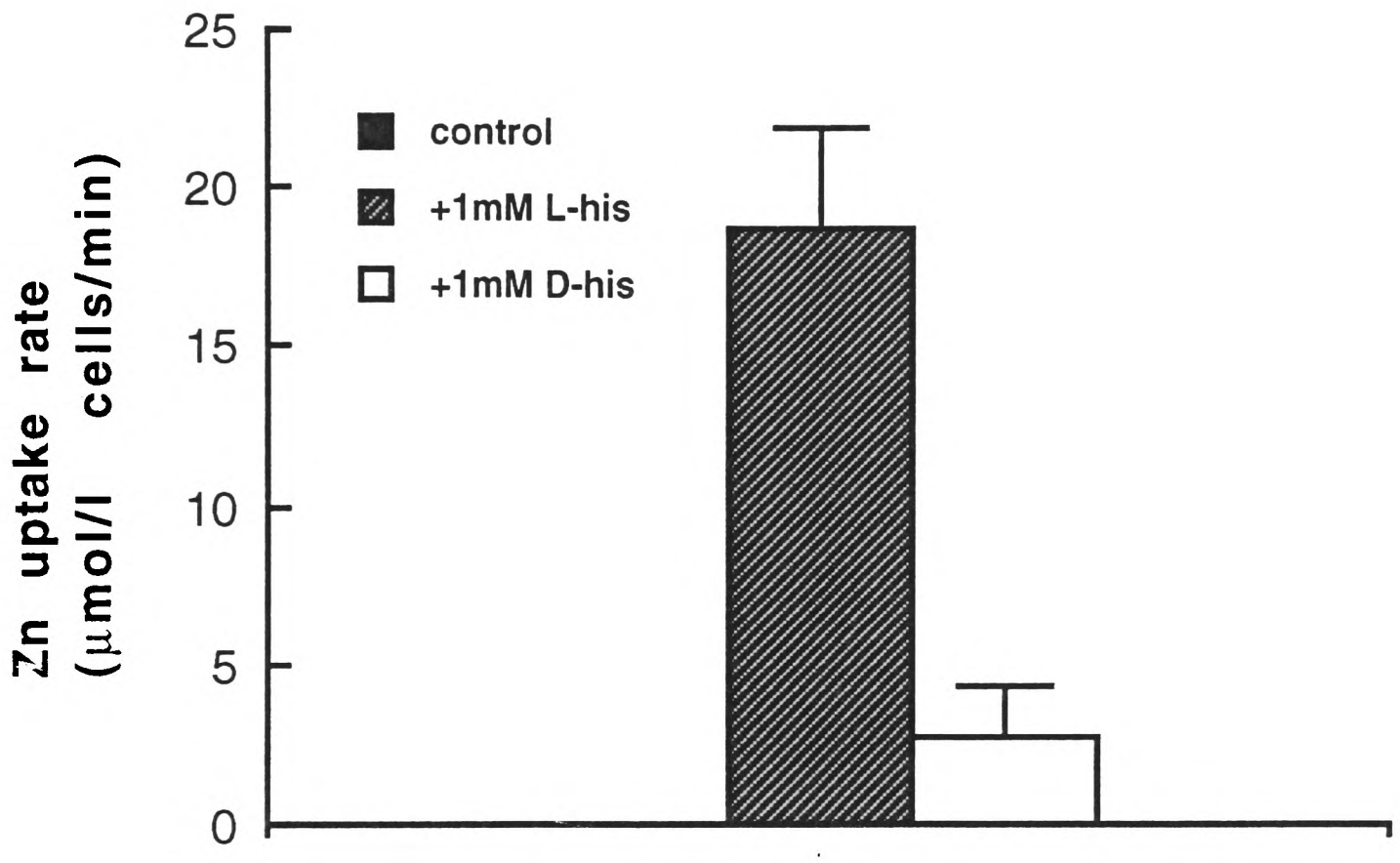


Figure 6.3

Effect of 1mM L,D-histidine on Zn uptake via a non-band 3 pathway. Sickle red cells were pretreated with 10µM DIDS and the experiments were carried out in bicarbonate-free saline. The uptake rates of the control, L-histidine containing group and D-histidine containing group were 0.03, 18.7±3.2, and 2.8±1.6 (±sem, n=3) µmol.l cell^{-1}.min^{-1}. The difference between the effects of L, and D-histidine was significant (p=0.01). The symbol for the control group was smaller than the sign of the x-axis.

6.4 Effect of BSA on the Histidine Effect

BSA (1%) was added to the medium to investigate the effect of albumin on this histidine linked zinc uptake. The uptake of zinc (30μM) was measured in sickle cells with the medium containing 1% BSA and 1mM L, or D-histidine. The result shown in Figure 6.4 demonstrates that in the presence of 1% BSA, the effect of L-histidine on zinc uptake occurs in both normal and sickle red cells. There was no difference observed in zinc uptake into normal or sickle red cells under these conditions.

Discussion

The results presented in this chapter show for the first time that zinc transport in sickle red cells is not different from that of normal red cells in terms of mechanism and transporter activity. In Chapter 3, we have reported two kinds of mechanism for zinc entry into red cells. Firstly, when the medium contains no zinc binding ligands, zinc is complexed with anions and transported via the band 3 anion exchanger. The evidence for this conclusion is that the uptake of zinc was inhibited by the treatment with DIDS and stimulated by the presence of bicarbonate. This proposal of an anion-dependent mechanism for zinc uptake across cell membranes agreed with the findings of others (Alda Torrubia and Garay, 1989; Kalfakakou and Simons, 1990) and was found consistently in the several cell types used in this thesis, including human and sheep red cells and bovine chondrocytes (see Chapter 3, 4, & 7). The second proposed mechanism for zinc transport into cells is that zinc can enter cells via an amino acid transport system in the form of zinc-histidine complex. The significance of this mechanism relies on the fact that amino acid bound

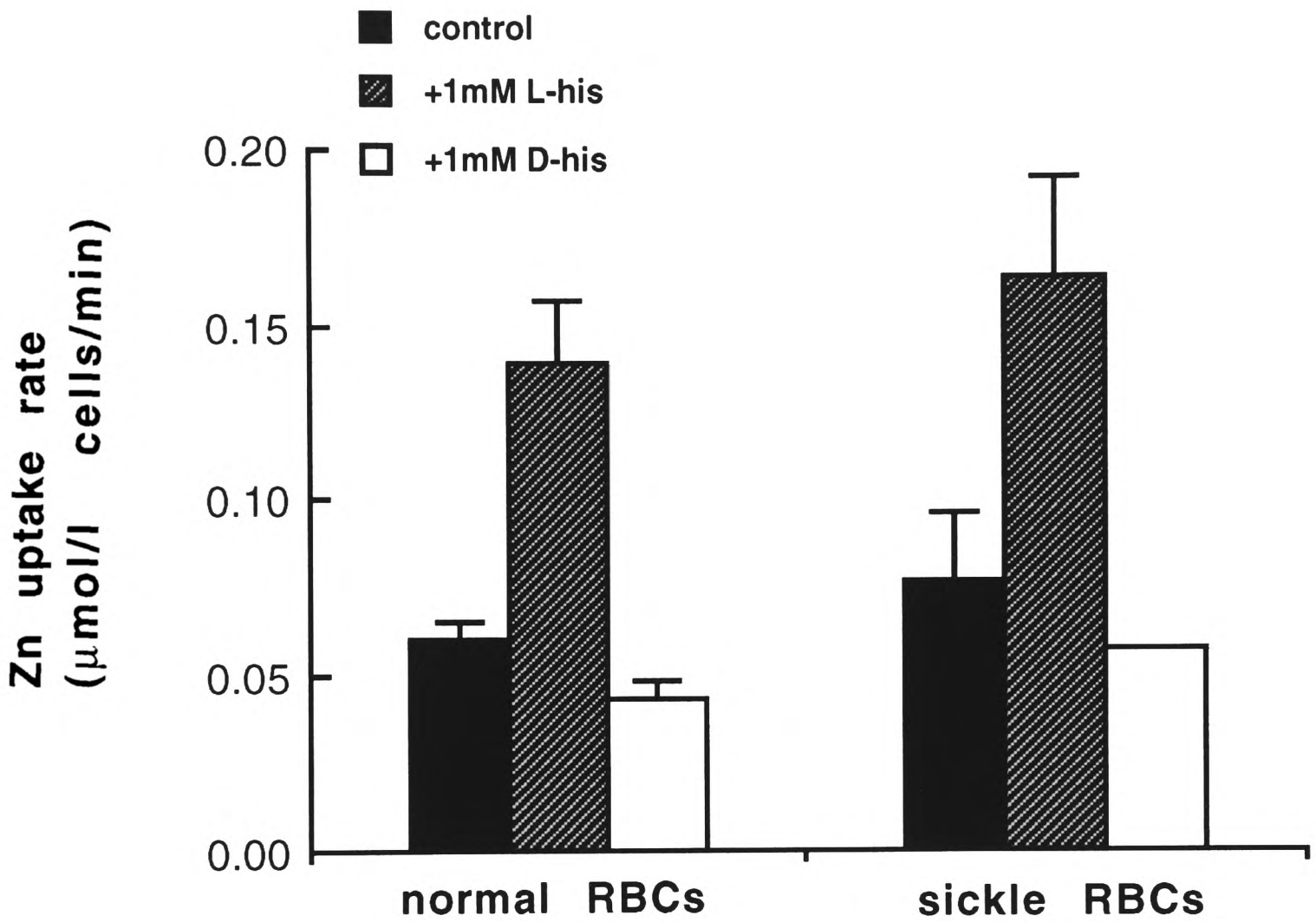


Figure 6.4

Effect of 1% BSA on the histidine effect in normal and sickle red cells. The uptake of Zn (30µM) was measured in bicarbonate-free medium which contained 1% BSA and L, or D-histidine. The uptake rates of Zn were 0.06±0.01 (n=8), 0.14±0.02 (n=6), 0.04±0.01 (n=3) (±sem) µmol.l cell^{-1}.min^{-1} in normal red cells and 0.08±0.02, 0.16±0.03, 0.06±0.00 (±sem, n=3) µmol.l cell^{-1}.min^{-1} in sickle red cells.

zinc is able to be exchanged with albumin-bound zinc; therefore such a transport mechanism may represent a physiological relevant and major uptake route for zinc entry into cells. In the present chapter, the results from Figure 6.2, 6.3 & 6.4 show that sickle cells possess both anion-dependent and histidine-linked transport mechanisms of very similar characteristics to those found in normal red cells. More importantly, the magnitude of transport was not significantly different from normal red cells which suggests that even though zinc deficiency has always been associated with sickle cell anaemia (Reed *et al.*, 1987), the ability of sickle red cells to transport zinc is not in any way defective when compared with normal red cells. It is noted that the uptake rates of zinc in sickle cells are actually slightly higher than those in normal human red cells. This may be because sickle blood contains a greater percentage of reticulocytes (Ellory *et al.*, 1991), which are known to have a higher amino acid transport activity than mature red cells (Johnstone *et al.*, 1987; Johnstone and Teny, 1989). Recently an extensive study has investigated the potential of using zinc as an anti-sickling agent because of its ability to increase oxygen affinity in HbSS (Hider *et al.*, 1990; Taylor, 1991). It was reported that red cells had to be loaded with an excess concentration of zinc (15-30 fold above normal) in vitro in order to be effective at improving sickle cell filterability (Taylor, 1991). To achieve the goal of raising intracellular zinc concentration, Hider et al (1990) have developed the use of an artificial carrier, ethylmaltol (EM), to transport zinc across sickle cell membranes. The idea is that EM could then help to achieve a greater and faster accumulation of zinc inside the red cells. Unfortunately, this approach seems to be unrealistic clinically. Under physiological conditions , ie. in the presence of higher affinity ligands such as albumin and histidine which have the binding constants 10^6 & 10^8 M^{-1} respectively, EM (which has a binding constant of $10^{4.5}$ M^{-1} for zinc) will not function as a competent binding ligand for zinc . In any case, sickle red cells possess the normal transport ability for zinc uptake across cell membranes. Therefore it seems unnecessary and impossible to use EM as a carrier to

facilitate zinc uptake into sickle red cells *in vivo*.

Since zinc supplementation is definitely beneficial in treating sickle cell anaemia both as a correction for zinc deficiency and as an anti-sickling agent at the cellular level, it would appear that studies on the problem of toxicity of high plasma and erythrocyte zinc are perhaps a more appropriate approach to research of the clinical state rather than the development of artificial zinc carriers *in vitro*.

CHAPTER SEVEN

ZINC TRANSPORT IN BOVINE CHONDROCYTES

Introduction

Chondrocytes are the only living cell type in cartilage. Their main physiological function is the synthesis of the extracellular matrix which consists of collagen and proteoglycan (Urban, 1992). Though chondrocytes occupy only 1-10% of matrix volume, their activity ultimately determines the structure and composition of the matrix (Stockwell, 1979). A recent methodological approach (Kuettner, 1982) has allowed the isolation of chondrocytes and thus enabled the study of their fundamental properties. Zinc is essential for collagen synthesis; it is a crucial cofactor of many important catalytic enzymes including DNA & RNA polymerase (Endre *et al.*, 1990). It has been reported that collagen and non-collagen protein are reduced in both the skin and the connective tissues of rats with dietary zinc deficiency (Endre *et al.*, 1990). Zinc also plays a significant role in the maintenance of the filamentous structures of collagen and proteoglycan by acting as a cross-link (Williams, 1987). However, despite its importance, the mechanism of zinc transport in chondrocytes is completely unknown. Therefore, the aim of this chapter is to investigate zinc transport in chondrocytes by examining the role of both the anion-exchanger pathway and the amino acid transporter system. Unlike the red cell which is a dead-end cell type with little metabolism within the cells, chondrocytes represent a functional cell type. It is hoped that by

using this cell type a better understanding of the roles of the band 3 pathway and amino acid transport systems in zinc transport into normal functional cells will be achieved.

Material & Methods

Bovine chondrocytes were prepared by Mrs Kathy Whittaker. The technique for chondrocyte isolation was adapted from Zanetti *et al.*(1985). The feet of 12-36 month old steers were collected from Reading Abattoir immediately after slaughter and taken to the laboratory. The metacarpophalangeal joints were opened under as aseptic conditions as possible. Shavings of hyaline cartilage (5x4x0.5mm) were removed from the outer two-thirds of the articular cartilage, so that contamination with bone cells or red blood cells could be avoided.

Isolated chondrocytes were then incubated in MOPS buffered saline for 2-3 hours to deplete the intracellular amino acids prior to the experiment. Flux measurement was carried out by employing the rapid washing technique (see section 2.3.1). The uptake rate is usually expressed in terms of nmol (or pmol). 10^6 $cells^{-1}$. min^{-1}. Cell counting was performed by the method described in section 2.3.2.2.

Results

7.1 Time Course

Time course experiments were carried out to determine the time period over which linear uptake occurs. Figure 7.1 shows the result of a typical experiment of this kind. At two chosen zinc concentrations (10 & 100 μM), the uptake of zinc appeared to be linear with time for up to an hour. Therefore, 15-30 min was chosen for the latter experiments to measure the initial zinc uptake rate. The uptake rates of Zn at the two concentrations (10 & 100μM) were 0.005±0.002 (±sem, n=3) and 0.030±0.016 nmol.10^6 cells.min^{-1} (±sem, n=9) respectively.

7.2 Surface Binding

All transport processes require energy. Therefore, uptake of a test substrate across cell membranes is likely to be more affected by a change of temperature than its binding on the cell surface. In order to differentiate actual uptake of zinc from its binding to the chondrocyte surface, the initial zinc uptake over a concentration range of 0.05-1mM was measured at 37 ^{0}C and 4 ^{0}C. The result of a typical experiment is shown in Figure 7.2. An assumption is made that the ^{65}Zn count which obtained at 4 ^{0}C should be mostly (if not completely) due to ^{65}Zn binding to the surface. After subtracting the latter count from the count obtained at 37 ^{0}C, the residual value should represent actual uptake of zinc across the chondrocyte membrane. The results of these experiments are summarized in Table 7.1.

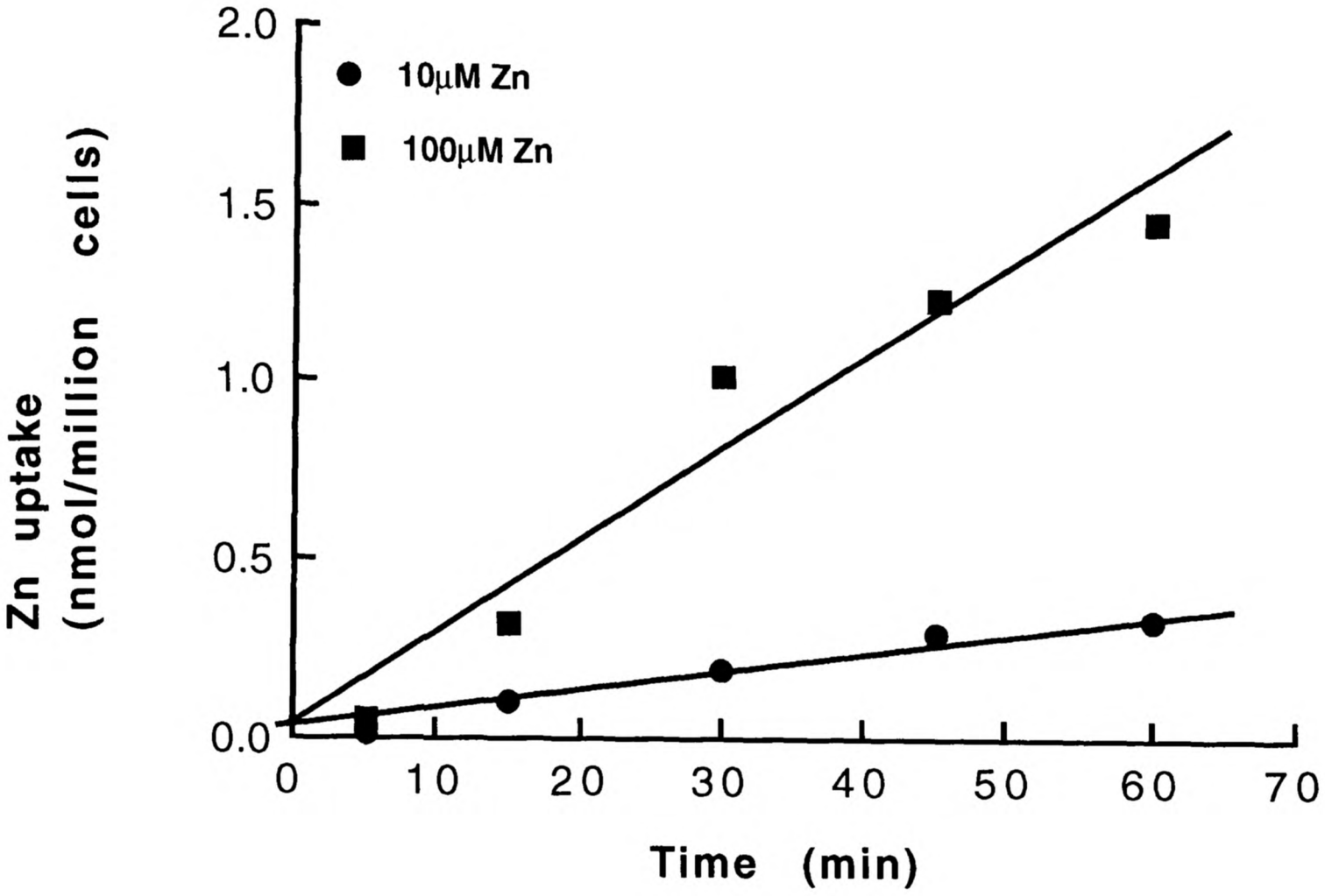


Figure 7.1

Time course for zinc uptake (10 & 100μM) in bovine chondrocytes. The data of this typical example fitted a linear regression with a slope of uptake rate 0.03 nmol. 10^6 $cell^{-1}$. min^{-1} (r=0.972) for 100μM zinc uptake and 0.01 nmol. 10^6 $cell^{-1}$. min^{-1} (r=0.990) for 10μM zinc uptake.

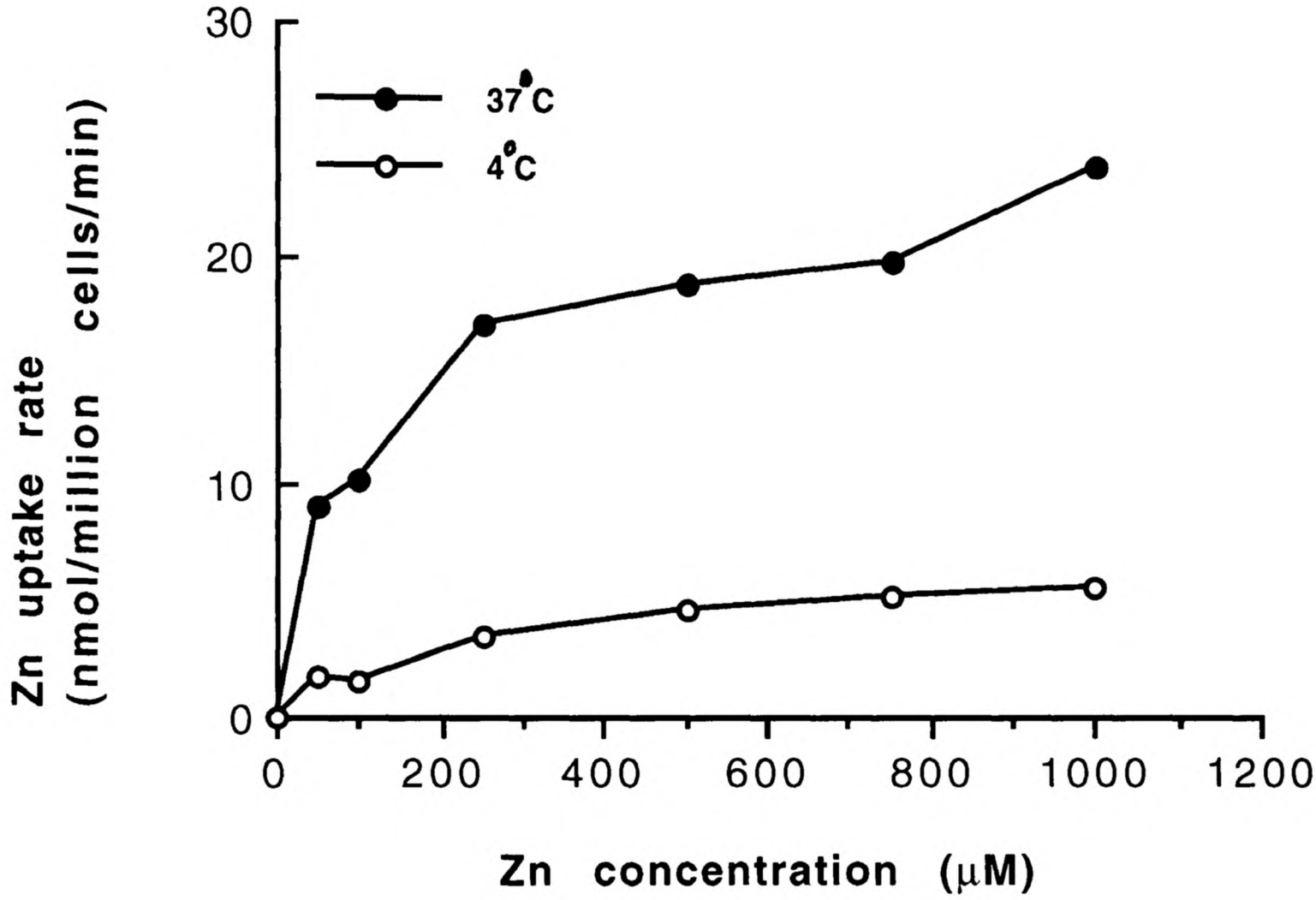


Figure 7.2

Effect of temperature on zinc uptake in bovine chondrocytes.

Zinc uptake (10-1000μM) was measured at 37^0 and $4\ ^0$C.

Table 7.1 Effect of Temperature on Zn uptake in Chondrocytes

	Zinc uptake rate (pmol. 10^6 cells^{-1}. min^{-1}) (±sem, n=3)	
External Zn (μM)	**4^{0}C**	**37^{0}C**
50	3.0± 1.8	11.5± 3.4
100	4.3± 1.4	15.4± 3.4
250	6.2± 1.5	23.5± 3.6
500	12.3± 3.9	35.5± 8.4
1000	15.3± 13.7	41.8± 9.0
1250	20.3± 13.1	38.0± 19.0

7.3 Concentration-dependent Uptake

The uptake of zinc (1-300μM) in chondrocytes was measured. The medium contained no bicarbonate. The time course of 5 min was chosen to measure the initial uptake rate. It was noticed in these experiments that chondrocytes became leaky at higher concentrations of external Zn (over 200μM). The uptake of zinc up to this concentration range was found to be saturable according to the external Zn concentration, which gave a good fit to the Michaelis-Menten equation. The kinetic parameters were estimated using the commercial program 'Enzyme-Fitter' as described in section 2.4.4. The results are listed in Table 7.2.

7.4 Effect of Na^+-replacement on Zinc Uptake

The effect of Na^+-replacement on zinc uptake in chondrocytes was investigated by measuring the zinc uptake (0.05-1mM) in standard saline and Na^+-free saline (replaced with NMDG). Table 7.3 summarizes the result of these experiments. In this concentration range, the uptake of zinc was slightly inhibited by Na^+-replacement.

7.5 Effect of SITS & Bicarbonate

The effect of SITS & bicarbonate was studied to identify the degree of band 3 anion exchanger transport of zinc across the chondrocyte membrane. Fresh 10mM SITS stock solution was

Table 7.2 Kinetic Constants for Zinc Uptake in Chondrocytes

	(±sem, n=3)
Cell Preparation 1	V_{max}= 23.1± 5.9
	K_m = 13.9± 7.5
	k_d = 0.00± 0.03
Cell Preparation 2	V_{max}= 14.0± 4.8
	K_m = 26.1± 19.6
	k_d = 0.03± 0.02
Cell Preparation 3	V_{max}= 7.7± 2.4
	K_m = 26.4± 17.3
	k_d = 0.03± 0.01

V_{max} is expressed in nmol. 10^6 $cells^{-1}$. min^{-1}.

K_m is expressed in μM per external Zn.

K_d is in sec^{-1}.

Table 7.3 Effect of Na^+-replacement on Zn Uptake in Chondrocytes

External Zn (μM)	% of Control Uptake rate	
	Exp1	**Exp2**
0.1	81.6	121.7
0.5	62.9	113.0
1.0	69.8	54.1
1.25	69.3	63.1
2.0	65.8	21.9

prepared for each experiment and several concentrations (10, 20, 100 μM) were tried to investigate the inhibitory effect of SITS on zinc uptake. Preliminary experiments showed that a high concentration of SITS or DIDS (over 100μM) seemed to have a damaging effect on chondrocyte membranes which became very leaky (data not shown). This resulted in difficulties of obtaining accurate measurements. Unlike human or sheep red cells, both treatment with SITS or the presence of bicarbonate seemed to have little effect on zinc uptake. Figure 7.3 shows a typical example of the effect of 20 μM SITS on the uptake of zinc (10μM) in chondrocytes. A summary of the results of these experiments is listed in the Table 7.4.

7.6 Effect of Histidine

The effect of L-, and D-histidine on zinc transport in chondrocytes was investigated to see if chondrocytes possess an amino acid linked mechanism for zinc uptake, as found previously in red cells. The uptake of zinc (5-100μM) was measured in chondrocytes which had been pretreated with 20μM SITS; experiments were carried out in the standard bicarbonate-free medium containing 1mM L, or D-histidine. The estimated values of external Zn concentration calculated according to the method described in section 2.4.6 under these condition were 0.02, 0.12, 0.21, 0.34, 0.48 μM respectively. Hence, the uptake rates of Zn at these concentrations could then be estimated to be 0.01, 0.07, 0.12, 0.20, 0.29 $pmol.10^6$ $cells.min^{-1}$ respectively. In the result presented in Figure 7.4, these uptake rates were used as the control uptake rates to compare with the histidine-containing groups. The uptake of zinc in chondrocytes was increased in the medium containing histidine. The effect of histidine did not differ between L, or D-histidine.

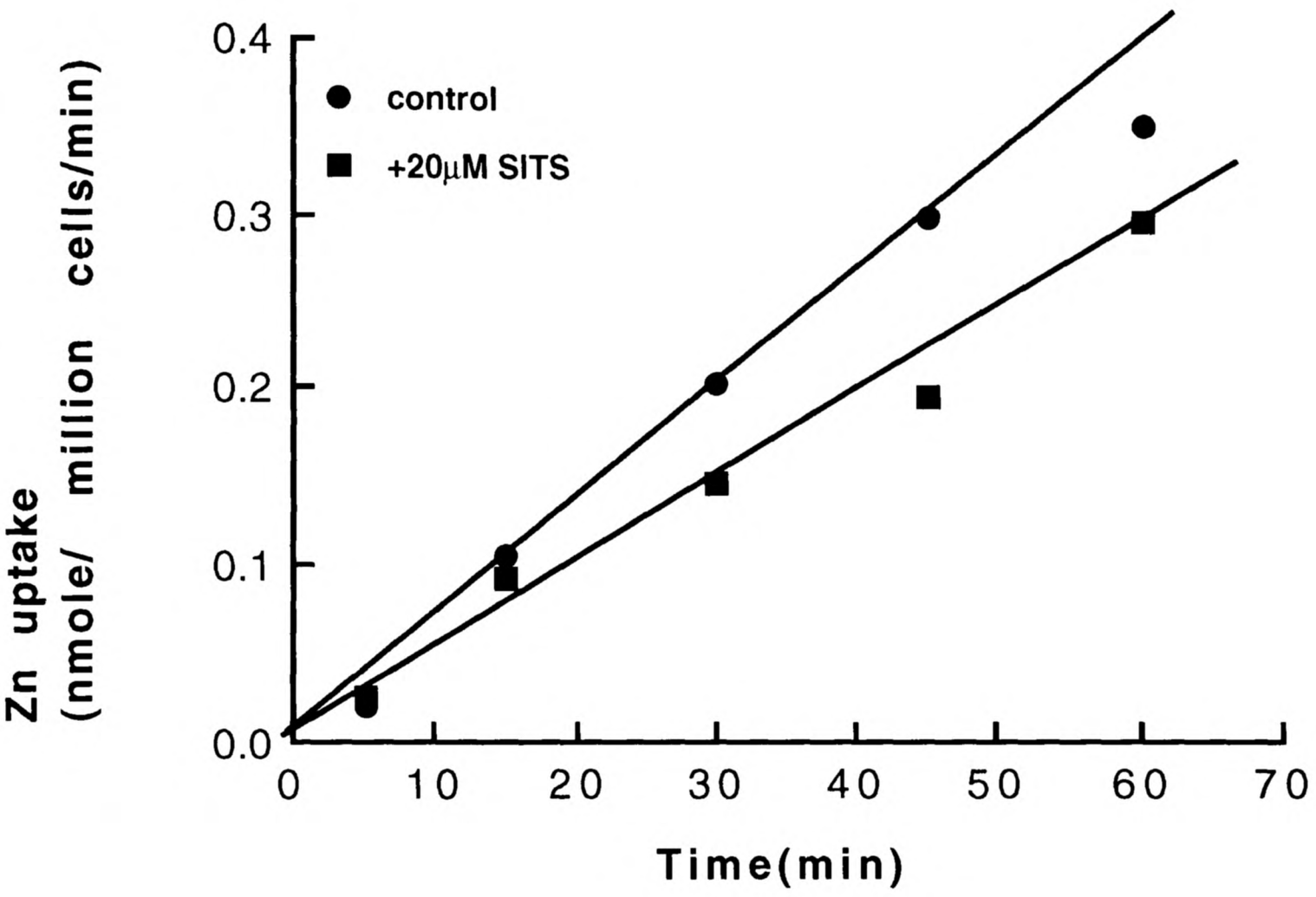


Figure 7.3

Effect of SITS (20μM) treatment on zinc uptake in bovine chondrocytes. The presence of SITS caused a slight decrease in zinc uptake. The data points of this typical experiment fitted a linear regression with a slope of 0.006 (r=0.990) nmol. 10^6 $cell^{-1}$. min^{-1} for the control group and 0.005 (r=0.990) nmol. 10^6 $cell^{-1}$. min^{-1} for the SITS treated group.

Table 7.4 Effect of SITS & HCO_3^- on Zn Uptake in Chondrocytes

	Zn Uptake Rate (pmol. 10^6 cells^{-1}. min^{-1})			
	10μM Zn		**100μM Zn**	
	Exp1	Exp2	Exp1	Exp2
Control	1	8	26	21
+20μM SITS	5	5	10	9
+5mM HCO_3^-	6	-	26	-

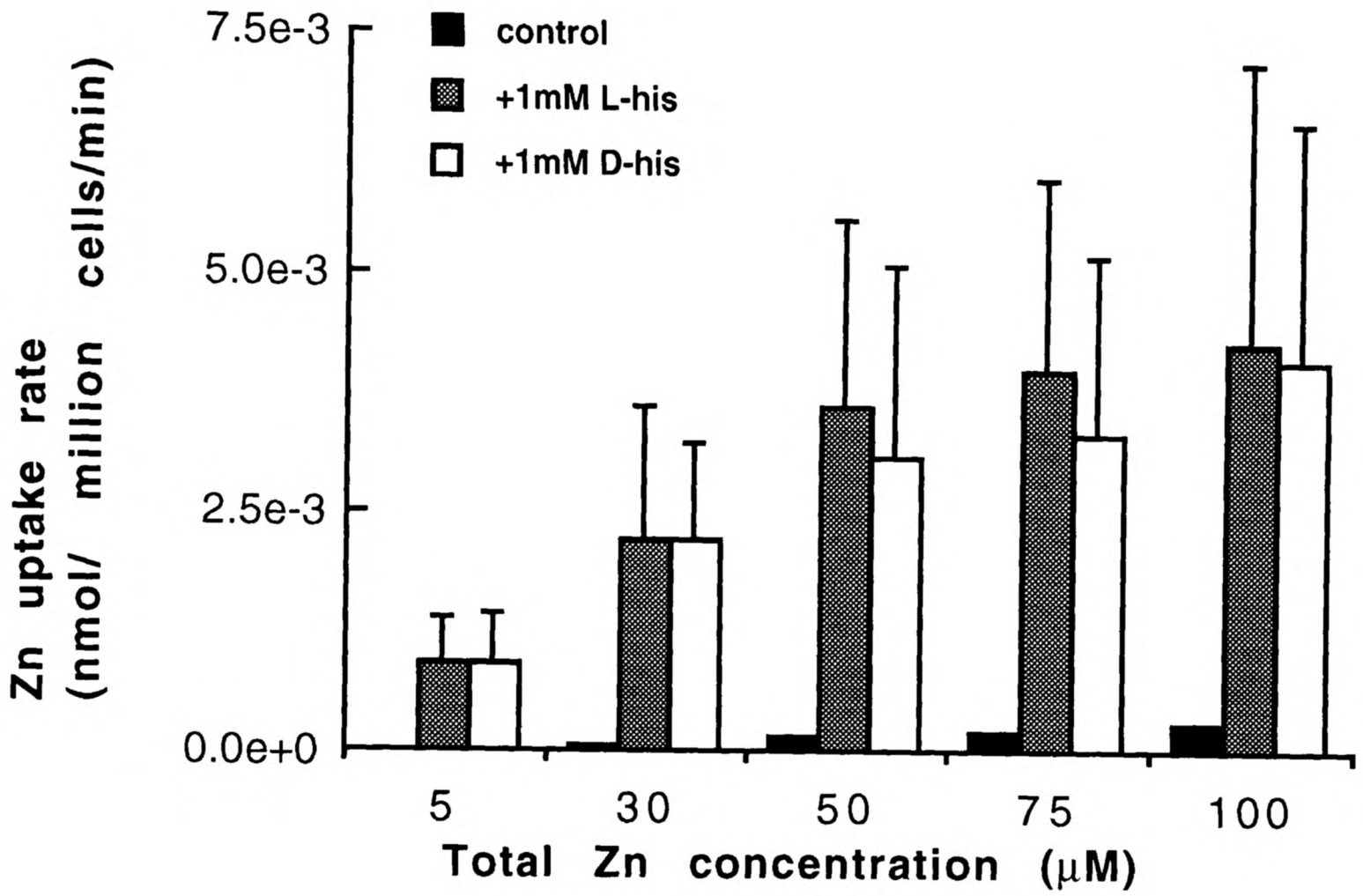


Figure 7.4

Effect of L-, or D-histidine (1mM) on zinc uptake in bovine chondrocytes.

Discussion

Free Zinc Uptake

Zinc uptake in bovine chondrocytes was saturable suggesting that zinc transport is via a carrier-mediated pathway with an apparent affinity constant about 22μM. Chondrocytes were reported to have band 3-like anion exchangers since sulphate transport was markedly DIDS-sensitive (Gehl and Ellory, 1991). The band 3-like transporter found in chondrocytes differs from the band 3 anion exchanger in red cells in that it might not operate Cl^--HCO_3^- exchange; in sulfate's case it was suggested to be exchanging with OH^- (Gehl and Ellory, 1992). Studies on pH regulation in chondrocytes show that there is little classical band 3 activity in this cell type (Wilkins personal communication). These pieces of evidence suggested the possibility that chondrocytes might have fewer copies of band 3-like transporter in the membrane than red cells or the property of exchanging anions was quite different from red cells' band 3 anion exchanger. Thus, the result of Table 7.4 indicated that in the chondrocyte, entry via band 3 anion exchanger in a form of anion zinc complex could not be the major route for zinc uptake. In order to investigate if zinc uptake was taking place in a cationic form, external Na^+ was replaced with NMDG with an intention to hyperpolarize the chondrocyte cell membrane. The membrane potential of chondrocytes was measured with the patch-clamp technique to be about -44 mV (Gehl and Ellory, 1991). The hyperpolarization should cause an increase in the uptake of zinc if it was cationic zinc which was transported across cell membrane. The result of Table 7.3 shows that in Na^+-free saline the uptake of zinc was slightly inhibited rather than enhanced. Thus, it remained to be resolved in what chemical form zinc was transported across chondrocyte membranes via the carrier-mediated pathway. In human fibroblasts, zinc uptake was also reported to be via a

saturable carrier-mediated pathway which had an apparent association constant of 1.1 * 10^7 M^{-1} (Ackland *et al.*, 1988). The same report suggested that this carrier-mediated pathway for zinc probably did not dependent on metabolic energy and concluded that zinc in human fibroblast was not taken up by a receptor-mediated endocytic pathway as had been described for transferrin and iron (Ackland *et al.*, 1988). The mechanism found in human fibroblasts may represent a similar transport process to that we found in the chondrocyte for zinc uptake when the medium contained no binding ligands. More work is needed to clarify further the detailed mechanism of this route for zinc transport.

Effect of Histidine

The result of Figure 7.4 shows clearly that the presence of histidine increased the uptake of zinc in chondrocytes, consistent with our findings in red cells. Over a concentration range of 5-100 μM total Zn, the presence of 1mM L, or D-histidine in the medium caused an over 35 fold increase in zinc uptake. This effect of histidine could be explained by proposing that chondrocytes operate the same mechanism as that found in red cells, ie zinc is transported via the histidine transporter in the form of zinc-histidine complex. Some amino acid transporters have been identified in chondrocytes; up to date there are at least five distinct transport systems for neutral amino acids have been studied. They consisted of four Na-dependent systems, system A, ASC, Gly and N and one Na-independent system, system L (Barker and Ellory, 1990). Although nobody has ever studied histidine transport *per se* in chondrocytes, in a study on the transport of glutamine and leucine in chondrocytes, histidine was used as a competitive inhibitor for glutamine and leucine entry via system N and L respectively (Barker, 1990). Since histidine proved to be

a competent inhibitor for both system N and L, it is fair to presume that histidine is a substrate for these two amino acid transport systems in chondrocytes. The substrate stereospecificity of these two amino acid transport systems, system N and L, has been investigated showing that system N in chondrocytes was highly stereospecific in favour of the L-isomer whereas system L had little stereoselectivity (Barker, 1990). Thus, of the two systems, system L seems to be the more plausible candidate for transporting the zinc-histidine complex since the transport of zinc-L-histidine & zinc-D-histidine was of the same activity in chondrocytes (Figure 7.4). It is not known whether chondrocytes possess any specific transport system for cationic amino acids such as system y^+ in red cells. Therefore, it remains unclear whether system L is the only system for the entry of zinc-histidine or whether there may be some other unknown transport systems. The main focus of this present chondrocyte study is to establish if the mechanism of zinc cotransport with histidine also exist in normal cell types. Thus it is more important to find out that the presence of histidine indeed has a similar effect on zinc uptake in chondrocytes as in red cells than to go into too much detail identifying histidine transporters in chondrocytes. In a study on human fibroblasts, it was suggested that no specific zinc binding ligands were essential for zinc transport into the fibroblast but only free zinc was required by the cell (Ackland and McArdle, 1991). However, this hypothesis could not explain our present result that an over 35 fold increase in zinc uptake by chondrocytes occurs when histidine is present in the medium.

Physiological Relevance

Very little is known about ion distribution either in the matrix or chondrocyte at the present time. Some studies report that chondrocytes are surrounded by a high concentration of free cations

(extracellular Na^+ concentration around 250-350mM, free Ca^+ 20mM and K^+ 10-15mM) and a low concentration of anions (Cl^- about 60-90mM) (Urban and Hall, 1992). The concentrations of zinc and zinc binding ligands in matrix are unknown. Presumably, with their very active and important physiological function of protein synthesis, chondrocytes need to have sufficient supply of zinc to maintain the normal cellular metabolism. At a molecular level, zinc was found to bind to the amino acid residues including cysteine and histidine in most of known zinc metalloenzymes and zinc metallothionein (Cousins, 1985). Hence, the cotransport of zinc and its binding amino acids is an important mechanism for providing precursors for highly active biosynthesis within the cell. The entry of the zinc-amino acid complex via an amino acid transport system may represent the physiologically significant route for zinc uptake into chondrocytes.

CHAPTER EIGHT

GENERAL DISCUSSION

8.1 Introduction

The main aim of this thesis has been to investigate the mechanisms by which zinc is transported across cell membranes. Red cells from different animals and bovine chondrocytes have been used as model membrane systems and for their special relevance to zinc studies. Zinc is predominantly (>99%) an intracellular ion (see Table 1.2). The transport mechanism for taking up zinc from the systemic circulation into the tissues is not clearly understood. Plasma zinc (total concentration 15μM) is either bound to proteins or amino acids. Two major mechanisms of zinc transport across cell membranes have been studied in this thesis; an anionic mechanism via the anion exchanger and an amino acid-linked mechanism via amino acid transporters. Both mechanisms have been investigated in all the cell types and their physiological significance tested by examining the effects of Zn-binding ligands.

8.2 Zinc Entry via the Band 3 Anion Exchanger

Zinc uptake is anion-stimulated, DIDS (or SITS)-inhibitable in all the cell types investigated except for lamprey red cells (see Table 8.1; Chapters 3-7). This mechanism of zinc uptake in

Table 8.1 Zinc Uptake (30μM) via the Anion Exchanger

Cell type	Zinc Uptake rate	DIDS Inhibition	HCO_3^- Stimulation
	(μmol.l cell^{-1}.min^{-1})	(% of control uptake rate)	
Human	2.6	>90	>500
Lamprey	6.3	-	-
Sheep			
normal	1.5	>80	>400
system C^-	2.2	>80	>400
Sickle cells	7.9	>50	>200

human red cells has been previously investigated by two groups (Alda Torrubia and Garay, 1989; Kalfakakou and Simons, 1990), and different chemical formulae for the transporter's substrate were suggested ($ZnCO_3Cl^-$, $Zn(HCO_3)Cl.OH^-$ and $Zn(HCO_3)_2Cl^-$). The anion exchanger is present in most cell types and is particularly abundant in red cells (Jennings, 1989). Zinc uptake via this pathway is very rapid and accounts for the dominant route of zinc uptake by red cells when no binding ligands are present in the medium. However, the result of the albumin effect (see Chapter 3) suggests that the binding to albumin may inhibit the formation of anionic complexes. Thus, under physiological conditions, this anionic mechanism is perhaps not representative of what happens *in vivo*. Nevertheless, this anionic mechanism may represent the facilitated process of zinc transport into cells when no ligands are present, which are well documented in many *in vitro* studies (Cousins, 1985; Ackland *et al.*, 1988; Bobilya *et al.*, Wensink *et al.*, Kalfakakou and Simons, 1990; Alda Torrubia and Garay, 1989).

8.3 Zinc Entry via Amino Acid Transport Systems

The effect of zinc-binding amino acids on zinc transport has been studied, using histidine as a model substrate. In addition to its chelating effect, histidine is found to have a specific stimulating effect on zinc uptake in all the cell types examined (see Table 8.2; Chapters 3-7). The facilitatory effect of histidine has been overlooked in other studies because of the apparent decrease in measured ^{65}Zn uptake rates. Further characterization has shown that the histidine effect is mostly likely to be due to the cotransport of a zinc-histidine complex via an amino acid transport system(s) or some unknown specific system(s) for zinc-histidine. The effect of histidine on zinc uptake was found to be stereospecific, favouring the transport of L-histidine over D-

Table 8.2

L-Histidine Effect on Zinc Uptake (30μM) and Possible Amino Acid Transport System for the Cotransport of (Histidine-zinc) complex

Cell Type	Stimulation by Histidine (n^{0}* control uptake rate)	Stereospecificity	Transporter
Human (1mM L-his; 37^{0}C)	>100	yes	L; y^{+}
Lamprey (0.1mM; 20^{0}C)	>6	No	L
Sheep (1mM; 37^{0}C)	>60	No	C; ?
HbSS (1mM; 37^{0}C)	>600	Yes	L; y^{+}
Chondrocytes (1mM; 37^{0}C)	>35	No	L

histidine, in normal human red cells and human sickle cells. The result of a *trans*-stimulus effect by internal leucine and lysine suggests that system L and y^+ are the possible candidates for such a cotransport process. The stereospecificity is not observed in lamprey or, sheep red cells and bovine chondrocytes; this can be explained by a lack of stereoselectivity in their amino acid transport system, namely an L-like system. In the case of lamprey red cells, that the flux of L-histidine-zinc can be *trans*-stimulated by internal leucine but not lysine, reinforces such a proposal.

In red cells from normal and system C-deficient sheep, this histidine effect is perhaps due to the cotransport of histidine-zinc via system C, which has a very low affinity for the (histidine-zinc) complex or simply via passive diffusion. In all the cell types studied, the histidine effect shows little Na^+-dependence which is consistent with the evidence that both system L and y^+ are Na^+-independent systems. The possibility of an unknown specific transport system, other than histidine transporters, for (histidine-zinc) is not completely ruled out. However, it is more difficult to explain why such a transport system should prefer L-histidine (in the case of human red cells) and more importantly, is *trans*-stimulated by the paradigm amino acids (leucine and lysine) for system L and y^+. A new transport system for cationic amino acids has been identified in human red cells recently (Deves *et al.*, 1991). It is possible that this transport system can contribute to the transport of the histidine-zinc complex.

In human red cells, this histidine effect is not abolished by the addition of albumin to the media, which suggests that this amino acid cotransport mechanism, unlike the anionic mechanism, is still operative when albumin is present. Such a transport process is therefore more likely to be a physiologically significant route for zinc uptake into cells. It is also interesting to note that lamprey red cells, which have a minimum activity of the anion exchanger but an exceptionally high activity of amino acid transport, have the greatest zinc uptake rate amongst all the cell types

tested. This may support the important role of such an amino acid-linked transport mechanism for zinc entry into cells. There are other amino acid which bind to zinc with a different affinity such as cysteine, glutamine, tyrosine and lysine (Cousins, 1985). The possibility that other binding amino acids can have a similar effect on zinc transport has not been studied in the present work. However, some preliminary results showed that cysteine also has a specific stimulating effect on zinc uptake into human red cells despite its chelating effect. Thus, the cotransport of zinc with amino acids may be a common mechanism and not only restricted to the transport of the zinc-histidine complex. The chemical form for the cotransport of zinc and histidine remains to be established. A complex of 1 zinc and 1 histidine is more probable since dipeptide transporters have not been found in red cells (Young *et al.*, 1987).

8.4 Physiological Relevance

Zinc homeostasis is known to be closely linked with the metabolism of proteins and amino acids (Cousins, 1985). The equilibrium between albumin-bound zinc and amino acid bound zinc plays a crucial role in determining the zinc status of the body. Zinc deficiency in many disease states often results from a disordered protein and amino acid metabolism (Endre, *et al.*, 1990). For example, hypozincaemia of protein energy malnutrition and hepatic cirrhosis may be caused by hypoalbuminamia (Cousins, 1988). However, our understanding of the effects of zinc binding ligands on zinc homeostasis is still very limited, particularly at a cellular level. From the present study on zinc transport, it may be concluded that in the two zinc transport mechanisms investigated, the cotransport of zinc and zinc binding amino acid accounts for a more important route under physiological conditions. This finding clearly demonstrates that amino acids not only

can act as binding ligands for zinc but actually provide a high affinity, specific transport mechanism by which a sufficient zinc supply into tissues is secured. In most of the known enzymes or zinc-containing protein, zinc is bound to histidine or cysteine residues (Williams, 1988). The cotransport of the (zinc-amino acids) complex may contribute precursors for protein biosynthesis.

Amino acid transport systems have been found in most cell types, though the characteristics of transporters vary in cells from different species or tissues (Ellory, 1987). In the present study amino acid linked zinc transport has been found in all the cell types examined including red cells from several different animals and bovine chondrocytes. It is important to investigate if such mechanism also operate in other major tissues.

In recent years, zinc supplementation has been of increasing importance in treating zinc deficiency-related diseases (Endre *et al.*, 1990). With the finding that histidine facilitates cellular zinc uptake, there may be a prospective clinical use for histidine supplementation to increase zinc body content and consequently improve zinc status. Histidine and cysteine are reported to enhance the absorption of zinc in small intestine (Cousins, 1985). The use of histidine supplementation increases the plasma histidine concentration. Consequently, a shift from albumin-bound zinc to histidine-bound zinc may occur, which results in an increase in the concentration of histidine-zinc in plasma. This will then enhance the cellular uptake of histidine-zinc. However, an increase in amino acid bound zinc is often accompanied by a greater loss of zinc through urine (Cousins, 1988). More work is needed to determine the appropriate ratio for the histidine supplementation.

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