ION GRADIENTS AND FLUXES IN ISOLATED LIVER CELLS

A thesis presented in part fulfilment of the requirement for the degree of Doctor of Philosophy of the University of Oxford

by

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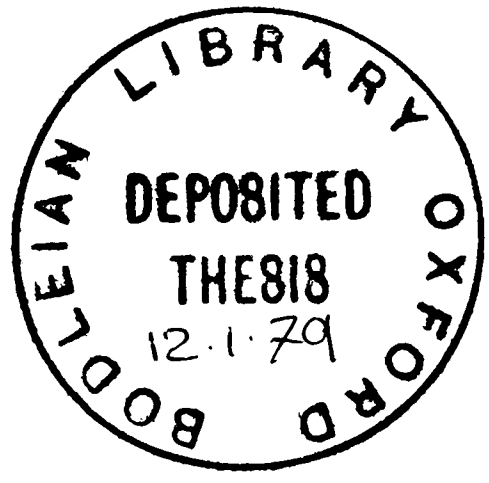

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DEFINITIONS AND ABBREVIATIONS

[...] indicates a concentration term in which:

[...] inside = μmol/ml cell H_2O

[...] outside = μmol/ml suspension medium

The X ratio abbreviates:

$$\frac{[X] \text{ inside}}{[X] \text{ outside}}$$

where inside = intracellular

outside = extracellular

P_K	permeability of the membrane to potassium (cm sec^{-1})
P_{Na}	permeability of the membrane to sodium (cm sec^{-1})
R	gas constant
T	absolute temperature
F	Faraday constant
K_m	Michaelis constant
Na-K ATPase	sodium-potassium adenosine triphosphatase
ADP	adenosine-5'-diphosphate
AMP	adenosine-5'-monophosphate
ATP	adenosine-5'-triphosphate
P_i	inorganic phosphate
NAD	oxidized nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
EDTA	ethylenadiamine tetra-acetic acid
EGTA	ethanedioxybis(ethylamine) tetra-acetic acid
MEM	minimum essential medium
BSA	bovine serum albumin
NMR	nuclear magnetic resonance

CHAPTER 1. INTRODUCTION

The aim of this thesis is to study the relationships in isolated rat liver cells between, on the one hand, metabolic processes such as glucose and urea synthesis, and, on the other, ionic content and rate of K^+ flux.

The general subject area within which my own work is located can usefully be classified as follows (papers refer to work on rat liver unless otherwise stated):

1. estimation of the ionic content and extracellular space in liver (see Heckmann & Parsons, 1959a & 1959b; Parsons & van Rossum, 1962; Williams & Woodbury, 1971).
2. relationships between energy metabolism and ion content of liver (see van Rossum, 1970, 1972; McLaughlin, 1973; Lambotte, 1977, in dog liver).
3. electrical properties of liver cell membranes by microelectrode and isotope flux studies (see Schanne & Coraboeuf, 1966; Claret & Mazet, 1972; Claret et al., 1973; Mazet et al., 1974; Graf & Petersen, 1974, in mouse liver).
4. effects of hormones such as glucagon and cyclic AMP on membrane potential in liver (see Dambach & Friedmann, 1974a; Petersen, 1974, in mouse liver; Berg & Iversen, 1976).
5. effects of pharmacological agents such as the catecholamines on membrane potential, ion movements and ion content in liver (see Haylett & Jenkinson, 1972a and 1972b in guinea pig liver).
6. effects of added substrates on membrane potential (see Dambach and Friedmann, 1974b; Folke, 1974).
7. amino acid transport in liver (see Tews & Harper, 1969a & 1969b; Kletzien et al., 1976; LeCam & Freychet, 1977).

My own work is located chiefly within areas 1, 6 and 7 above. The connection between my work and that listed above is described in

more detail in the appropriate chapters. The preliminary stage of experiments in this study (see chapter 3) was to determine endogenous values of Na^+ and K^+ in isolated rat hepatocytes and to measure the effect of inhibitors of metabolism and the Na-K ATPase on these values. In addition, preliminary experiments were made on the effects of added substrates and of rates of metabolic processes such as glucose and urea synthesis on K^+ content and turnover in hepatocytes. It was not possible to establish a relationship between rates of metabolic processes and rates of K^+ turnover and K^+ content since under certain conditions rates of metabolic processes varied independently of changes in K^+ content and turnover. However, the work raised further interesting problems. In particular, experiments in Chapter 3 suggested the importance of investigating cell volume of hepatocytes, which is discussed in chapter 4. The question of cell volume was of importance not only for calculation of intracellular concentrations of solutes, but also in revealing the complexity of factors affecting ion content of hepatocytes.

The physiological importance of alanine as a gluconeogenic substrate in the liver made it seem especially suitable for study (see Chapter 5), and the results substantiate the suggestion, already present in experiments of Chapter 3 section 4, that ion content and K^+ turnover changes are more intimately connected with transport and distribution of substrates than with rates of metabolic processes.

The problem of whether there is a gradient of ammonia between liver cells and extracellular fluid arose as a distinct issue from those so far discussed. This problem resulted from the observation that the tissue concentration of ammonia was frequently considerably greater than that of the plasma (see Lund et al., 1970; Hindfelt, 1975; Brosnan, 1976). However, since the free base form of ammonia, NH_3, is thought to diffuse through membranes very readily (Jacobs, 1940), it is not clear what mechanism could maintain an ammonia gradient. In this

thesis, isolated hepatocyte intracellular ammonia content was examined in several different conditions in an attempt to elucidate the mechanism by which the unequal distribution of ammonia is maintained (see Chapter 6). Fructose was used in the investigation of the above problem, and the chance observation that administration of fructose to hepatocytes was associated with changes in K^+ content led to the work described in Chapter 7.

The isolated hepatocyte preparation was used throughout the work reported in this thesis. The perfused liver preparation, first proposed by Claude Bernard (1855), and the liver slice preparation (Warburg, 1923) have been extensively used in the study of metabolic processes and electrophysiology of liver. The rat liver slice technique has limitations in that intracellular $[K^+]$ tends to be lower and $[Na^+]$ inside higher than *in vivo* (Krebs et al., 1951). Liver perfusion has the disadvantage that only a small amount of information can be extracted from each liver perfused. Estimation of extracellular space in liver perfusions is problematic in that equilibration of the extracellular marker takes at least 15 minutes and inward transport of the marker may occur in this period (McIver & Macknight, 1974). By contrast, equilibration of the extracellular space marker for isolated hepatocytes takes less than one minute. A further advantage of isolated hepatocytes is that the preparation contains chiefly parenchymal cells, since other cells present in liver, such as Kupffer cells and erythrocytes, are almost entirely removed during preparation (see Krebs et al., 1974).

Existing techniques for the separation of isolated cells from the suspension medium proved unsatisfactory for the work in hand. A substantial part of this project turned out to involve locating and overcoming the limitations of existing techniques. This is described in Chapter 2.

CHAPTER 2. MATERIALS AND EXPERIMENTAL PROCEDURES

1. Rats

Females of the Wistar strain, starved for 48 hours with water *ad libitum*, and weighing approximately 200 g were used unless otherwise stated. Rats were housed in conditions of controlled lighting in which the lights were on from 8.0 a.m. to 8.0 p.m. daily.

2. Reagents

Standard analytical grade reagents were obtained commercially. Enzymes were the products of Boehringer Corporation (London) Ltd., except for Urease (type VI) which was the product of Sigma Chemical Co., (London S.W.6). Tritiated water, Inulin [^{14}C] carboxylic, Urea [^{14}C], Potassium Chloride [^{42}K] (code PES 1P), were obtained from the Radiochemical Centre, Amersham, England. Insulin (glucagon and glycerol free) was given by Dr N.Z.Lazarus (Wellcome Research Laboratories, Beckenham, Kent, England). Silicone oil fluids were obtained from Hopkins and Williams, Ltd., Chadwell Heath, Essex. 'Norit' (activated charcoal) was obtained from Harrington Bros., London, S.W.12, England. Ouabain was obtained from Sigma Chemical Co., (London S.W.6).

Ouabain and metabolic substrates such as amino acids were made up in Krebs-Henseleit solution (1932) before addition to cell incubations, in order to avoid dilution of the incubation medium. Lactic and pyruvic acid were added as the sodium salt.

3.Preparation of Isolated Hepatocytes

Hepatocytes were prepared essentially as described by Berry & Friend (1969), incorporating the modifications described by Cornell et al. (1973), and Krebs et al. (1974), and the modifications described below. Collagenase (0.02% w/v) was used in the liver perfusion, but hyaluronidase was omitted. Perfusion time of the liver was decreased to 20 minutes. Hepatocytes were used immediately after preparation.

Cell viability was assessed by the method of Mapes and Harris (1975), which is based on the fact that healthy hepatocyte membranes are not permeable to succinate. Oxygen uptake of cells incubated with succinate is measured with an oxygen electrode before and after digitonin treatment. Digitonin destroys the integrity of the hepatocyte membrane and renders it permeable to succinate. The comparison of rates of succinate oxidation before and after digitonin treatment thus gives an indication of cell viability. By this method, viability ranged from 70% to 95%.

4. Incubation and Treatment of Isolated Hepatocytes

In each experiment, incubation was carried out in one of the following types of vessel:

(a) 25 ml Erlenmeyer flasks, when about 80 mgs wet weight of liver cells were incubated at 37°C in a final volume of 4.0 ml in Krebs-Henseleit saline (1932) containing 2.5% dialysed bovine serum albumin fraction V (Miles Limited). Flasks were gassed for 10-15 seconds with 5% CO_2 and 95% O_2 unless otherwise stated. Incubation times are stated for each set of experiments.

(b) 50 ml Erlenmeyer flasks with centre wells were used for incubations with phosphorus, when about 80 mgs of cells were incubated at 37°C in a final volume of 4.0 ml and gassed for 30 seconds with 5% CO_2 and 95% N_2.

(c) 50 ml Erlenmeyer flasks without centre wells were used for experiments in which cell particulate matter was separated from cytosol (Chapter 6 section viii), when about 300 mgs wet weight of hepatocytes were incubated at 37°C in a final volume of 4.0 ml and were gassed as for the 25 ml flasks.

(d) Warburg manometer vessels were used when oxygen uptake was to be measured (Chapter 3 section 2). These incubations were carried out as described by Krebs et al. (1974), in which bicarbonate-CO_2 was

used as a buffer. The wet weight of cells was 80 mgs in a final volume of 4.0 ml, and incubation was at 37°C for 60 minutes.

5. Separation Techniques

Cells were separated from the medium by two different methods because no single method was satisfactory for the estimation of all intracellular substances and for wet and dry weight. Conical centrifuge tubes (Method I) were used for measuring Na^+, K^+, alanine, urea and wet and dry weights, whereas separation devices (Method II) (Hems et al., 1975) were used for estimating all intracellular substances except Na^+, urea, wet and dry weight, and ^3H_2O. The reasons for this cumbersome arrangement, which involved incubation of parallel flasks within the same experiment for separation by Methods I and II, are of some interest and will be discussed in detail. First, separation by Method I is discussed, and included in this is measurement of wet and dry weight and estimation of intracellular water; then separation by Method II, and problems arising from this technique, are considered.

5A. Method I

(a) The Conical Centrifuge Tube

Cells were rapidly poured from the incubation flasks into 15 ml conical centrifuge tubes and were centrifuged for one minute at 1700 × g in an M.S.E. 'Minor' centrifuge. Immediately after centrifugation, the supernatant (3.0 ml) was pipetted from the tube into 0.3 ml perchloric acid (20% v/v) for analysis. The remains of the supernatant were poured off from the tube. The tube was dried with absorbent paper and the pellet was resuspended in 1.5 ml perchloric acid (4% v/v). The time between centrifugation and deproteinization of the pellet in perchloric acid was between one and three minutes. For this reason, this method was not suitable for measuring cellular metabolites such as lactate which may undergo changes during the one to three minutes before deproteinization.

(b) Estimation of Wet and Dry Weight of Cells

Method I was used in all experiments for estimation of total wet

weight, dry weight and inulin space because it was impossible to dry the separation devices (Method II) before weighing the wet cells. After incubation, cells and suspension medium were poured into conical centrifuge tubes which had been previously weighed dry and stored in a desiccator. After centrifugation at 1700 × g for two minutes, the supernatant was poured off and the tube and pellet surface were dried with absorbent paper. The cell pellet and the tube were then weighed. From this, a total wet weight of cells was obtained. The cells were then dried for at least four hours at 100-110°C. Tubes containing dry cells were allowed to cool in a desiccator and were then reweighed. This gave a value for dry weight of cells. By this method, 95% of wet cells were transferred from the incubation flask to the conical tube, while 5% of cells remained adhering to the incubation flask. Final wet weight values were corrected for this incomplete transfer of cells.

(c) Estimation of inulin space

Radioactive inulin (^{14}C-inulin) was added to the cell suspension (0.5 µc/4 ml suspension) after incubation and prior to separation by both Methods I and II to estimate the amount of medium carried down with the cells during centrifugation. Inulin was not incubated with the cells because it is thought to be slowly taken up by liver cells (McIver & Macknight, 1974). This 'carry down' or adhering fluid is equivalent to an inulin space or extracellular space measurement. A sample (0.1 ml) of medium and of the pellet extract (0.1 ml) were counted in a Beckman Scintillation Counter in vials containing 10 ml scintillation fluid containing 5.5 g Permablend III (PPO 91%, bis MSB 9%) from Packard Instrument Co., 60 g naphthalene, 600 ml toluene, and 400 ml methoxyethanol. ^{14}C-inulin gave a value of 25% of the total wet weight of cells for the extracellular water in cells separated by Method I. The value obtained by Krebs et al. (1974) for hepatocytes

was also 25%. This can be compared with the value of 26% found in liver slices (Hems et al., 1968), and with the value of 22.3% found in the fasted perfused rat liver (Hems and Krebs, unpublished). For calculating intracellular water volume, inulin space was never estimated in the dried pellet used for measuring dry weight, because McIver & Macknight (1974) found that drying rat slices prevented subsequent extraction of a considerable amount of inulin.

Correction was made for solutes in adhering fluid in cases where the intracellular metabolite being measured was in high concentration in the suspension medium, for example, Na^+. In cases where the major proportion of the metabolite or ion was intracellular, as for example in the case of K^+, no correction was necessary.

(d) Calculation of Intracellular Water Volume from Wet Weight, Dry Weight and Inulin Space

The total wet weight measured by the conical tube separation (Method I) is composed of the following components:

$$\left\{\begin{matrix}\text{Total wet wt.}\\ \text{of cells}\end{matrix}\right\} = \left\{\begin{matrix}\text{Dry wt. of}\\ \text{cells}\end{matrix}\right\} + \left\{\begin{matrix}\text{Extracellular}\\ H_2O\ \text{(inulin space)}\end{matrix}\right\} + \left\{\begin{matrix}\text{Intracellular}\\ H_2O\end{matrix}\right\}$$

Therefore:

$$\left\{\begin{matrix}\text{Intracellular}\\ H_2O\end{matrix}\right\} = \left\{\begin{matrix}\text{Total wet wt.}\\ \text{of cells}\end{matrix}\right\} - \left\{\begin{matrix}\text{Dry wt. of}\\ \text{cells}\end{matrix}\right\} - \left\{\begin{matrix}\text{Extracellular}\\ H_2O\end{matrix}\right\}$$

(The simplifying assumption is made that 1 mg cells is equivalent to 1 µl of H_2O.)

Results of 18 experiments in which wet and dry weight, and inulin space, were estimated within the same experiment are given in table 2:1, which shows that intracellular water was about twice that of the dry weight (or 50% of the total wet weight). It was found that in conditions with no added substrates, the larger the wet weight to dry weight ratio, the larger was the inulin space (H_2O outside), so that the calculated intracellular water content was remarkably constant. Intracellular water content was also estimated more directly from the activity of ^3H_2O.

Table 2:1. Wet wt., dry wt., extracellular water and intracellular water content of isolated hepatocytes

Experimental details are described in this chapter. Cells were separated from medium in 2 sets of parallel flasks, one for measurement of wet and dry wt. and one for measurement of inulin space (extracellular fluid), in conical centrifuge tubes (Method I). Values are means of 18 observations ± S.E.M. The simplifying assumption is made that 1 mg cells is equivalent to 1 µl water.

	mg	µl	% of wet wt.	% of dry wt.
Total wet wt. of cells	77.7 ± 2.68	-	-	-
Dry wt. of cells	19.9 ± 0.69	-	-	-
$\frac{\text{Wet wt.}}{\text{dry wt.}}$ ratio	3.91 ± 0.041	-	-	-
H_2O outside	-	19.3 ± 0.94	25.0 ± 0.94	97.0 ± 3.4
H_2O inside	-	38.4 ± 1.05	49.4 ± 0.77	193 ± 2.8

(e) Measurement of Intracellular Water with ^3H_2O

Within the same experiment intracellular water content was measured both by the distribution of ^3H_2O and by the method described in section (d) above.

Pairs of flasks were incubated for 40 minutes with no added substrates. One pair was used to measure wet and dry weight as described in section (b) above, and another pair was used to determine inulin space and ^3H_2O content. Tritiated water was added to the flasks of cells after 20 minutes of incubation and 1.5 minutes before centrifugation to allow equilibration with intracellular water. Inulin was added 30 seconds before centrifugation, as described in section 5A (c) above. Tritiated water and ^{14}C-inulin were counted in a scintillation counter and correction was made for quench. The tritium values were corrected for the inulin space value. In four experiments intracellular ^3H_2O gave a mean value of 36.0 µl H_2O in 19.5 mgs dry wt. of cells. Water content estimated from wet wt., dry wt., and inulin space gave a mean value of 36.9 µl for 19.5 mgs dry wt. of cells. Thus there was a good correlation between the two methods. However, both methods rely in part on estimation of inulin space. The validity of inulin as a marker for extracellular H_2O is questionable (for discussion of this, see the review by Macknight and Leaf (1977) pp 512-13). From data shown in table 2:3, it is possible to adduce indirect evidence that the estimated water content of hepatocytes is in the right order of magnitude. Urea should theoretically be distributed equally across hepatocyte membranes to give a urea ratio of 1.0. Urea ratios in table 2:3 rely on a correction for inulin space, yet are in the order of 0.9. Furthermore, this ratio of 0.9 is an underestimation by 5%, since determining urea in pellet samples involves using Norit which absorbs between 4% and 6% of the added urea (see section 9 below); no correction was made for this. Thus the urea ratio indicates that cell water estimations are in the right order. Throughout this thesis,

the assumption has been made that the total volume of cell water is available as solvent for intracellular solutes. The validity of this assumption has been questioned (see Kleinzeller, 1972). For example, Kleinzeller & Knotková (1967) estimated that 90% of the cell water was available as solvent for propylene glycol in kidney cortex cells, and similar results were obtained by Ling & Nagendank (1970) by a vapour-equilibration method. The estimated urea ratio in hepatocytes reported in this thesis indicates that the proportion of water available as solvent in liver is probably large (95%), and for this reason the assumption that the total volume of cell water is available as solvent was thought to be acceptable.

(f) Calculation of Concentration Terms

In all experiments, separation by Method I was used to estimate intracellular water content. Intracellular concentration terms ([...] or mM) were calculated with a value for intracellular water content derived from estimation of total wet weight of cells, dry weight of cells and inulin space (see section 5A (b), (c) and (d)) for almost all conditions and time points in each experiment. Intracellular concentrations are thus expressed as µmol/ml cell water.

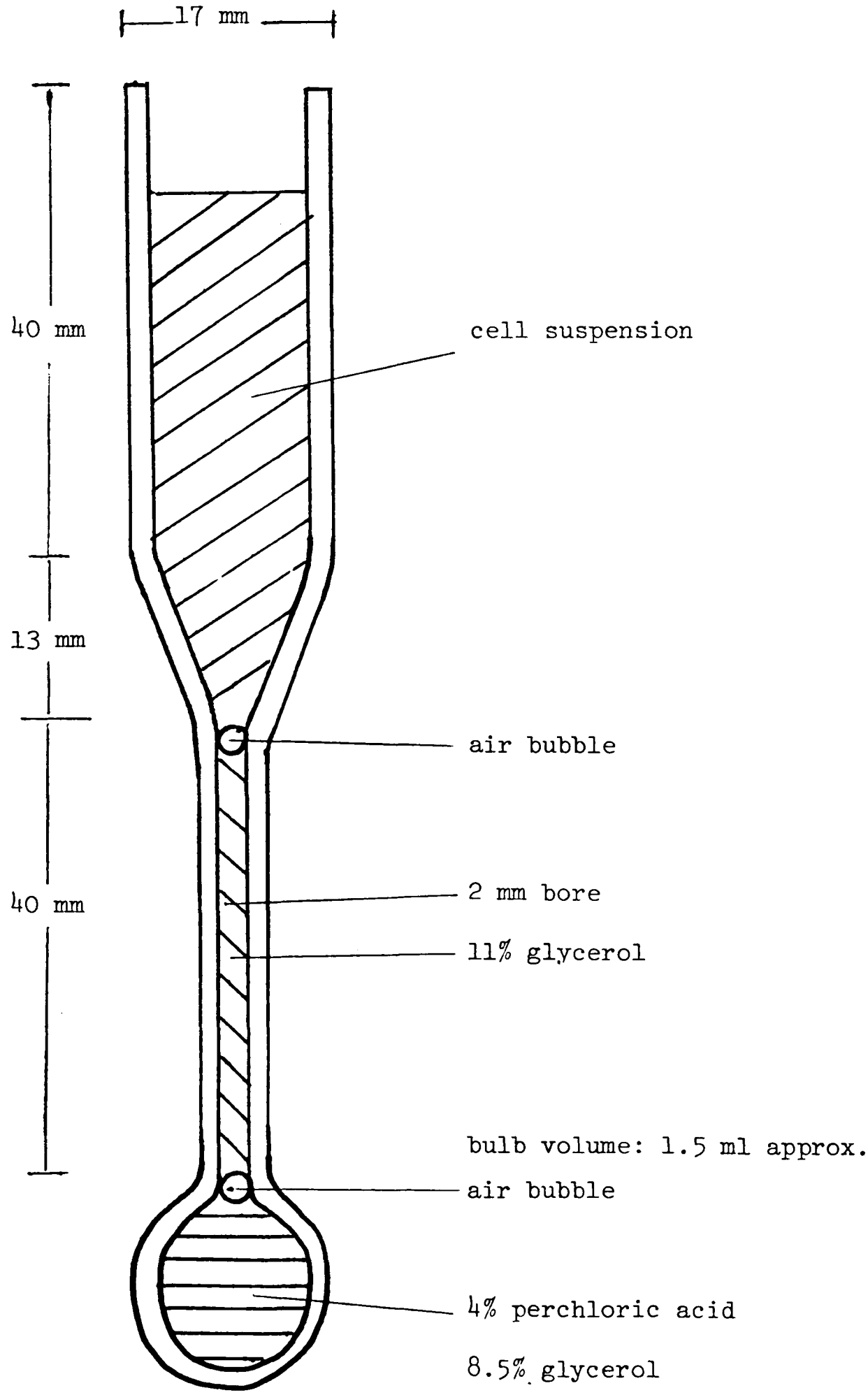
Extracellular concentration terms ([...] or mM) were estimated for µmol/ml suspension medium. For purposes of simplification, no correction was made for solid matter such as the 2.5% BSA in the medium to convert these molar concentrations to molal ones.

5B. Method II

(a) The Separation Device

A separation device (fig 1) for rapid separation of cells from medium as described by Hems et al. (1975) was used in addition to conical centrifuge tubes in all experiments, unless the contrary is explicitly stated. The fluid in the stem and bulb of the device was modified to allow measurement of ions; NaCl was replaced by 11% v/v glycerol in the stem, and NaCl with perchloric acid was replaced by

FIGURE 1. Separation Device for Separating Cells and Medium (after Krebs et al., 1975)



8.5% v/v glycerol and 4% v/v perchloric acid in the bulb of the device. The concentrations of perchloric acid and glycerol were so adjusted as to maintain an increase in the specific gravity in the direction of the bottom of the tube. In consequence, the three layers remained separate during centrifugation.

The cell suspension is poured into the upper chamber of the separating device and centrifuged at 1700 × g for one minute in a bench top centifuge (M.S.E. 'Minor'). The cells migrate rapidly through the glycerol in the stem, into the bulb, where they are de-proteinized. The cell free suspension medium remains in the upper chamber.

The separating devices are in two sizes, one which takes 4.0 ml cell suspension containing about 80 mgs wet weight of cells, and one which takes 16 ml suspension containing about 300 mgs wet weight of cells. The smaller tubes were used in all experiments except those reported in Chapter 6 when cell cytosol was separated from plasma membranes, lysozomes and mitochondria, when the larger tubes were used.

(b) Estimation of Inulin Space

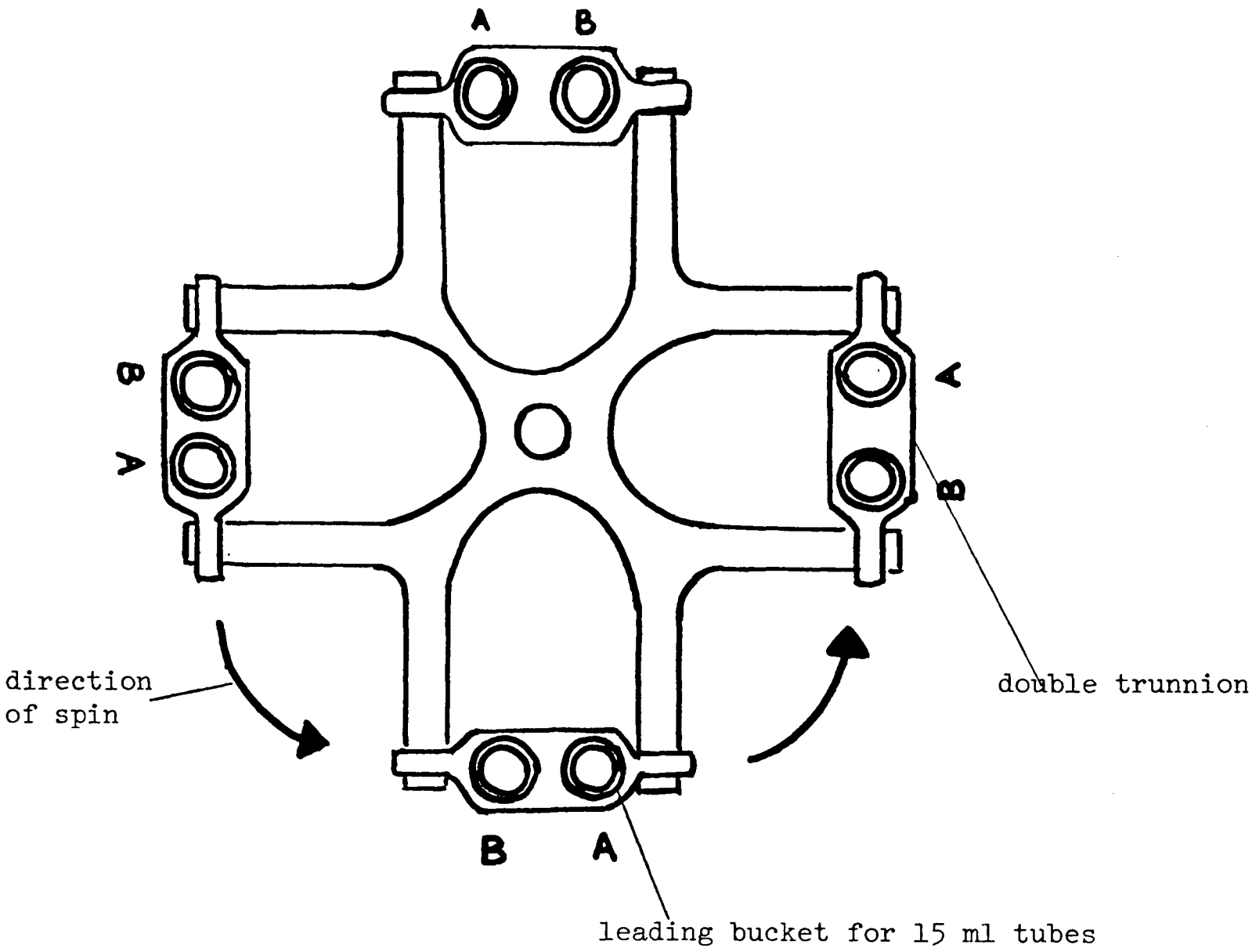
^{14}C-inulin was used to estimate 'carry down' (inulin space, adhering or extracellular fluid) in separation devices, in a similar manner as was used for Method I. The amount of adhering fluid (inulin space) tended to be in the order of four times higher in the conical tube method of separation (Method I) than in the separation device method (Method II), hence it was not possible to extrapolate the inulin space found by one method to the other method. Inulin gave a value of about 7% of the total wet weight for the inulin space of cells separated from the medium in separation devices (Method II). A possible reason for the difference between this value and that obtained by Method I is that cells passing through the stem solution of the separation device are washed by the stem solution and thus carry

down smaller quantities of cell suspension medium. Since the inulin space measurements in cells separated by Method II were solely used for correction for the solutes in the adhering fluid, and not for estimation of true extracellular water, this washing effect was hot a disadvantage. Extracellular space volume for estimation of intracellular water content was always estimated in conical centrifuge tubes, as were wet and dry weights.

(c) Effects of Centrifugation on Inulin Space

The bench top centrifuge (M.S.E. 'Minor') used for the rapid separation technique (see 5B Method II above) contained a swing out head with four arms, each with a double trunnion carrying a pair of buckets (fig 2). The leading member of a pair of buckets is defined as that which, relative to the direction of spin, travels ahead of the other member. The leading bucket is marked A in fig 2. Cells separated in devices placed in the leading buckets carried down a smaller extracellular adhering fluid (inulin space) than those placed in non-leading buckets (B). The mean inulin space of the (A) group of separation devices in four experiments was 6 µl of extracellular solution per 78 mgs wet wt. of cells (6 µl ±0.048 µl S.E.M.). By contrast, the mean inulin space in the (B) group of separation devices was 21 µl of extracellular solution per 78 mgs wet wt. of cells (21 µl ±0.09 µl S.E.M.). The difference between the two groups was highly significant ($P<0.005$). Another centrifuge (M.S.E. 'Super Minor') with four trunnions, each carrying four buckets placed in pairs, exhibited the same behaviour. I know of no explanation of this phenomenon, which appears not to have been reported by other workers, although it is likely in some cases to have led to the assignment of incorrect values to intracellular concentrations of metabolites. The errors will be very marked in cases where inulin space was not measured on every separation tube, and especially where the intracellular concentration of the metabolite

FIGURE 2. Plan of M.S.E. 'Minor' Swing-Out Head Centrifuge



being measured is low while the extracellular concentration is high, for example with Na^+. In all experiments reported in this thesis, separation devices were placed in the leading buckets (fig 2, A buckets). To avoid measuring inulin space on all tubes it was necessary to use either only leading buckets or only non-leading ones. Leading buckets were selected in virtue of the smaller standard error of the mean inulin space in the leading tube. Inulin space was measured in at least one separation device in each experiment.

(d) Loss of Intracellular Solutes During Centrifugation by Method II

The discovery that some intracellular solutes were lost from hepatocytes during centrifugation in separation devices was intially prompted by the finding that the urea ratio ([urea] inside divided by [urea] outside) was substantially less than 1.0, which meant that [urea] inside was less than [urea] outside. For example, if 5.5 mM urea was present in the medium, then the concentration found inside the cell was in the order of 1.5 mM, which gives an apparent gradient of 0.27 inside to outside. Urea is thought to permeate most mammalian membranes readily (see Kaplan et al., 1975), and thus a concentration gradient would be surprising. The low urea ratio in hepatocytes was found when urea was estimated both enzymatically and with ^{14}C urea; thus it was not an artifact of measurement (see table 2:2). Maintenance of concentration gradients usually requires energy, so an attempt was made to decrease the urea gradient by inhibiting supply of metabolic energy within the cells by incubation with 5% CO_2 and 95% N_2 together with yellow phosphorus in the centre well of the Erlenmeyer flask. The ratio of urea inside to outside did not approach 1.0 as would be expected if metabolic energy were utilized for the maintainance of a urea gradient. The possibility then arose that the urea ratio was an artifact of the separation technique. It was possible that the cells lost or exchanged intracellular water and thus lost solutes during their migration through 11% v/v glycerol in the stem of the separating devices.

Table 2:2. Comparison of the estimation of urea by the enzyme assay system and by distribution of ^{14}C urea

Experimental details are described in this chapter. Cells were separated using the separation devices (Method II). Cells were incubated for 20 min. Inulin space was measured in a parallel flask and wet and dry wt. in a further parallel flask. The data are derived from a single representative experiment.

	Intracellular Urea mM	Extracellular Urea mM	Ratio
Urea estimated using enzyme assay system	2.17	10.22	0.21
Urea estimated by ^{14}C distribution	2.23	9.51	0.23

Since glycerol contained none of the other solutes found inside the cells, the migration stage of separation presented enormous gradients of solutes inside to outside. To test the hypothesis that the urea ratio was an artifact of separation by Method II, cells from the same preparation were incubated with 4.5 mM urea for 20 minutes in parallel flasks. Cells were then separated from the medium by both Methods I and II. Inulin space was measured in all flasks and wet and dry weights were estimated in parallel flasks. Table 2:3 shows that the urea ratio was 0.86 in cells separated by Method I, but was only 0.26 in cells separated by Method II. This confirmed that the low urea ratio by Method II was an artifact of centrifugation.

If urea was lost from cells as they passed through the stem of the separation device, it was likely that other intracellular substances were also lost. Table 2:3 shows a comparison of some solutes inside and outside cells separated by Methods I and II. Solutes such as ammonia, alanine, K^+, aspartate and glutamate appeared to be unaffected by the method of separation, whereas 75% of intracellular [Na^+] and [urea] was lost when cells were separated by separation devices (Method II). On the other hand, [lactate] inside was less in the conical tubes than in the separation devices. Lactate is likely to undergo rapid changes in the one to three minute period that was taken before deproteinization of the cell pellet in the conical tubes, and so the Method I value may not be reliable. The next question to be answered was that of the intracellular water content of the hepatocytes after separation by Method II, since movement of urea outwards during centrifugation was likely to be accompanied by a movement of cell water. Parallel flasks of cells were incubated in ^3H_2O for two minutes before separation by Methods I and II. Activity of ^3H_2O in the cell sample was corrected for adhering fluid (inulin space) (see sections 5A (c) and 5B (b) above). Table 2:4 shows intracellular

Table 2:3. The effect of the method of separation of cells from medium on the intracellular and extracellular concentrations of some metabolites in hepatocytes

Experimental details are described in this chapter. Values are from one representative experiment. Initial concentrations of added substrates were: urea, 4.5 mM; alanine, 10 mM and lactate, 5.0 mM. Aspartate was generated from incubation of cells with 10 mM ethanol, 10 mM lactate and 10 mM NH_4Cl, glutamate was generated from incubation with 10 mM proline and 2.0 mM ornithine. Cells were incubated for 40 min. Method II refers to separation of cells from medium in separation devices; Method I refers to separation of cells from medium in conical centrifuge tubes.

	Method I			Method II		
	Inside mM	Outside mM	Ratio inside/ outside	Inside mM	Outside mM	Ratio inside/ outside
Urea	3.87	4.49	0.86	1.09	4.26	0.26
Ammonia	0.57	0.10	5.70	0.54	0.09	6.00
Alanine	24.87	6.80	3.66	21.80	6.68	3.26
Lactate	1.46	2.75	0.53	2.37	2.46	0.96
Aspartate	55.5	0.22	252	54.4	0.22	247
Glutamate	58.0	0.27	215	57.7	0.26	222
K^+	166	5.97	27.6	164	5.97	27.5
Na^+	21.5	145	0.15	5.51	145	0.038

Table 2:4. Intracellular water content in cells separated by Methods I and II

Experimental details are as described in this chapter. Values are means ± S.E.M. and the number of observations is in parenthesis. Values are expressed as a percentage of dry wt. of cells. Method I refers to separation of cells from medium in conical tubes. Method II refers to separation of cells from medium by separation devices.

Intracellular H_2O as % of cell dry wt.		
Method I	Method II	
	Glycerol in stem of device	Silicone oil in stem of device
185 ± 11.1 (14)	10.4 ± 2.9 (14)	114 ± 9.4 (14)

Table 2:4. Intracellular water content in cells separated by Methods I and II

Experimental details are as described in this chapter. Values are means ± S.E.M. and the number of observations is in parenthesis. Values are expressed as a percentage of dry wt. of cells. Method I refers to separation of cells from medium in conical tubes. Method II refers to separation of cells from medium by separation devices.

Intracellular H_2O as % of cell dry wt.		
Method I	Method II	
	Glycerol in stem of device	Silicone oil in stem of device
185 ± 11.1 (14)	10.4 ± 2.9 (14)	114 ± 9.4 (14)

water given as a % of dry weight of cells. Column two of table 2:4 shows that with glycerol in the stem of the separation device only 10.4% of intracellular ^3H_2O was present by the time the cells had reached the bulb of the separation device, whereas when cells were separated by Method I (column one) intracellular ^3H_2O as a % of dry weight was 185%. Intracellular water content calculated from wet weight, dry weight and inulin space by Method I was in good agreement with the intracellular ^3H_2O by Method I. Low ^3H_2O content of the pellet sample by Method II may have been caused by exchange of intracellular ^3H_2O with extracellular H_2O as the cells migrated through the stem of the device and/or may have been a result of mechanical loss of net intracellular H_2O caused by gravitational force on the cell membrane.

It was of interest to substitute glycerol in the stem of the separation device with a non-aqueous substance, silicone oil, of the appropriate specific gravity (a mixture of 42.8 g MS 550 and 8.7 g MS 200/2CS silicone fluid). In addition, the effect of NaCl in the stem was compared with that of glycerol (NaCl was the solution used by Hems et al., 1975). Table 2:4 shows that silicone oil decreased the loss of intracellular ^3H_2O (expressed as % of dry wt. of cells) but intracellular ^3H_2O was still 40% less than that found in cells separated by Method I ($P<0.001$). Table 2:5 compares urea, Na^+, K^+ and lactate ratios of cells separated by Methods I and II. The urea ratio was increased when silicone oil was present in the separation device stem, but was still lower (by 25%) than that found by Method I. The urea ratio also tended to be lower when NaCl rather than glycerol was present in the stem. The K^+ ratio was unaffected by the method of separation, but the Na^+ ratio was not significantly increased by the presence of silicone oil rather than glycerol. This was surprising, since it means that the same amount of Na^+ was lost both from cells from which there had been a 40% intracellular water loss (silicone

Table 2:5. The effect of NaCl, glycerol and silicone oil in the stem of separation devices on ratios of urea, lactate, Na^+ and K^+ in hepatocytes. Comparison of the values with those obtained from cells separated from the medium by Method I

Experimental details are described in this chapter. Values are means ± S.E.M. and the number of observations is in parenthesis. NaCl, glycerol and silicone oil refer to the substance in the stem of the separation device; concentrations of NaCl and glycerol were 4% and 11% respectively. Method I refers to the separation of cells from medium in conical centrifuge tubes. Incubation time was for 20 min. Urea and lactate were added to initial concentrations of 5.0 mM.

	Ratio [inside]/[outside]			
	Method I	Method II		
		NaCl	Glycerol	Silicone oil
Urea	0.88 ± 0.08 (4)	0.18 (1)	0.28 ± 0.025 (3)	0.64 ± 0.014 (3)
Lactate		0.56 (1)	0.96 (1)	
Na^+	0.17 ± 0.011 (16)	-	0.056 ± 0.008 (11)	0.045 ± 0.009 (3)
K^+	25.8 ± 0.56 (12)	24.0 (1)	25.7 ± 0.67 (12)	-

oil in stem) and from which 94% intracellular water was lost or exchanged (glycerol in stem). This finding is consistent with the idea that intracellular Na^+ is compartmentalized and that the Na^+ compartment looses Na^+ with water more readily during centrifugation by Method II than does the non-Na^+ compartment. Compartmentalization of Na^+ has been suggested in other contexts, for example, by McLaughlin & Hinke (1966) in striated muscle of the giant barnacle; by Pietrzyk & Heinz (1974) in nuclei of Ehrlich cells; and by Hooper & Dick (1976) in rat hepatocytes. The finding that intracellular H_2O is lost in the presence of silicone oil, a non-aqueous substance, is not surprising, since M. Spry (personal communication) has shown that silicone fluid can take up water. The water lost from cells into silicone oil must represent net loss of H_2O since exchange of intracellular H_2O with extracellular H_2O is not possible when silicone oil is present. Thus during migration of cells through glycerol in the stem of the separation device the measured changes in intracellular ^3H_2O may have two components: an exchange of ^3H_2O inside for H_2O from outside, and a net loss of H_2O plus ^3H_2O from inside.

(e) Separation of Cytosol and Particulate Cell Matter

Separation of cytosolic and mitochondrial components of the cell were made by the method of Zuurendonk & Tager (1974), which depends on making the plasma membrane permeable by treatment with digitonin for 40 seconds at about 5°C, and centrifuging the suspension. The mitochondria, plasma membranes, nuclear membranes and other insoluble material appear in the pellet, while all soluble contents of the cytosol appear in the supernatant. By this method, Siess et al. (1977) estimate that carry over from cytosolic and nuclear metabolites contributes less than 5% of the amount of metabolite in the mitochondrial and membrane pellet. In experiments reported in chapter 6 section viii below, lactate dehydrogenase and glutamate dehydrogenase were used as cytosolic and mitochondrial markers respectively. If

more than 80% LDH, and less than 15% GLDH were found in the cytosol, separation was considered inadequate.

6. Determination of K^+ and Na^+

Na^+ and K^+ were estimated on acid perchloric extracts of the tissue and medium with a Perkin-Elmer (Norwalk, Connecticut, USA) Atomic Absorption Spectrophotometer, model 103. Samples and standard solutions were diluted in lithium carbonate solution (1.5 g/l H_2O) to overcome ionization effects. Na^+ and K^+ were read at wavelengths of 589 and 766 nm respectively with hollow cathode lamps and an air/acetylene flame. Distilled deionized water was used in the preparation of all samples and solutions. Values for reagent blanks were deducted from gross sample values.

(a) Problems in the Measurement of Na^+

Oguro (1974a and 1974b) demonstrated that perchloric acid in concentrations as low as 10^{-3}M increased the absorption of Mg^{2+} and Ca^{2+} and decreased the absorption of Fe^{2+}, Ni^{2+} and Co^{2+} in an air/acetylene flame. The absorption of K^+ and Na^+ were not tested by Oguro. For this reason, experiments were undertaken to ascertain whether perchloric acid affected the absorption of Na^+.

Three stock solutions of Na^+ (0.43, 0.85 and 1.67 mM) were diluted with 5 ml lithium carbonate solution and 0.2 ml perchloric acid solution (20% v/v) to give final solutions of 0.016, 0.032 and 0.062 µmol/ml Na^+. Values of $[Na^+]$ obtained by atomic absorption spectrophotometry were compared with a control set of samples in which 0.2 ml perchloric acid was replaced by 0.2 ml lithium carbonate solution. There was no significant difference between control and perchloric acid sample values of Na^+ in three separate experiments. This indicates that perchloric acid does not interfere with Na^+ estimation.

(b) Problems in the Measurement of K^+

1. Incomplete Extraction of K^+ from Tissue Samples

When cells were separated from medium, it was found that on

ashing the cell debris in H_2O_2 and resuspending it in 0.1 M nitric acid, a mean value of 4.77% ±0.14 (S.E.M. when N=3) of total cell K^+ content remained in the cell debris after the cell extract had been removed from the separation device. The loss was relatively constant (the S.E.M. was small). The content of K^+ in the cell debris was similar in cells separated into perchloric acid or nitric acid. No correction of pellet values was made for this small and relatively constant loss.

2. The Use of Perchloric Acid

Perchloric acid could interfere with the measurement of K^+ in two separate ways:

1. By interference with absorption during spectrophotometry.

2. By precipitating with K^+ to form potassium perchlorate.

To test for precipitation of K^+ with perchloric acid, and to ascertain whether perchloric acid interfered with absorption, various concentrations of KCl were suspended in 4% perchloric acid to give final concentrations of 1.25 to 25 mM KCl at room temperature. Aliquots (0.1 ml) of these samples were suspended in lithium carbonate solution (10 mls) and measured on an atomic absorption spectrophotometer. No loss of K^+ was found in this range of concentrations. The actual concentration of K^+ in cell extracts in 1.5 ml of 4% perchloric acid is in the order of 4.3 mM (aliquots (0.1 ml) of these samples were also suspended in lithium carbonate solution (10 mls) for measurement). Cell extracts of K^+ (4.3 mM) are well below the maximum concentration of K^+ used in the above experiment. This indicates that there was no precipitation of K^+ with perchloric acid at room temperature and no interference of perchloric acid on absorption.

7. Calculation of the Rate of Turnover of Cell K^+ with $^{42}K^+$

(a) Incubation of Cells and Preparation of Samples

Turnover of cell K^+ was determined by measurement of the distribution of $^{42}K^+$ between cells and medium over a time course. Cells were preincubated without $^{42}K^+$ for 20 minutes in the presence of metabolic substrates (unless otherwise stated) to allow K^+ content of the cells to reach a steady state following perturbations caused by cell preparation. $^{42}K^+$ (0.05 µcuries of radioactivity and 0.078 µmol KCl per 1 ml suspension medium) was added at zero time (t=0). After incubation with $^{42}K^+$, cells were separated from medium by Method II (see section 5B (a) above). Acid samples of medium (0.1 ml) and pellet extract (0.1 ml) were counted in a Beckman Scintillation counter in vials containing 10 ml scintillation fluid containing 5.5 g Permablend III (Packard Instrument Co.), 60 g naphthalene, 600 ml toluene and 400 ml methoxyethanol. Correction was made for decay of $^{42}K^+$, and for carry down of $^{42}K^+$ during separation of cells from medium (see section 5B (b) above). Inulin space for correction of $^{42}K^+$ in carry down was measured on parallel samples.

(b) Calculation of Rate of Turnover of Cell K^+

The calculation of rate of turnover of cell K^+ used in these experiments was described by Krebs et al. (1951):

$$v = \frac{ab}{t(a+b)} \ln \frac{bx_o}{x(a+b) - ax_o}$$

where:

v = quantity of total K^+ passing from cell to medium and in the opposite direction per unit (min) time

a = quantity of total K^+ in medium

b = quantity of total K^+ in tissue

x = quantity of $^{42}K^+$ in medium at time t

y = quantity of $^{42}K^+$ in tissue at time t.

The total quantity of $^{42}K^+$ in the whole system is assumed to remain constant: $x + y = x_o$.

$^{42}K^+$ (x_o) was added to medium at t = 0.

The turnover rate, expressed as % of tissue K^+ turned over per minute was:

$$\frac{v}{b} \times 100.$$

(c) Limitations of This Calculation

The calculation is valid only in steady state conditions, i.e. when the quantity of K^+ moving into the tissue per minute equals the quantity of K^+ moving out of the tissue per minute. This means that in a number of situations where K^+ influx exceeds efflux or vice versa the calculation is invalid.

8. Enzyme Assays

Glutamate dehydrogenase was assayed according to Schmidt (1963), with modifications (see Tomkins et al., 1963); lactate dehydrogenase was assayed according to Bergmeyer et al. (1963), and adenylate kinase according to Bergmeyer (1974).

9. Determination of Metabolites

Metabolites were determined on neutralized extracts of the tissue and medium by methods described as follows:

glucose (Slein, 1963);

lactate and pyruvate (Hohorst et al., 1959);

aspartic acid (Pfleiderer, 1963);

glutamate and alanine successively in the same cuvette by a modification of the method of Bernt & Bergmeyer (1963) as described by Cornell et al. (1974);

glutamine (Lund, 1970);

3-hydroxybutyrate and acetoacetate (Williamson at al., 1962);

ATP (Lamprecht & Trautschold, 1963);

ADP and AMP (Adam, 1963);

P_i (Martin and Doty, 1949).

Ammonia and urea were measured successively in the same cuvette in the assay system described by Bernt and Bergmeyer (1970) for urea. Determination of ammonia and urea on tissue samples required two modifications. EDTA was added to the assay buffer, and all tissue samples were treated with 'Norit' (activated charcoal) essentially as described by Brosnan and Williamson (1974). Tissue extract (2.0 ml) was pipetted into centrifuge tubes which contained 25 mg Norit. These tubes were mixed by means of a vortex mixer and centrifuged at 10,000 × g in an Eppendorf Zentrifuge 3200 centrifuge for two minutes; blanks containing $HClO_4$ were treated identically. The supernatant was used for ammonia determination. There was 5% loss of urea in samples treated with Norit. No correction of urea values was made for this loss. These two modifications to the ammonia assay were necessary because not only did non-enzymatic oxidation interfere with the assay, but also some agent removed by Norit inhibited the enzymatic reaction. In all experiments where ammonia was to be determined, distilled deionized water was used for preparation of reagents and for rinsing apparatus. Acid samples were kept covered to avoid contamination by ammonia from the atmosphere.

10. Use of Statistics

Throughout this thesis, numbers in tables are means ± S.E.M., where the numbers represent different rats. Student *t*-test was used throughout, except where stated, when a paired *t*-test was used.

11. Electron microscopy

Cells were incubated as described in section 4 above. Equal parts of cell suspension and 4% glutaraldehyde in P_i buffer, pH 7.3, were mixed and kept at room temperature for one hour. The fixed cells were then sedimented (not centrifuged), and the supernatant was removed. The cell pellet was combined with an equal quantity of 2% Agar to form Agar blocks. These blocks were then washed in P_i buffer, pH 7.3, and post fixed for two hours in 1% osmium and 99% P_i buffer, pH 7.3.

The cells in the Agar blocks were then embedded in 'Araldite' epoxyresin. Ultrathin sections cut at approximately 600 to 800 Å intervals were mounted on bare copper grids and double stained in 2% aqueous uranyl acetate and lead citrate. Sections were examined on a Phillips 301 electron microscope.

CHAPTER 3. THE ENDOGENOUS K^+ AND Na^+ CONTENT OF ISOLATED HEPATOCYTES AND ITS MODIFICATION BY INHIBITORS AND ADDED METABOLIC SUBSTRATES

General Introduction to Chapter 3

The first section of this chapter presents the endogenous values of Na^+ and K^+ in isolated hepatocytes and compares these values with those found *in vivo* by other workers. Section 2 shows the effects of inhibitors on the Na^+ and K^+ content of isolated hepatocytes. In addition, endogenous rates of O_2 consumption and K^+ turnover are compared with those when ouabain is present. Section 3 compares the effects of various added metabolic substrates on K^+ content, K^+ turnover and cell volume in hepatocytes. Results from these three sections provide the starting point for work which is developed in more detail in Chapters 4,5,6 and 7 of this thesis.

Section 1. The Intracellular Content of K^+ and Na^+ in Hepatocytes.

Introduction

i. Values of K^+ and Na^+ in Whole Liver Tissue

Whole male fed rat liver intracellular $[K^+]$, $[Na^+]$ and $[Cl^-]$ were estimated by Williams and Woodbury (1971) to be 165 mM, 22 mM and 22 mM respectively. These workers found no significant difference between male and female rat liver ion values, whilst the effect of starvation (24 hrs) was to increase $[K^+]$ slightly. Similar values were obtained by Claret & Mazet (1972) in whole rat liver (166 mM, 23.5 mM and 36.5 mM for $[K^+]$, $[Na^+]$ and $[Cl^-]$ respectively). In whole liver, there is considerable difficulty in obtaining accurate values for concentrations of intracellular ions because of problems of estimation of extracellular space (for discussion see Parsons & van Rossum, 1962; Williams & Woodbury, 1971; and McIver & Macknight, 1974). Correct estimation of extracellular space is required both for calculation of intracellular water volume and for correction of total ion values for contamination by extracellular ions. No ideal extracellular marker has been discovered, and the inaccessibility of

extracellular space in whole liver means that a marker such as inulin must be perfused through liver for as much as one hour (Williams & Woodbury, 1971). Not only does inulin enter liver cell water during perfusion, but drying of tissues can prevent subsequent extraction of inulin (McIver & Macknight, 1974). Isolated hepatocytes have the advantage that inulin can equilibrate with extracellular space within seconds and accurate estimation of intracellular water is less problematic. It is of interest to compare the K^+ and Na^+ values found in isolated hepatocytes with those given above.

ii. Values of K^+ and Na^+ Content in Isolated Hepatocytes

Berg and Iversen (1976) found intracellular $[K^+]$ values of 172 mM and $[Na^+]$ of 25 mM in starved rat hepatocytes prepared in Hanks solution by a modification of Seglen's procedure (1973). Intracellular water in their preparation was 60% of the wet weight of cells. Claret et al. (1976) found a K^+ content of 315 µmol/g dry wt. in isolated hepatocytes, which was similar to their in vivo value of 320 µmol/g dry wt. liver (Claret & Mazet, 1972). However, since their isolated hepatocytes were swollen (4.92 ml H_2O/g dry wt.) as compared with whole tissue (3.0 ml H_2O/g dry wt.), $[K^+]$ was only 103 mM. Claret et al. (1976) used a modification of Seglen's technique (1973) for preparation of cells, which were then incubated in oxygenated MEM (Eagle). Baur et al. (1975) found similar $[K^+]$ values to those of Claret, namely 109 mM $[K^+]$ and 47.0 mM $[Na^+]$. They also calculated a liver cell membrane potential of 36.4 mV, assuming that Cl^- was passively distributed. Barnabei et al. (1974) reported a K^+ content of 102 µmol/g wet wt. for female rat hepatocytes. No details were given for cell water content so $[K^+]$ cannot be calculated. Tomasi et al. (1975) compared cells prepared by the modified method of Seglen (1973) in which livers were perfused for the first five minutes with Ca^{2+} free Hanks solution, for the next five minutes with Ca^{2+} free Hanks solution plus 0.5 mM EGTA, and for the final seven to eight

minutes with Hanks solution plus 2.5 mM Ca^{2+}, 0.03 mM collagenase and 1% BSA, with those prepared by the original method of Berry & Friend (1969). They found the K^+ content of hepatocytes prepared by Berry & Friend's method to be 49 µmol/g wet wt., and suggested that Ca^{2+} in the medium of the final stage of the perfusion was important in maintainance and recovery of K^+ content. Dickson & Pogson (1977) compared the K^+ content of hepatocytes in prolonged incubations in two different incubation mediums. Cells incubated for two hours in MEM (Eagle) plus 10% foetal calf serum and 2 g $NaHCO_3$/l had a K^+ content of 270 µmol/g dry wt., whereas those in Krebs-Henseleit bicarbonate buffer containing 2% fatty acid free dialysed BSA contained only 210 µmol/g dry wt. K^+. Furthermore, cells incubated in MEM and serum maintained their K^+ content for 8 hours. Thus the reported range of intracellular $[K^+]$ in isolated hepatocytes is wide: from 172 mM (Berg & Iversen) to 103 mM (Claret et al.). This variation appears to depend in part on the difference in intracellular water content of hepatocyte preparations, since K^+ content (µmol/g dry wt.) is less variable. In addition, the mode of preparation and incubation of isolated hepatocytes appears to affect K^+ content considerably.

The intracellular $[Na^+]$ value of 25 mM (Berg & Iversen, 1976) for isolated hepatocytes is similar to the <u>in vivo</u> value of 22 mM (Williams & Woodbury, 1971).

Throughout this thesis, Na^+ values are given in terms of concentrations (mM) and content (µmol/g dry wt.). However, it may not be justifiable to express intracellular Na^+ in terms of concentrations, since there is evidence that intracellular Na^+ is not equally distributed thoughout the cell interior. Hooper & Dick (1976) estimated by flame photometry and histological analysis that liver nuclei $[Na^+]$ was as high as 131 mM, which is a similar value to that of plasma $[Na^+]$. This means that cytosolic $[Na^+]$ is very low. Monoi (1974) suggests on the basis of nuclear magnetic resonance (NMR) analysis that 61% of intracellular Na^+ is not in

solution in the cytosol of rat liver, which is compatible with his hypothesis that Na^+ is secreted around negatively charged DNA. Lev & Armstrong (1975), using ion sensitive electrodes, found only half the predicted Na^+ activity intracellularly. However, K^+ activity was similar to that predicted if K^+ was distributed equally through the cell interior. Further evidence suggests that K^+ is free in solution in most cells. Pietrzyk & Heinz (1974) suggest that K^+ is equally distributed throughout Ehrlich cells, and Claret & Mazet (1972), on the evidence of $^{42}K^+$ efflux, suggest that K^+ ions are uniformly distributed in only one compartment in liver cells. (For review of intracellular ionic activity measurements with selective microelectrode techniques, see Walker & Mack-Brown (1977).)

In this thesis, values of endogenous K^+ and Na^+ from fed and starved rat hepatocytes are given both as concentration terms (mM) and as µmol/g dry wt. Two different methods of preparation of cells are compared.

Results and Discussion

Table 3:1 shows intracellular and extracellular contents (µmol/g dry wt.) and concentrations (mM) of K^+ and Na^+ in isolated hepatocytes prepared from starved rats (48 hours) and incubated at 37°C in Krebs-Henseleit medium containing 2.5% BSA and gassed with 95% O_2 and 5% CO_2. Concentration values were corrected for cell water as described above (Chapter 2 section 5A (f)), and Na^+ values were corrected for adhering fluid (Chapter 2 section 5A (c)). During preparation of isolated hepatocytes intracellular K^+ content decreased and Na^+ content increased so that at zero time of incubation $[K^+]$ was 132 mM and $[Na^+]$ was 35.8 mM. During the first 10 minutes of incubation $[K^+]$ and $[Na^+]$ were restored to values of 160 mM and 28 mM respectively. These values remained relatively constant for the following 80 minutes of incubation. Both $[Na^+]$ and $[K^+]$ values were similar to those found in whole liver

Table 3:1. Endogenous K^+ and Na^+ content of isolated hepatocytes prepared from starved rats

Experimental details are described in Chapter 2 and the text. Values are means ± S.E.M., and the number of observations is in parenthesis. mM values are corrected for intracellular water. Extracellular $[K^+]$ was 5.98 ± 0.031 (44) and extracellular $[Na^+]$ was 146 ± 1.07 (10) µmols/ml medium.

Incubation (min)	Intracellular mM		Intracellular µmol/g dry wt.		
	$[Na^+]$	$[K^+]$	Na^+	K^+	$Na^+ + K^+$
0	35.8 ± 4.5 (5)	132 ± 5.7 (11)	72.9 ± 8.3	258 ± 9.2	331
10	28.4 ± 2.7 (8)	160 ± 2.9 (23)	55.6 ± 5.1	306 ± 5.0	362
20	23.2 ± 2.4 (10)	157 ± 1.8 (44)	45.5 ± 4.8	297 ± 3.7	343
30	-	164 ± 3.5 (12)	-	307 ± 6.5	-
40	24.1 ± 2.2 (10)	155 ± 1.8 (38)	47.0 ± 4.5	295 ± 3.9	342
60	28.1 ± 2.2 (4)	155 ± 2.3 (28)	53.5 ± 4.6	298 ± 3.8	352
80	26.4 ± 3.3 (5)	151 ± 3.3 (14)	53.3 ± 7.1	296 ± 6.3	349
100	27.3 ± 6.6 (4)	145 ± 5.7 (6)	54.9 ± 13.7	294 ± 10.9	349

(Williams & Woodbury, 1971; Claret & Mazet, 1972).

Table 3:2 gives values for intracellular K^+ content from fed rats prepared both by the method described in Chapter 2 section 3 (A on table 3:2), and by the following method (B on table 3:2), which is similar to that described by Barnabei et al. (1974). Rat liver was perfused for 10 minutes in Ca^{2+} free Krebs-Henseleit solution containing 0.1 mM EGTA. Perfusion was continued for the subsequent 10 minutes with 0.02% w/v collagenase and 2.5 mM Ca^{2+}. After this, the method was similar to that of method A. There was no significant difference in intracellular $[K^+]$ between the two methods. Cells prepared from fed rat liver had a lower K^+ content than those from starved liver (266 µmol/g dry wt. as compared with 297 µmol/g dry wt.). In addition, liver cells from fed rats were larger in cell volume. When both these factors were taken into account, $[K^+]$ of fed liver cells was significantly lower than that of starved cells (135 mM as compared with 157 mM at 20 minutes of incubation). Cells from fed rats contained less K^+ than reported by Williams & Woodbury (1971) from whole fed rat liver.

The values of intracellular K^+ content reported throughout this thesis are underestimated, because 5% of the cell K^+ remained in the cell pellet debris after extraction with perchloric acid. No correction was made for this (see Chapter 2 section 6 (b)1).

Section 2. The Effect of Ouabain on Rat Hepatocytes

Introduction

In physiological conditions the function of the sodium pump (Na-K ATPase) is to exchange cell Na^+ for extracellular K^+ at the expense of energy derived from hydrolysis of ATP at the inner surface of the cell membrane. In 1953, Schatzmann observed that Strophanthin K inhibited the active movement of K^+ and Na^+ in the erythrocyte without interfering with energy yielding reactions of the cell.

Table 3:2. Endogenous values of K^+ in hepatocytes prepared from fed rats

Experimental details are described in Chapter 2 and the text. The values are means ± S.E.M., and the number of observations is in parenthesis. Intracellular concentration values (mM) are corrected for cell water. Incubation time was 20 min in all cases. Method A and B are described in Chapter 2 and in the Results section of this chapter respectively.

Method	Intracellular K^+		Extracellular K^+
	mM	µmol/g dry wt.	µmol/ml medium
A	134 ± 4.3 (3)	266 ± 8.3	6.26 ± 0.16 (3)
B	135 ± 6.1 (7)	263 ± 8.6	6.28 ± 0.094 (7)

Since that time it has been established that cardiac glycosides such as ouabain are highly specific inhibitors of the Na-K ATPase. Reviews on the sodium pump are numerous, and include those by Skou, 1965; Whittam & Wheeler, 1970; Dahl & Hokin, 1974; and Glynn & Karlish, 1975.

The binding properties of ouabain on the ATPase receptor vary between species and between tissues. Rates of association of ouabain with the Na-K ATPase do not appear to differ greatly between different species or tissues. However, rates of dissociation of ouabain with the enzyme vary: for example, the rate is higher in cat than in dog, and in guinea pig than in cat (Tobin et al., 1972). Rat tissue has a high rate of dissociation of the ouabain Na-K ATPase complex, thus rat tissue is relatively insensitive to cardiac glycosides (Repke et al., 1965; Macknight et al., 1974). In addition, liver is less sensitive than other tissues: for example, brain ATPase has been found to be about 100 times more sensitive to ouabain than liver tissue (Judah & Ahmed, 1964). Because of the variation in the response of preparations to ouabain, a preliminary study was undertaken on hepatocytes in which the effect of various concentrations of ouabain on the distribution of K^+ and Na^+ between cells and medium was determined. In these experiments the medium was not supplemented with metabolizable substrates. Furthermore, the effect of ouabain on oxygen consumption, glucose synthesis and urea formation was measured both in the presence and the absence of additions. In work to be discussed in later chapters, ouabain was used to ascertain the effect of the Na-K ATPase on the ammonia content of hepatocytes (Chapter 6). It was also used to elucidate the mechanism by which substances which modify liver metabolism, such as amino acids and fructose, alter the K^+ and Na^+ distribution in hepatocytes (Chapters 4, 5 and 7).

Results

i. The Effect of Ouabain on the K^+ and Na^+ Content of Hepatocytes

Table 3:3 shows the effects of 0.1 to 16 mM ouabain on intracellular $[K^+]$ over a time course of 100 minutes. The values in table 3:3 are normalized taking as 100% values of K^+ in conditions without ouabain. Diagram 3:1 is derived from data in table 3:3. In all case the value for intracellular $[K^+]$ at zero time was lower than the endogenous $[K^+]$ value for the subsequent incubation. This is discussed in section 1 of this chapter. At low concentrations of ouabain (0.1 mM), some recovery of intracellular K^+ was made. This recovery decreased with increasing concentration of ouabain. A maximal effect of ouabain was seen at a concentration of 2.0 mM.

Table 3:4 shows the effect of ouabain (2.0 mM) on both Na^+ and K^+ content of hepatocytes. Since ouabain had no effect on the wet weight to dry weight ratio of hepatocytes or on inulin space (see Chapter 4 section ii), calculation for cell water is similar both in cells with and without ouabain. Table 3:4 shows that loss of K^+ in the presence of ouabain was accompanied by a gain in a similar number of Na^+ ions (199 µmol/g dry wt. Na^+ gained, and 186 µmol/g dry wt. K^+ lost, from 0 - 100 minutes). The sum of Na^+ plus K^+ inside (µmol/g dry wt.) was slightly lower in the presence of ouabain. A similar effect was found in the presence of 4.0 mM ouabain (not shown). Since intracellular ionic balance in maintained, this implies that there was either an increase in another intracellular cation or a loss of anion. The question remains to be answered.

ii. The Effect of Ouabain on the Turnover of K^+ in hepatocytes

Table 3:5 shows the % turnover of K^+ in hepatocytes which had been preincubated with and without ouabain (0.5 mM) for 20 minutes before addition of $^{42}K^+$. Cells without additions had a K^+ turnover of 2.14% of tissue K^+ per minute at 40 minutes of incubation. Assuming a steady state in which K^+ uptake equals K^+ output, the K^+ uptake can

Table 3:3. The effect of various concentrations of ouabain on K^+ distribution between hepatocytes and medium

Experimental details are described in Chapter 2 and the text. $[K^+]$ inside values are means and are normalized with cells with no additions as 100%. The number of observations is in parenthesis. $[K^+]$ inside values are corrected for cell water. $[K^+]$ outside are expressed as µmols/ml suspension medium.

Ouabain (mM):	None		0.1		0.25		0.5		1.0		2.0		4.0		8.0		16.0	
	$[K^+]$ in	$[K^+]$ out	$[K^+]$ in	$[K^+]$ out	$[K^+]$ in	$[K^+]$ out	$[K^+]$ in	$[K^+]$ out	$[K^+]$ in	$[K^+]$ out	$[K^+]$ in	$[K^+]$ out	$[K^+]$ in	$[K^+]$ out	$[K^+]$ in	$[K^+]$ out	$[K^+]$ in	$[K^+]$ out
Incubation (min)																		
0	132 (11)	6.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10	156 (15)	6.0	144 (1)	6.1	112 (1)	6.2	113 (3)	6.3	110 (3)	6.5	107 (5)	6.6	102 (4)	6.8	123 (2)	6.3	105 (1)	6.2
20	156 (37)	6.0	131 (2)	6.3	112 (1)	6.2	101 (11)	6.4	103 (5)	6.5	95.7 (6)	6.5	85.9 (4)	6.8	94.9 (2)	6.6	90.2 (1)	6.6
40	154 (29)	5.9	138 (2)	6.1	104 (1)	6.3	93.6 (11)	6.5	82.6 (5)	6.6	72.6 (6)	6.7	69.6 (4)	6.9	71.9 (2)	6.8	66.4 (1)	6.7
60	152 (20)	6.0	-	-	-	-	88.1 (8)	6.7	75.2 (3)	6.7	-	-	-	-	-	-	-	-
80	151 (13)	6.0	136 (1)	6.0	108 (1)	6.3	84.2 (4)	6.6	65.2 (2)	6.8	52.3 (5)	6.9	50.0 (4)	7.1	46.2 (2)	7.0	44.4 (1)	6.8
100	145 (6)	6.0	-	-	-	-	88.0 (2)	6.5	-	-	45.7 (1)	7.1	41.9 (4)	7.4	41.6 (2)	7.2	40.9 (1)	6.9

DIAGRAM 3:1. The Effect of Ouabain (0 - 16 mM) on Intracellular $[K^+]$ in Hepatocytes

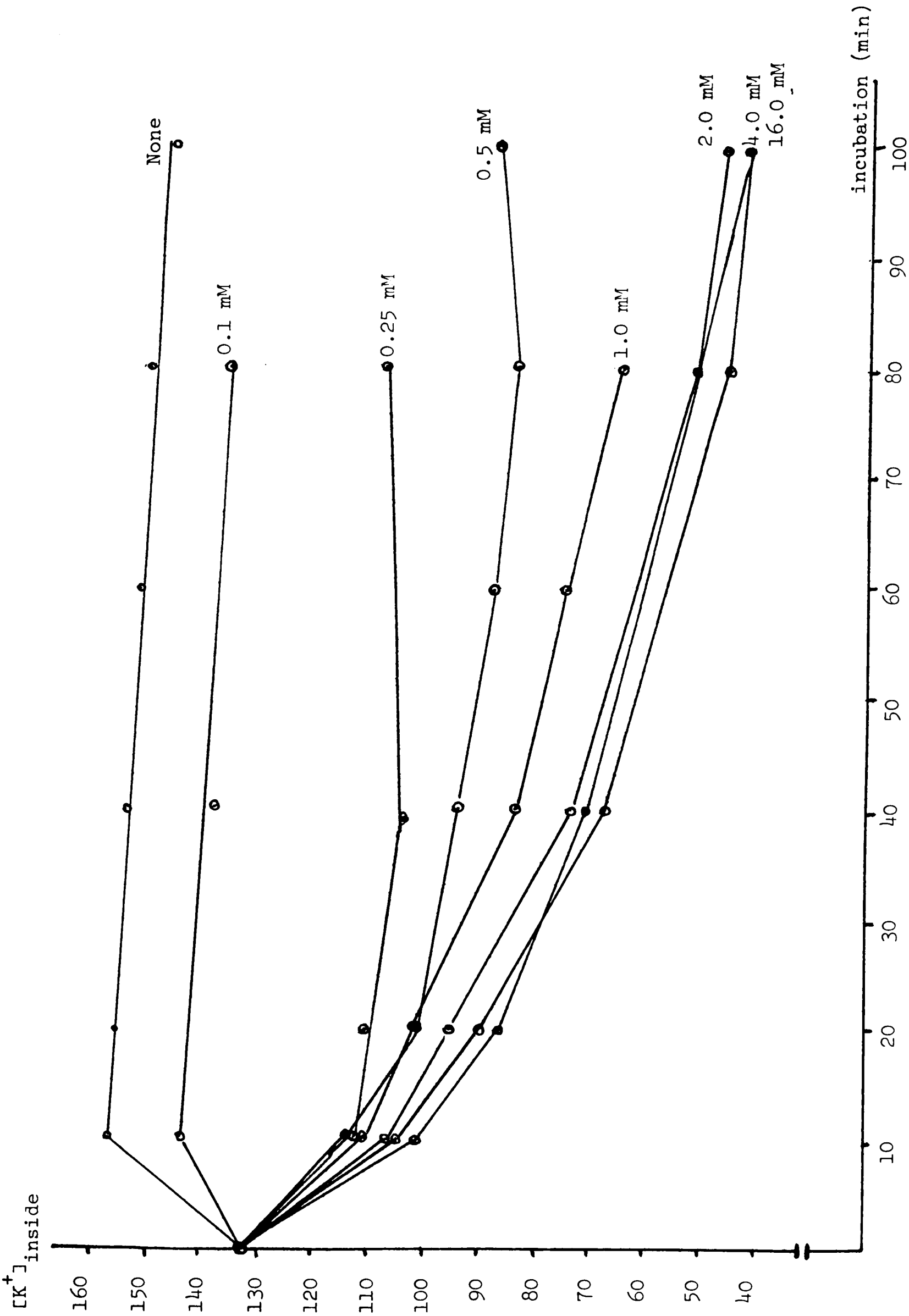


Table 3:4. The effect of ouabain on the distribution of Na^+ and K^+ between hepatocytes and medium

Experimental details are described in Chapter 2 and the text. Ouabain was added to the appropriate flasks to an initial concentration of 2.0 mM. Values are means ± S.E.M. and the number of observations is in parenthesis. There was no difference between the wet wt. to dry wt. ratios of control and ouabain treated cells. The concentration of Na^+ in the medium was 145 mM.

	Additions	Incubation (min) 0	10	20	40	80	100
K^+ inside µmol/g dry wt.	None	273 ± 14.0 (4)	322 ± 9.9 (5)	326 ± 7.8 (6)	320 ± 9.7 (6)	311 ± 11.2 (5)	296 ± 5.0 (3)
	Ouabain	-	218 ± 4.7 (5)	200 ± 6.9 (6)	151 ± 7.4 (6)	108 ± 8.9 (5)	87.1 ± 8.2 (3)
$[K^+]$ in mM	None	134 ± 6.2	159 ± 4.2	161 ± 2.1	159 ± 2.7	155 ± 4.1	148 ± 0.9
	Ouabain	-	108 ± 2.2	98.7 ± 2.7	75.0 ± 3.2	53.6 ± 3.8	43.1 ± 4.3
$[K^+]$ out	None	6.2 ± 0.08	6.0 ± 0.06	6.0 ± 0.05	6.0 ± 0.05	6.0 ± 0.06	6.0 ± 0.09
	Ouabain	-	6.4 ± 0.14	6.5 ± 0.08	6.7 ± 0.10	6.9 ± 0.11	7.0 ± 0.15
Na^+ inside µmol/g dry wt.	None	72.6 ± 10.7 (4)	54.7 ± 7.4 (5)	46.8 ± 5.9 (6)	50.3 ± 5.8 (6)	53 ± 7.1 (5)	66.7 ± 9.8 (3)
	Ouabain	-	152 ± 13.7 (5)	175 ± 12.7 (6)	198 ± 14.7 (6)	235 ± 15.9 (5)	272 ± 13.2 (3)
$[Na^+]$ in mM	None	35.9 ± 5.8	27.2 ± 3.6	23.1 ± 2.8	25.1 ± 2.8	26.4 ± 3.3	33.0 ± 4.7
	Ouabain	-	75.3 ± 6.4	86.2 ± 5.7	98.6 ± 7.6	117 ± 8.0	135 ± 8.8
$K^+ + Na^+$ µmol/g dry wt.	None	346	377	373	370	364	363
	Ouabain	-	370	375	349	343	359

Table 3:5. The effect of ouabain on the turnover of K^+ in hepatocytes

Experimental details are described in Chapter 2, section 7. Values are means ± S.E.M. of four observations. Ouabain was added to an initial concentration of 0.5 mM. The rate of turnover calculation is described in Chapter 2, section 8. Cells were preincubated with ouabain for 20 min before addition of $^{42}K^+$.

Incubation (min) after addition of $^{42}K^+$	Rate of turnover of K^+ (expressed as % of tissue K^+ turned over per min)	
	No additions	Ouabain
10	2.24 ± 0.34	1.44 ± 0.16
20	2.14 ± 0.40	1.13 ± 0.21
30	2.04 ± 0.36	1.35 ± 0.25

be calculated as approximately 1.70 µmol/min/g wet wt. This is a somewhat higher value than that of 1.07 µmol/min/g found by Claret et al. (1973) in perfused liver. Ouabain (0.5 mM) decreased K^+ turnover by 36% after 30 minutes of incubation (10 minutes of incubation with $^{42}K^+$), which indicates that there was continued K^+ influx in the presence of ouabain. Since the Na-K ATPase was not totally inhibited by 0.5 mM ouabain, some of this inward K^+ movement may be accounted for by the Na-K ATPase. In addition, there is a ouabain insensitive K^+ inward movement. Claret et al. (1973) found that 28% of the total inward K^+ movement was ouabain insensitive, whilst Russo et al. (1977) found that in liver slices 12% of the total K^+ influx was ouabain insensitive. The magnitude of the ouabain insensitive influx of K^+ in isolated hepatocytes could be ascertained from similar experiments to those in table 3:5, in which 4.0 mM ouabain was substituted for 0.5 mM ouabain. This remains to be done.

iii. The Effect of Ouabain on Glucose and Urea Synthesis, Lactate, Alanine and ATP Concentrations, and Oxygen Consumption in Hepatocytes

Table 3:6 shows the effect of ouabain (0.5 mM) on endogenous glucose and urea production in hepatocytes. In the presence of ouabain, glucose production was about 65% of that in the absence of ouabain. Urea production was less inhibited than glucose production, namely 92% and 77% of that without ouabain at 20 and 60 minutes respectively. Endogenous oxygen consumption in isolated hepatocytes was in the order of 2.5 µmol/min/g wet wt. Oxygen consumption was decreased by 18% in the presence of ouabain (table 3:6). Endogenous lactate production was decreased, whereas endogenous alanine production was increased, in the presence of ouabain (table 3:6). Decrease in lactate production in the presence of ouabain was also found by Whittam et al., 1964, and van Rossum et al., 1975.

Table 3:7 gives the results of two similar experiments in which glucose synthesis was stimulated by addition of lactate (10 mM),

Table 3:6. The effect of ouabain on glucose and urea synthesis, ATP, lactate and alanine concentrations and oxygen consumption in hepatocytes

Experimental details are described in Chapter 2 and the text. Values are means ± S.E.M. and the number of observations is in parenthesis. Ouabain was added to an initial concentration of 0.5 mM. Cells were incubated without added substrate.

Incubation (min)	Ouabain mM	Glucose µmol/g wet wt.	Urea µmol/g wet wt.	Lactate µmol/g wet wt.	Alanine µmol/g wet wt.	ATP µmol/g wet wt.	Oxygen consumption µmol/min/g
20	None	8.74 ± 1.05 (6)	5.87 ± 0.56 (5)	2.51 ± 0.79 (3)	0.52 ± 0.089 (3)	1.84 ± 0.074 (3)	-
	0.5	5.80 ± 0.64 (6)	5.39 ± 0.53 (5)	1.66 ± 0.29 (3)	0.77 ± 0.094 (3)	1.71 ± 0.14 (3)	-
60	None	13.23 ± 1.56 (4)	10.78 ± 0.83 (4)	2.48 ± 0.31 (3)	0.60 ± 0.059 (3)	1.95 ± 0.054 (3)	2.53 ± 0.21 (4)
	0.5	8.49 ± 1.08 (4)	8.14 ± 0.53 (4)	2.06 ± 0.34 (3)	0.83 ± 0.14 (3)	1.71 ± 0.15 (3)	2.08 ± 0.09 (4)

Table 3:7. The effect of ouabain on urea and glucose synthesis and oxygen consumption in conditions of stimulated glucose and urea synthesis

Experimental details are described in Chapter 2 and the text. Values are means of two similar experiments. The initial concentration of lactate was 10 mM except where stated, that of NH_4Cl was 10 mM, that of pyruvate was 0.1 mM and of oleate and ornithine was 1.0 mM. Ouabain was added to an initial concentration of 0.5 mM. Incubation time was 60 min.

Substrates added	Rate of urea synthesis µmol/min/g wet wt.	Rate of glucose synthesis µmol/min/g wet wt.	Oxygen consumption µmol/min/g wet wt.
NH_4Cl Lactate (2 mM) Pyruvate Oleate Ornithine	5.90		8.96
NH_4Cl Lactate (2 mM) Pyruvate Oleate Ornithine Ouabain	4.41		7.16
Lactate Pyruvate Oleate		2.22	6.00
Lactate Pyruvate Oleate Ouabain		1.19	5.51

pyruvate (0.1 mM) and oleate (1.0 mM), with and without ouabain. After 60 minutes of incubation, glucose synthesis was 46% less in cells with ouabain. Oxygen consumption was decreased by 8% in these conditions in the presence of ouabain. Table 3:7 also gives the results of two experiments in which urea synthesis was stimulated by the presence of NH_4Cl (10 mM), lactate (2.0 mM), pyruvate (0.1 mM), ornithine (1.0 mM) and oleate (1.0 mM). After 60 minutes, urea synthesis was 25% less in cells incubated with ouabain, whilst oxygen consumption was 20% less in these conditions in the presence of ouabain.

It is of interest to note that urea synthesis from alanine (5.0 mM) was inhibited in the presence of ouabain (0.5 mM) by 32% after 40 minutes, whereas glucose production was unaffected (see Chapter 5, table 5:6). No explanation has yet been proposed for the different effects of ouabain on glucose and urea production in conditions of added alanine on the one hand, and of added lactate, pyruvate, NH_4Cl and oleate on the other.

Discussion

i. The Concentration of Ouabain Used in Experiments with Liver Tissue

There is considerable variation between concentrations of ouabain used by different workers in rat preparations. For example, van Rossum (1970) reported that in rat liver slice preparations 0.5 mM ouabain was sufficient to give maximal inhibition of ion transport and respiration. Claret et al. (1973) used a concentration of 1.0 mM in rat perfusions, and 1.0 mM ouabain in isolated hepatocyte preparations (Claret et al., 1976). Bakkeren & Bonting (1968) and Macknight et al. (1974), working with rat liver slice preparations, used 10 mM ouabain to ensure a maximal effect on the Na-K ATPase. Results in this thesis indicate that 2.0 mM ouabain gives a maximal effect in isolated rat hepatocytes. It is not clear whether the higher concentration required for maximal inhibition in hepatocytes (this thesis) as compared with that required for liver slices (van Rossum) is due to a change in the sensitivity of the Na-K ATPase

following preparation of hepatocytes or to an interspecies or sex difference between rats. Van Rossum used male albino rats (200-250 g), Macknight et al. used adult male black and white hooded rats, whilst in this laboratory female Wistar rats (180-220 g) were used.

In much of the work reported in this thesis, 0.5 mM ouabain was used in experiments because at this concentration changes in cell ion content were found to reach an almost steady state by 20 minutes of incubation (frequently this was the preincubation period). This permitted the application of the calculation of rate of turnover of cell K^+ detailed above (Chapter 2 section 7 (b)). Although greater effects could perhaps have been obtained with 2.0 mM ouabain, the effects of ouabain on hepatocyte ammonia distribution and alanine content were clearly demonstrable in the presence of 0.5 mM ouabain.

ii. The Effects of Ouabain on the Rate of Loss of K^+ and Gain of Na^+ in Hepatocytes

In this thesis, K^+ loss from hepatocytes in the presence of ouabain (2.0 mM) was accompanied by approximately a stoichiometric gain in Na^+ (one for one). This stoichiometry was also found by Macknight et al. (1974) in liver slice preparations treated with ouabain (10 mM). Diagram 3:2 (in section 3 (ii) below) shows that the gain of Na^+ by hepatocytes on addition of ouabain was not linear.

The rate of gain of Na^+ content by hepatocytes in conditions of maximum inhibition of the Na-K ATPase by ouabain will depend upon:

1. The electrochemical inward driving force on Na^+.
2. The passive permeability of the membrane to Na^+ (P_{Na} = 4.0×10^{-8} cm sec^{-1}; Claret & Mazet, 1972).
3. The presence of an active ouabain-insensitive Na^+ efflux mechanism, such as proposed by Macknight et al. (1974) and Russo et al. (1977) for regulation of cell volume.

The rate of loss of K^+ from hepatocytes in conditions of maximum

inhibition of the Na-K ATPase will depend upon:

1. The permeability of the membrane to Na^+, since Na^+ permeates liver cell membranes more slowly than K^+ ($P_K = 7.6 \times 10^{-8}$ cm sec^{-1}, Claret & Mazet, 1972) and intracellular electroneutrality is maintained.
2. The presence of a ouabain-insensitive K^+ inward transport mechanism such as is suggested by the work of Claret et al. (1973), who found that 15% of active K^+ inward transport in liver slices was independent of the Na-K ATPase.

Because it is likely that active transport systems other than the Na-K ATPase are involved in the transport of K^+ and Na^+, it is not possible to deduce the rate of passive net inward permeation of the membrane by Na^+ from the changes in Na^+ content of cells seen on addition of ouabain.

iii. The Effect of Ouabain on Oxygen Consumption

The rate of oxygen consumption in cells incubated without additions in experiments reported here (2.5 µmol/min/g wet wt.) was similar to that found by Krebs et al. (1974) (2.4 µmol/min/g wet wt. at 10 minutes and for subsequent incubation of isolated cells without added substrates). The 18% inhibition of this rate by ouabain was similar to that found by Russo et al. (1977) in liver slices (20% in the presence of 1.0 mM ouabain).

Experiments with mitochondria indicate that ouabain itself has no direct effect on respiration (Blond & Whittam, 1964). However, inhibition of the Na-K ATPase by ouabain leads indirectly to a reduction in oxygen consumption in liver. The difference between the rate of respiration when transport is active and inactive, the so-called supra-basal oxygen consumption, has frequently been taken to represent the energy requirement of the transport process. Whittam (1964) suggests that 20-45% of the resting respiration in mammals is used for the Na-K ATPase. However, it is unlikely that the value of 18% decrease

in oxygen consumption reported here represents the supra-basal oxygen consumption, since not only was active ion transport inhibition incomplete in conditions of 0.5 mM ouabain, but also other energy requiring processes, such as urea and glucose production, were inhibited by ouabain.

iv. The Effect of Ouabain on ATP Content of Hepatocytes

Active transport of Na^+ and K^+ by the Na-K ATPase and glucose and urea production require energy in the form of ATP. Inhibition of these processes by ouabain would tend to decrease ATP utilization. At the same time, O_2 consumption is decreased by ouabain. This should result in similar ATP contents of cells with and without ouabain. This was found to be the case in experiments reported here and in those reported by van Rossum (1971).

Section 3. The Effect of Anaerobiosis and Cyanide on the Distribution of K^+ and Na^+ Between Hepatocytes and Medium

Introduction

The effects of anoxia and metabolic inhibitors such as cyanide have been examined from the point of view of membrane potential (Lambotte, 1970 and 1977, in perfused dog liver, and Heller & van der Kloot, 1974, in whole guinea pig liver), and from that of the relationships between energy metabolism and cation transport (see van Rossum (1976) in rat liver slices and isolated mitochondria). However, little has been done to establish the effects of anoxia and cyanide on isolated hepatocyte cation distribution. For this reason, although anaerobiosis and cyanide were primarily used in this thesis as tools in examining the mechanisms by which cell volume and cell ammonia content are maintained, it was also necessary to establish their effects on K^+ and Na^+ distribution in isolated hepatocytes. It was also of interest to compare effects of cyanide and anaerobiosis with those of ouabain.

Results

i. The Effect of Anaerobiosis on the Distribution of Na^+ and K^+ Between Cells and Medium

Hepatocytes incubated in the presence of phosphorus and gassed with 95% N_2 and 5% CO_2 lost intracellular K^+ and gained Na^+ throughout incubation (tables 3:8 and 3:9). During the first 20 minutes of incubation, the rate of loss of K^+ was in the order of 1.7 µmol/min/g dry wt. During the subsequent 20 minutes this rate of loss was increased to 1.9 µmol/min/g dry wt, and over the next 40 minutes the rate was linear and was in the order of 3.0 µmol/min/g dry wt. (table 3:8). The lower rate of K^+ loss during the first few minutes of incubation may be partly explained by the fact that a small amount of residual oxygen may be trapped in the cells initially, and this may support some respiration. The phosphorus 'fumed' for the first 10 minutes, which indicated that some oxygen was still present, despite gassing with nitrogen for 30 seconds before sealing the flasks. The rate of loss of K^+ is calculated with values expressed in µmol/g dry wt., since values expressed as mM take changes in cell volume into account and do not give a correct value for the number of ions lost from the cell. In anaerobiosis, cell volume increased steadily over the period 40-80 minutes, whereas the dry weight did not change during this time (see Chapter 4 for discussion of this). Sodium values for table 3:8 were inaccurate due to the use of Method II for separating cells from medium, and are thus omitted.

Table 3:9 gives the results of one experiment in which K^+, Na^+, inulin space, wet weight and dry weight were estimated. Cells were incubated anaerobically as described for the experiments shown in table 3:8. Parallel flasks were used for measurement of wet and dry weight. Table 3:9 shows that, as in the experiments of table 3:8, intracellular K^+ content decreased linearly during the second half of incubation. However, Na^+ increase was not as great as K^+ decrease, so that the total Na^+ plus K^+ decreased throughout anaerobic incubation.

Table 3:8. The effect of anaerobiosis on the distribution of potassium between hepatocytes and medium

Experimental details are described in Chapter 2 and the text. The values are means ± S.E.M., and the number of observations is in parenthesis. Anaerobiosis was attained by the addition of phosphorus placed in the centre well of 50 ml Erlenmeyer flasks and gassed with 95% N_2 and 5% CO_2. Control flasks (50 ml Erlenmeyer flasks without centre well) contained no phosphorus and were gassed with 95% O_2 and 5% CO_2.

Incubation (min)	K^+ inside µmol/g dry wt.		$[K^+]$ inside mM		$[K^+]$ outside mM	
	Control	Anaerobiosis	Control	Anaerobiosis	Control	Anaerobiosis
0	262 (1)	262 (1)	125	125	6.0	6.0
20	276 (2)	228 (2)	148	112	6.0	6.4
40	294 ± 6.6 (5)	191 ± 13.7 (5)	152 ± 2.8	96.8 ± 5.0	5.8 ± 0.09	6.6 ± 0.11
50	290 (1)	165 (1)	148	84.4	6.0	6.6
60	289 ± 8.3 (5)	133 ± 3.5 (5)	152 ± 4.4	64.9 ± 2.4	6.0 ± 0.03	6.7 ± 0.09
80	290 ± 6.7 (5)	72.3 ± 7.9 (5)	148 ± 3.4	34.4 ± 3.3	5.9 ± 0.05	7.0 ± 0.11
100	281 (1)	66.3 (1)	149	31.2	5.9	7.2

Table 3:9. The effect of anaerobiosis on the distribution of K^+ and Na^+ between hepatocytes and medium

Experimental details are described in Chapter 2 and the text. The values are from one experiment. Anaerobiosis was attained by addition of phosphorus placed in the centre well of 50 ml Erlenmeyer flasks and gassed with 95% N_2 and 5% CO_2. Control flasks (50 ml Erlenmeyer flasks without centre wells) contained no phosphorus and were gassed with 95% O_2 and 5% CO_2. Na^+ concentration outside was 145 mM. Con.= control, An.=anaerobiosis.

		Incubation (min)					
		0	10	20	40	60	80
K^+inside µmol/g dry wt.	Con.	262	262	278	274	270	285
	An.	262	236	218	167	138	89.1
$[K^+]$ in mM	Con.	142	142	150	148	146	146
	An.	142	125	115	88.1	65.0	42.0
$[K^+]$ out mM	Con.	6.1	6.0	6.1	5.9	6.0	6.0
	An.	6.1	6.2	6.4	6.7	6.9	7.1
Na^+ in µmol/g dry wt.	Con.	153	72.3	65.3	57.4	60.0	35.2
	An.	153	81.7	98.0	117	133	145
$[Na^+]$ in mM	Con.	53.5	39.0	35.3	31.0	30.7	18.0
	An.	53.5	43.1	51.8	62.0	62.7	68.3
$K^+ + Na^+$ µmol/g dry wt.	Con.	415	334	343	331	330	320
	An.	415	318	316	284	271	234
H_2O in % wet wt.	Con.	-	48	48	49	-	46
	An.	-	47	46	43	-	40
H_2O out % wet wt.	Con.	-	25	26	26	-	32
	An.	-	27	30	36	-	43

ii. The Effect of Cyanide on the Distribution of Na^+ and K^+ Between Cells and Medium

The effects of cyanide on K^+ content of hepatocytes incubated either in the presence of air or 5% CO_2 and 95% N_2 are compared in this section. In early experiments, air was used for incubation. However, it became clear that without the presence of 5% CO_2, the pH of the system was elevated, and this introduced a second variable into the experiments. In subsequent experiments, CO_2 was used and the results were very different.

The effect of cyanide (added as 1.0 mM HCN) incubated with cells in the presence of air is shown in table 3:10. Loss of K^+ from the cells occurred more rapidly than in the anaerobic experiments of the previous section (table 3:8), and those with cyanide in the presence of 5% CO_2 (table 3:11). The rate of K^+ loss was in the order of 10 µmol/min/g dry wt. When cyanide was used, cells not only increased their volume by 40% but also decreased their dry weight in the first 20 minutes of incubation. It is probable that cell lysis occurred. In these experiments, change in dry weight was taken into account in the calculation of µmol/g dry wt. After 40 minutes, $[K^+]$ inside cells treated with cyanide was nearly equal to that outside ($[K^+]$ inside = 8.3 mM, $[K^+]$ outside = 7.3 mM), taking into account changes in wet weight, dry weight and inulin space that occurred in these circumstances. However, since inulin space was probably inaccurate, concentration values are probably also inaccurate (see Chapter 4, section iii).

The effect of cyanide on K^+, Na^+ and water content of hepatocytes incubated in the presence of 5% CO_2 and 95% N_2 is shown in table 3:11. Nitrogen was used in these experiments to inhibit microsomal respiration, since van Rossum (1976) suggests that the 30% cyanide resistant respiration probably represents the activity of microsomal oxidases. Hepatocytes lost K^+ and gained Na^+ in a manner similar to that when treated anaerobically, except that total Na^+ plus K^+ content of cells treated

Table 3:10. The effect of incubation of hepatocytes with cyanide in the presence of air on potassium distribution between cells and medium

Experimental details are described in the Methods section. The values are means ± S.E.M., and the number of observations is four. Cyanide was added to appropriate flasks as HCN to an initial concentration of 1.0 mM. Control flasks contained no added substrates and were gassed with 95% O_2 and 5% CO_2. Flasks containing cyanide were incubated in the presence of air.

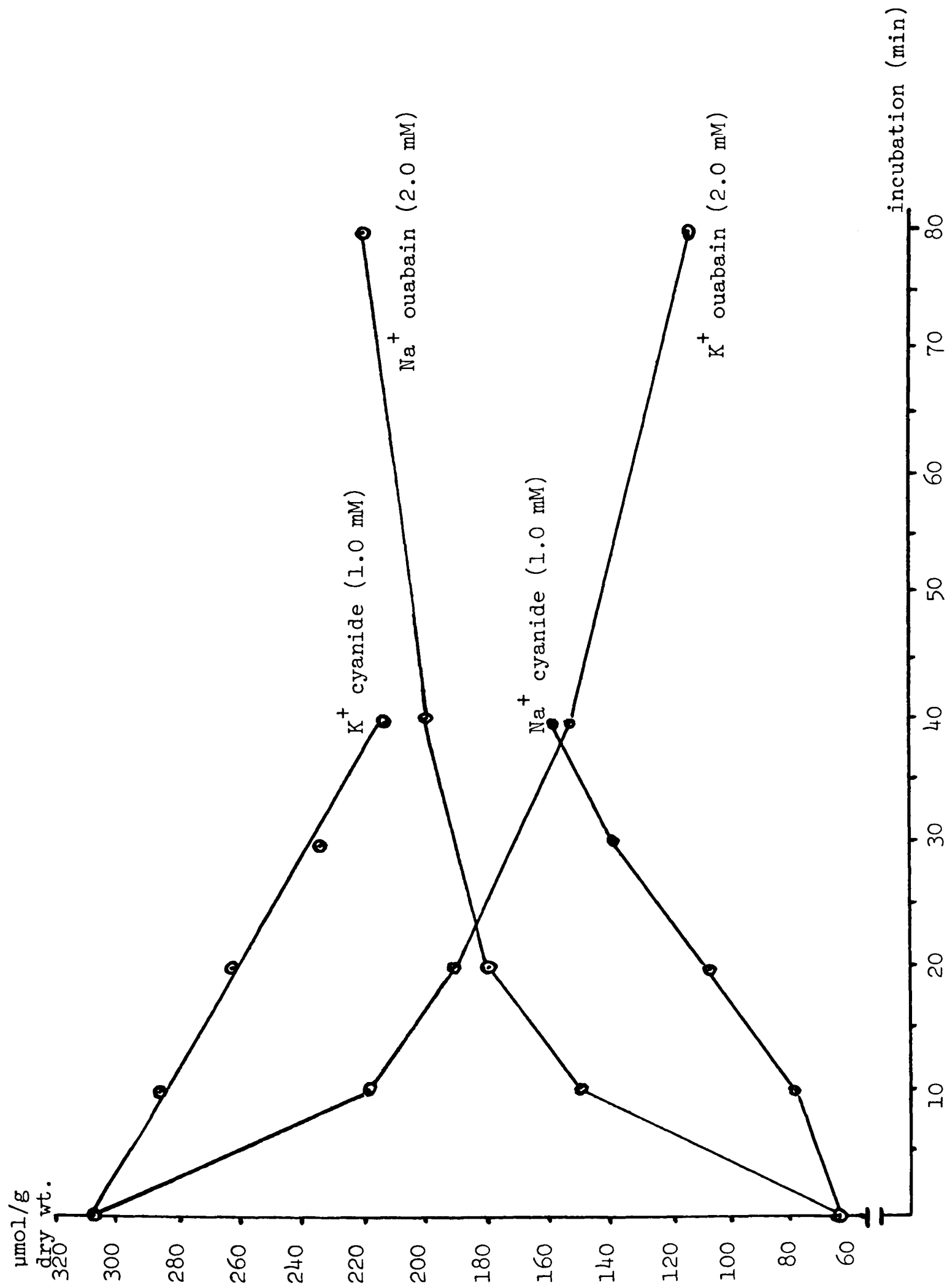
		Incubation (min) 20		40	
K^+ inside µmol/g dry wt.	Control	276	± 14.2	280	± 14.0
	Cyanide	57.2	± 7.9	28.2	± 2.0
$[K^+]$ inside mM	Control	137	± 11.5	136	± 9.7
	Cyanide	17.3	± 2.3	8.3	± 0.55
$[K^+]$ outside mM	Control	5.8	± 0.032	5.8	± 0.031
	Cyanide	7.0	± 0.16	7.3	± 0.15
$[K^+]$ ratio inside/ outside	Control	23.6		23.5	
	Cyanide	2.5		1.1	

Table 3:11. The effect of incubation of hepatocytes with cyanide in the presence of 5% CO_2 on K^+ and Na^+ distribution between cells and medium

Experimental details are described in the methods chapter. The values are means ± S.E.M., and the number of observations was three. Cyanide was added to the appropriate flasks as HCN to an initial concentration of 1.0 mM. Control flasks contained no added substrates and were gassed with 95% O_2 and 5% CO_2. Flasks containing cyanide were gassed with 95% N_2 and 5% CO_2.

		Incubation (min)			
		10	20	30	40
K^+ inside µmol/g dry wt.	Control	321 ± 10	318 ± 11	319 ± 9.1	318 ± 9.4
	Cyanide	287 ± 7.9	265 ± 10	235 ± 2.0	217 ± 6.9
$[K^+]$ inside mM	Control	165 ± 4.9	164 ± 4.2	165 ± 3.5	164 ± 5.8
	Cyanide	158 ± 5.0	126 ± 5.7	103 ± 5.6	82.3 ± 4.4
$[K^+]$ outside mM	Control	6.0 ± 0.04	5.9 ± 0.03	6.0 ± 0.02	6.0 ± 0.03
	Cyanide	6.0 ± 0.02	6.2 ± 0.04	6.4 ± 0.04	6.6 ± 0.04
$[K^+]$ ratio inside/ outside	Control	27.5	27.8	27.5	27.3
	Cyanide	26.3	20.3	16.1	12.5
Na^+ inside µmol/g dry wt.	Control	52.2 ± 3.7	47.3 ± 6.0	54.8 ± 4.7	49.4 ± 6.7
	Cyanide	75.5 ± 6.3	104 ± 2.7	137 ± 10	154 ± 12
$[Na^+]$ inside mM	Control	26.8 ± 1.4	24.3 ± 2.8	28.2 ± 2.0	25.3 ± 2.6
	Cyanide	38.9 ± 3.0	49.5 ± 3.2	59.4 ± 3.0	54.8 ± 4.8
$Na^+ + K^+$ µmol/g dry wt.	Control	373	365	374	367
	Cyanide	363	369	372	371
Wet wt./dry wt. ratio	Control	3.79	-	-	3.76
	Cyanide	3.80	4.01	4.31	4.83
H_2O inside as % of dry wt.	Control	191	189	186	189
	Cyanide	188	191	206	263

DIAGRAM 3:2. Effect of Ouabain and Cyanide on the K^+ and Na^+ Content of Hepatocytes



with cyanide remained relatively constant throughout incubation. Loss of K^+ was again linear, and was in the order of 2 to 3 µmol/min/g dry wt. during the 10-40 minute period. K^+ lost from the cells was accompanied by a stoichiometric gain (one for one) of Na^+.

Comparison of tables 3:10 and 3:11 shows that the rate of loss of K^+ from hepatocytes greatly increased when cells were incubated in air rather than with 5% CO_2, which suggests that pH increases are damaging to cell membranes.

Diagram 3:2 above shows the effect of ouabain and of cyanide on the K^+ and Na^+ content of hepatocytes. Ouabain values are derived from table 3:4 (above), but are normalized taking as 100% the control values of the cyanide experiments of table 3:11. During the first 20 minutes of incubation, the Na-K ATPase was clearly not totally inhibited in the presence of cyanide: the rate of loss of K^+ was only in the order of 2.5 µmol/min/g dry wt., whereas in the presence of ouabain, when the Na-K ATPase was totally inhibited, the rate was as great as 9 µmol/min/g dry wt.

Discussion

i. The Rate of Loss of K^+ and Gain of Na^+ by Hepatocytes During Anaerobiosis and During Treatment with Cyanide in the Presence of CO_2

Cyanide and anaerobiosis are thought to affect ion distribution by limiting the supply of ATP for active transport. Van Rossum (1972) has shown that ATP values have to decrease below 0.48 µmol/ml intracellular H_2O before Na^+ extrusion from cells is affected. Thus, in the experiments with cyanide in the presence of CO_2 and anaerobiosis in this chapter, it is likely that the residual ATP and ATP from the small amount of anaerobic ATP synthesis occurring in cells prepared from starved livers, was sufficient to allow some activity of the Na-K ATPase during the first 40 minutes of incubation. Cell volume changes in the presence of cyanide were not apparent until 30 minutes of

incubation. Both low rate of K^+ loss and slow increase in cell volume are in contrast to the findings of Seidman & Cascarano (1966), who suggest that anaerobic metabolism alone is insufficient to allow ion transport by rat liver slices. However, my findings are consistent with those of Macknight et al. (1974), who suggest that, in rat liver slices, energy derived from anaerobic metabolism could contribute to the maintainance of both cellular volume and cellular K^+.

The rate of K^+ loss and Na^+ gain by hepatocytes in the presence of cyanide and CO_2 was remarkably linear (diagram 3:2 above). This was perhaps the result not only of increasing inhibition of the Na-K ATPase as incubation proceeded, but also of changes in the permeability of the membrane to Na^+ and/or K^+. Lambotte (1977) found that, in hypoxic conditions, dog liver P_{Na}/P_K ratio was decreased and cell membrane potential increased. He suggested that changes in P_{Na}/P_K were related to the stretching of the cell membrane which resulted from volume increases during hypoxia.

Section 4. The Effect of Various Added Substrates on % K^+ Turnover, $[K^+]$, K^+ Content and Wet Weight to Dry Weight Ratios in Hepatocytes

Introduction

The aim of the work reported in this section was to demonstrate, concerning isolated hepatocytes, whether or not rates of metabolic processes such as glucose synthesis were linked to alterations in rates of K^+ turnover or K^+ content. In some cases, such as on addition of glutamine, the justifiability of applying the K^+ turnover calculation is questionable, since K^+ content was not in a steady state (K^+ influx did not equal K^+ efflux). In these circumstances, K^+ turnover values nonetheless give some indication of increased K^+ influx. In following chapters, conditions in which there was steady, or nearly steady, K^+ state were chosen for more detailed study. K^+ influx calculations using $^{42}K^+$ data depend on knowledge of cell surface area. Hepatocytes are not spherical but are covered with microvilli

(see plates in Chapter 4 section iii). The calculation of surface area could not be undertaken, and therefore this method of K^+ influx calculation was avoided.

Results and Discussion

Table 3:12 shows the effects of various concentrations of added metabolic substrates on the turnover of K^+ ($\frac{\mu mol\ K^+\ exchanged/min}{total\ tissue\ K^+} \times 100$): $[K^+]$ (mM); K^+ content (μmol/g dry wt.); and wet to dry weight ratios. Turnover was estimated with the addition of $^{42}K^+$ as described in Chapter 2 section 7. Throughout this section, numbers in braces refer to conditions given in table 3:12. For example, {6} refers to conditions of added alanine.

A. The Effects of Added Substrates on the Rates of K^+ Turnover in Hepatocytes

The results of table 3:12 can be categorized as follows (increases and decreases in turnover are given as % above and below the value (2.25 %/min) for no additions ({0})).

Increase in % turnover of K^+ occurred in hepatocytes on addition of:

{1} lactate (10 mM) (27% increase above {0})

{2} lactate (10 mM), NH_4Cl (10 mM), ornithine (1.0 mM), oleate (1.0 mM) (13% increase)

{3} lactate (10 mM), oleate (1.0 mM), lysine (2.0 mM) (15% increase)

{4} lactate (7.5 mM) NH_4Cl (10 mM), ethanol (10 mM) (56% increase)

{5} histidine (2.0 mM) (47% increase)

{6} alanine (10 mM) (89% increase)

{7} glutamine (10 mM) (64% increase)

{8} asparagine (10 mM) (146% increase)

Decrease in % turnover of K^+ occurred in hepatocytes on addition of:

{9} NH_4Cl (10 mM) (32% decrease)

{10} NH_4Cl (10 mM), lactate (2.0 mM), ornithine (1.0 mM), oleate (1.0 mM) (21% decrease)

{11} oleate (1.0 mM) (21% decrease)

Table 3:12. The effect of various added substrates on % K^+ turnover, K^+ content and wet wt. to dry wt. ratio in hepatocytes

Experimental details are described in the Methods section. Values are normalized. Cells were preincubated with added substrates for 20 min before the addition of $^{42}K^+$. NH_4Cl, lactate, dihydroxyacetone (DHA), alanine, glutamine, asparagine, ethanol and fructose were all added to initial concentrations of 10.0 mM unless stated. Ornithine and oleate were added to an initial concentration of 1.0 mM and histidine and lysine to 2.0 mM. Values are for 20 min after the addition of $^{42}K^+$.

	Conditions	K^+ turnover %/min	K^+ inside µmol/g dry wt.	$[K^+]$ inside	Wet wt./dry wt. ratio
{0}	No additions	2.25	287	152	3.77
{1}	Lactate	2.85	312	167	3.74
{2}	NH_4Cl, lactate, ornithine and oleate	2.54	320	168	3.80
{3}	Lactate, oleate and lysine	2.58	295	157	-
{4}	Ethanol, lactate (7.5 mM) and NH_4Cl	3.50	334	153	4.38
{5}	Histidine	3.31	327	165	3.96
{6}	Alanine	4.24	309	142	4.36
{7}	Glutamine	3.70	286	135	4.24
{8}	Asparagine	5.53	261	123	4.21
{9}	NH_4Cl	1.53	243	130	3.73
{10}	NH_4Cl, lactate (2.0 mM), ornithine and oleate	1.78	268	142	-
{11}	Oleate	1.77	288	156	3.70
{12}	Lactate (7.5 mM) and NH_4Cl	2.29	273	146	3.73
{13}	DHA	2.02	299	159	3.87
{14}	Ethanol	2.33	286	157	3.64
{15}	Histidine, ornithine, oleate and NH_4Cl	2.11	303	157	3.86
{16}	Fructose	2.05	299	158	3.78

No change in % turnover of K^+ occurred in hepatocytes on addition of:

{12} lactate (7.5 mM), NH_4Cl (10 mM)

{13} dihydroxyacetone (DHA) (10 mM)

{14} ethanol (10 mM)

{15} histidine (2.0 mM), ornithine (1.0 mM), oleate (1.0 mM), NH_4Cl (10 mM)

{16} fructose (10 mM)

B. Cell Volume Changes

In all cases where inulin space and wet and dry weights were measured in the experiments of table 3:12, inulin space was similar in all conditions despite alteration in wet weight. It can reasonably be assumed, therefore, that change in wet to dry weight ratio gives an indication of change in cell volume. Of the group of conditions in table 3:12 in which increased K^+ turnover occurred, cell volume increased on addition of the following:

{4} lactate, NH_4Cl, ethanol

{5} histidine

{6} alanine

{7} glutamine

{8} asparagine

In conditions where there was no change in K^+ turnover, cell volume was slightly increased on addition of:

{15} histidine, ornithine, oleate, NH_4Cl.

In all conditions in which cell volume changes occurred there was a concomitant accumulation of one or more amino acids. For example, in {4} aspartic acid accumulation occurred (see Chapter 4, Introduction, for an explanation of this). However, in conditions of table 3:12 where added substrates gave no accumulation of amino acids, no increase in cell volume occurred.

C. K^+ Content Changes

K^+ content varied in all conditions in which both cell volume

changed and K^+ turnover increased, except on addition of {7} (glutamine), when K^+ content (μmol/g dry wt.) was similar to that of {0}, despite increased cell volume and increased K^+ turnover. As a result, $[K^+]$ in {7} was lower than in {0}. In {8} (asparagine), K^+ content was lower than in {0}, and as cell volume had increased, $[K^+]$ was considerably lower than in {0}. In {6} (alanine), K^+ content was increased, but $[K^+]$ was lower than in {0}. In {1}(10 mM lactate), K^+ content was increased and, since no volume change occurred, $[K^+]$ was also increased above {0}. The same was probably true of pyruvate (not shown), but cells were of poor quality in these three experiments, so the results are omitted from table 3:12. In {5} (histidine), K^+ turnover, $[K^+]$, K^+ content and cell volume increased.

In {9} (NH_4Cl) and {10} (NH_4Cl, 2.0 mM lactate, ornithine and oleate), there was a decrease in K^+ turnover, K^+ content and $[K^+]$, while cell volume was unchanged. In {11} (oleate), cell volume and K^+ content were unchanged, but K^+ turnover decreased below {0}.

D. Relations Between K^+ Turnover, K^+ Content and Rate of Metabolic Processes

It is clear that no single factor, such as the processes of glucose or urea synthesis, can be responsible for changes in K^+ turnover. For example, the rate of glucose formation in {12} (NH_4Cl and lactate) was 1.31 μmol/min/g wet wt., as compared with 0.12 μmol/min/g wet wt. in {0}, while K^+ turnover was the same as in {0}. However, glucose formation in {1} (lactate) was 0.56 μmol/min/g wet wt., but K^+ turnover was 27% above {0}. Furthermore, glucose production in {2} (lactate, NH_4Cl, ornithine and oleate) was high, 1.95 μmol/min/g wet wt., but K^+ turnover was only 13% above {0}, which was less than in {1}. A similar lack of correlation between rates of glucose production and K^+ turnover is seen in {5} (histidine), when K^+ turnover was increased by 47% but glucose formation was the same as in {0}. By contrast, in {15} (histidine, ornithine, oleate and NH_4Cl), the rate was 1.33 μmol/min/g wet wt., but there was no change in the rate of K^+ turnover. Rates of glucose formation from amino acids, as in {6}

(alanine), {7} (glutamine) and {8} (asparagine) were low, 0.39, 0.36 and 0.49 µmol/min/g wet wt. respectively, yet rates of K^+ turnover were dramatically increased above {0}. By contrast, rates of glucose formation in {13} (DHA) were high (2.48 µmol/min/g wet wt.), yet K^+ turnover was similar to {0}. There was also a lack of correlation between rates of K^+ turnover and urea synthesis. The rate of urea formation in {2} (lactate, NH_4Cl, ornithine and oleate) was 5.40 µmol/min/g wet wt., and in {10} (2.0 mM lactate, NH_4Cl, ornithine and oleate) was 5.35 µmol/min/g wet wt.; yet in the former case the rate of K^+ turnover was slightly increased (by 13%), whereas in the latter it was decreased (by 21%). This last example is worth examining. The only difference between these two sets of conditions was that in {2} [lactate] was 10 mM whereas in {10} [lactate] was 2.0 mM. It is thus possible that addition of lactate itself was partly or wholly responsible for increase in K^+ turnover. Lactate is 99% negatively charged at physiological pH, and it is reasonable to suppose that addition of negative charge to the interior of the cells would lead to a redistribution of other ions, perhaps, in this case, K^+. Indeed, in most conditions where lactate was added in high concentrations (7.5 - 10 mM) ({1}, {2}, {3}, {4}). K^+ turnover and content were increased. NH_4Cl could produce a similar effect, except that, since ammonia is 99% positively charged at physiological pH, K^+ would move out of the cell instead of inwards. Following from this, it is not surprising that on addition of similar concentrations of lactate and NH_4Cl together ({12}), there was no change in rate of K^+ turnover, for in this case cationic ammonia taken into the cell would be balanced by anionic lactate. Parallel movements of K^+ and lactate in and out of muscle were also noted by Odashima (1932). Similarly, Gollwitzer-Meier (1925) recorded high plasma K^+ after NH_4Cl injection into man.

E. Conclusions

From the above discussion of table 3:12 a pattern emerges:

(a) K^+ turnover and content were increased on addition of large concentrations of lactate (7.5 - 10 mM). Lactate was not accumulated against a gradient (not shown) and cell volume was not increased.

(b) K^+ turnover and content were decreased when NH_4Cl (10 mM) was added to hepatocytes. $[Ammonia]_{inside}$ was only slightly greater than $[Ammonia]_{outside}$ (see Chapter 6) and cell volume was not increased.

(c) When NH_4Cl and lactate were added in the presence of other substrates, for example in {2} and {4}, these other substances may have exerted additional effects on K^+ content and/or K^+ turnover. For example, in {12}, lactate and NH_4Cl gave no increase in K^+ turnover, but in {4}, when ethanol was also present, K^+ turnover increased by 56%. Ethanol alone gave no such changes ({14}). However, as will be seen in Chapter 4, ethanol in the presence of lactate and NH_4Cl leads to a large intracellular accumulation of aspartic acid (see Stubbs & Krebs, 1975).

(d) K^+ content, K^+ turnover and cell volumes all altered in conditions where amino acids accumulated within the cells, whether these amino acids were generated within the cells, as in {4}, or whether they were accumulated by the cell from the medium, for example in {6} (alanine), {7} (glutamine) and {8} (asparagine). There may be at least two mechanisms affecting K^+ behaviour in these conditions. First, inward transport of certain neutral amino acids in many tissues is associated with inward movement of Na^+, which in turn will lead to redistribution of other intracellular ions (see Heinz, 1972). Secondly, large accumulations of amino acids within hepatocytes was associated with increases in cell volume, which in turn may increase the permeability of the membrane to K^+ and Na^+. Consistent with this hypothesis is the finding that

in all conditions with substantially increased cell volume, K^+ turnover was increased. Increased K^+ turnover would occur if P_K was increased and the Na-K ATPase was stimulated by decreased $[K^+]$ inside.

(e) Oleate presents a special case, since it gave decreased K^+ turnover whilst K^+ content and cell volume were unaltered. It is tempting to suggest that oleate may alter the permeability of the membrane to K^+. This would be consistent with the finding of Dambach & Friedmann (1974b) that the addition of palmitate (1.0 mM) led to increased membrane potential in perfused rat liver. However, this issue has yet to be investigated.

To sum up, I have presented data demonstrating that in the conditions described it was not possible to establish a relationship between rates of metabolic processes such as glucose synthesis and rates of K^+ turnover and content. K^+ turnover and content changes appear to be chiefly related to transport and accumulation of metabolic substrates within hepatocytes.

If increased K^+ turnover is accompanied by increased P_K (see above), then, assuming that P_{Na} is unchanged, increase in K^+ turnover will be concomitant with hyperpolarization of the cell membrane (P_{Na}/P_K will decrease). If this assumption is correct, the conclusions of Dambach & Friedmann (1974b) concerning the relationship between substrate induced metabolic processes and membrane hyperpolarization are in contrast to those above concerning relations between K^+ turnover and rates of metabolic processes. Dambach & Friedmann (1974b) found that, on addition of metabolic substrates such as lactate, pyruvate, fructose and alanine to perfused rat liver, hyperpolarization of the membrane occurred. Since no such hyperpolarization occurred in the presence of the non-metabolizable amino acid α-aminoisobutyric acid (AIB), they suggested that 'substrate induced hyperpolarization

may be the result of substrate metabolism rather than an effect correlated with substrate transport'. Two points arise here. First, though they did not show rates of glucose formation, in my data (see section D above) the rate of glucose formation from alanine is less than that from lactate. However, Dambach & Friedmann's experiments show that increase in membrane hyperpolarization on addition of alanine was two times greater than with lactate. This shows that Dambach & Freidmann's hypothesis should not be interpreted as linking rate of metabolic process with degree of hyperpolarization, but rather as the claim that some non-zero rate of metabolic process is sufficient for some non-zero degree of hyperpolarization. Secondly, the finding of Dambach & Friedmann that there was no increase in membrane hyperpolarization with AIB is surprising, since AIB is accumulated against a gradient by liver (Le Cam & Freychet, 1977); this should result in increased cell volume. In all circumstances in which I measured increased cell volume there was concomitant increase in K^+ turnover, and hence membrane hyperpolarization is likely to occur. The differences between my conclusions and those of Dambach & Friedmann cannot be resolved unless membrane potential and Na^+ and K^+ are measured within the same liver preparation in the presence and absence of AIB, lactate, pyruvate and alanine.

The results of table 3:12 were the starting point for work which is more fully developed in the following chapters of this thesis.

CHAPTER 4. FACTORS AFFECTING CELL VOLUME, AND RELATIONSHIPS BETWEEN CELL VOLUME AND K^+ AND Na^+ IN HEPATOCYTES

Introduction

An energy dependent water pump for maintenance of cell volume by extrusion of H_2O was postulated by Robinson (1950). However, it has now become established that increase in cell water content is accompanied by increase in solute. For example, in 1951 Mudge demonstrated that on inhibition of metabolism in rabbit kidney slices there was gain of isosmotic extracellular fluid. Thus an H_2O pump would not be sufficient to regulate cell volume.

There is now evidence to support the view that the volume of mammalian cells is determined by the balance between the influx of Na^+ down its electro-chemical gradient and the energy dependent extrusion of Na^+ from the cells (Woodbury, 1965). Originally, the cardiac glycoside sensitive Na-K ATPase was thought to be the Na^+ transport mechanism involved (Wilson, 1954). The pump was thought to prevent the continual entry of extracellular fluid into the cells which would otherwise occur as the result of the colloid osmotic force generated by intracellular macromolecules to which the cell membrane is impermeable (Leaf, 1956). However, work in kidney cortex slices (Kleinzeller & Knotková, 1964), and liver slices (Macknight et al., 1974), indicates that a ouabain insensitive, energy dependent Na^+ extrusion mechanism may exist. Russo et al. (1977) give evidence to suggest that liver slices have two energy dependent mechanisms operating to control volume: a ouabain sensitive and a ouabain insensitive mechanism. (Recent reviews on regulation of cell volume include Kleinzeller, 1972, and Macknight & Leaf, 1977.) In this thesis, it was of interest to ascertain whether isolated hepatocytes retained the ability to maintain cell volume in the presence of ouabain.

Cell swelling is known to occur in the absence of energy supply (see Robinson, 1950). For this reason, wet and dry weight, inulin

space and ion content were analysed in conditions of added cyanide and anaerobiosis. Electron microscopy was used to attempt to ascertain reasons for changes in inulin space seen in early stages of incubation of isolated hepatocytes in the presence of cyanide.

In feeding and starvation the liver accumulates and disposes of stores of metabolic fuels such as glycogen and amino acids. Accumulations of these metabolic substances are likely to lead to alterations in intracellular osmotic pressure. Indications that this was indeed the case were seen in Chapter 3 (table 3:12): for example, hepatocytes prepared from fed rats were shown to have increased wet weight to dry weight ratios. Accordingly, it was of interest to examine changes in cellular ion and water content in conditions of accumulated amino acids, such as alanine and aspartic acid. For example, according to Stubbs and Krebs (1975), additions of ethanol (10 mM), lactate (10 mM) and NH_4Cl (10 mM) to isolated hepatocytes led to large cytosolic accumulation of aspartic acid (50 µmol/60 mins/g wet wt.). The underlying metabolic events leading to this accumulation are summarized as follows. In conditions of gluconeogenesis from added lactate alone, glutamate and oxaloacetate are generated in the mitochondrial matrix. These in turn are transaminated in the matrix to form aspartic acid and α-oxoglutarate. Aspartic acid accompanied by an equimolar amount of α-oxoglutarate moves out of the matrix into the cytosol on the aspartic acid shuttle (Borst, 1963). A cytosolic aminotransferase reaction disposes of aspartic acid and α-oxoglutarate. However, when NH_4Cl is added to the system, NH_4^+ and NADH react with α-oxoglutarate in the matrix to form glutamate. Transamination of aspartic acid is limited by the supply of α-oxoglutarate, and aspartic acid is accumulated to a small degree. Formation of glutamate from NH_4^+, NADH, and α-oxoglutarate is limited by the supply of NADH. Hence when ethanol is added in addition to NH_4Cl and lactate, oxidation of ethanol and and acetaldehyde provides a source of NADH, and the formation of

glutamate from α-oxoglutarate and NH_4^+ is increased. A large accumulation of aspartic acid occurs.

Large increases in wet weight to dry weight ratio were seen on addition of any one of alanine, glutamine and asparagine to hepatocytes (see Chapter 3 table 3:12). According to Lund and Watford (1976), glutamine, when added to hepatocytes in concentrations above 2.0 mM, was rapidly accumulated against a concentration gradient. It was likely, therefore, that increase in osmotic pressure occurred in these circumstances. It was of interest to determine whether alanine was also accumulated against a concentration gradient and, if so, to relate changes in intracellular [alanine] to those of ion and water content.

Results

Table 4:1 shows that the wet weight to dry weight ratio was not constant in hepatocytes, but increased in the following circumstances:

1. In hepatocytes prepared from fed rats.
2. In cells in which an intracellular accumulation of amino acids had occurred against a concentration gradient, for example, on addition of alanine, glutamine, asparagine, histidine, and ethanol with lactate and NH_4Cl (aspartic acid accumulation).
3. When cells were subjected to anaerobiosis or were treated with cyanide.

In all but the last case above, inulin space (H_2O outside) did not vary between control and experimental conditions within any one experiment, so that increase in wet weight to dry weight ratio was directly related to increase in intracellular H_2O content. For example, from table 4:1, cells with no additions at 20 minutes of incubation had a wet weight to dry weight ratio of 3.77, which means that for every 1 g of dry cells the total wet weight will be 3.77 g. H_2O inside can be calculated thus:

Table 4:1. The effect of added substrates on the wet wt. to dry wt. ratios in hepatocytes

Experimental details are described in Chapter 2. Values are means ± S.E.M. and the number of observations is in parenthesis. Initial concentrations of added substrates are in parenthesis. Anaerobiosis was achieved by placing yellow phosphorus in the centre well of 50 ml Erlenmeyer flasks and gassing with 95% N_2 and 5% CO_2. Cyanide was added as HCN.

Conditions	Wet wt./dry wt. ratio during incubation (min)					
	0	20	30	40	60	80
No additions (starved rats)	3.62 ± 0.038 (12)	3.77 ± 0.042 (26)	3.64 ± 0.055 (13)	3.78 ± 0.023 (48)	3.82 ± 0.047 (23)	3.81 ± 0.095 (6)
No additions (fed rats)	-	3.93 ± 0.066 (7)	-	-	-	-
Lactate (10 mM)	-	3.74 ± 0.12 (3)	-	4.02 (2)	3.74 (2)	-
Lactate (20 mM)	3.57 ± 0.082 (3)	3.72 ± 0.068 (7)	3.78 (1)	-	-	-
NH_4Cl (10 mM)	-	3.60 (2)	3.73 ± 0.12 (4)	3.86 ± 0.049 (8)	3.98 ± 0.071 (4)	-
NH_4Cl (20 mM)	-	3.70 (1)	-	3.93 ± 0.056 (4)	-	-
Ethanol (10 mM)	-	3.64 (1)	3.50 ± 0.073 (3)	3.67 ± 0.092 (6)	3.96 ± 0.086 (4)	3.62 (1)
Lactate (7.5 mM) NH_4Cl (10 mM)	-	3.59 (1)	3.73 ± 0.076 (4)	3.82 ± 0.065 (4)	3.85 ± 0.074 (4)	3.68 (1)
Lactate (7.5 mM) Ethanol (10 mM) NH_4Cl (10 mM)	-	4.33 (1)	4.38 ± 0.18 (3)	4.56 ± 0.048 (5)	4.62 ± 0.16 (3)	4.07 (1)
Glutamine (10 mM)	-	-	4.24 ± 0.010 (4)	4.40 ± 0.049 (5)	4.55 ± 0.060 (5)	-
Alanine (5 mM)	-	4.18 ± 0.076 (5)	-	4.35 ± 0.038 (5)	4.05 (2)	-
Alanine (10 mM)	-	4.51 (2)	4.36 ± 0.062 (5)	4.47 ± 0.10 (7)	4.44 ± 0.14 (6)	-
Asparagine (10 mM)	-	-	4.21 ± 0.037 (7)	4.25 ± 0.054 (7)	4.23 ± 0.067 (7)	-
Histidine (2 mM)	-	-	3.86 ± 0.067 (6)	3.96 ± 0.052 (7)	4.08 ± 0.092 (6)	-
DHA (10 mM)	-	-	3.87 (1)	3.80 ± 0.057 (3)	3.89 (1)	-
Urea (5.0 mM)	-	-	3.81 (1)	3.82 ± 0.050 (5)	3.83 (1)	-
Oleate (1.0 mM)	-	-	3.26 (2)	3.51 (2)	-	-
Fructose (10 mM)	-	3.91 (1)	3.88 (2)	3.96 ± 0.102 (3)	3.88 (1)	-
Anaerobiosis	-	4.19 (1)	-	4.10 ± 0.28 (3)	5.00 ± 0.30 (5)	5.44 ± 0.40 (4)
Cyanide (1.0 mM) (incubated in air)	-	6.01 ± 0.14 (6)	-	6.48 ± 0.14 (5)	-	-
Cyanide (1.0 mM) (incubated with 5% CO_2)	-	4.03 ± 0.020 (3)	4.31 ± 0.011 (3)	4.84 ± 0.032 (3)	-	-
Ouabain (0.5 mM)	-	3.78 ± 0.073 (7)	-	3.87 ± 0.078 (7)	3.77 ± 0.052 (7)	-

(1) H_2O inside = {total wet weight - H_2O outside - dry weight}

1.79 g = { 3.77 g - 0.98 g - 1.0 g }

(For estimation of H_2O outside, see Chapter 2 sections 5A (c) and 5B (b).)

Cell swelling occurs under conditions of added alanine (10 mM) (table 4:1): at 20 minutes of incubation the wet weight to dry weight ratio had increased to 4.51:

(2) H_2O inside = {total wet weight - H_2O outside - dry weight}

2.35 g = { 4.51 g - 0.98 g - 1.0 g }

Thus there was a 31% increase in H_2O inside and a 20% increase in the total wet weight in example (2) as compared with (1).

Measurement of total wet weight to dry weight ratio did not give direct indication of increase in H_2O inside in the case of cells kept anaerobically, or treated with cyanide, because inulin space (H_2O outside), and also in some cases dry weight, were not constant. For this reason, data from these experiments will be given in full, whereas those from experiments with constant inulin space will be expressed as wet weight to dry weight ratios and % change in H_2O inside. The results given in table 4:1 will now be discussed in more detail.

i. Cell Volume in Hepatocytes Incubated Without Added Substrates

There was no significant difference in wet weight to dry weight ratio of isolated hepatocytes prepared from starved rats during incubation from 0 to 80 minutes. The mean value of all the means of the ratios from 0 to 80 minutes was 3.74 ±0.036, which is similar to that of 3.70 used by Krebs et al. (1974).

Wet weight to dry weight ratios in hepatocytes prepared from fed rats were significantly larger than those from starved rats ($P<0.05$). The value for wet weight to dry weight ratio (table 4:1) of 3.93 is similar to that for cells from fed rats found by Krebs et al. (1974) of 3.95. The higher ratio found in cells from fed rats may

be related to the increased glycogen content of these cells. Fenn (1939) suggested that glycogen cannot be deposited unless accompanied by appropriate amounts of K^+ and H_2O. However, intracellular K^+ did not increase in isolated hepatocytes prepared from fed rats in experiments reported in Chapter 3.

ii. The Effect of Ouabain on Hepatocyte Volume

Table 4:2 shows the effect of ouabain (0.5, 1.0 and 2.0 mM) on wet weight to dry weight ratios in hepatocytes at 20, 40 and 60 minutes. Values for ouabain treated cells are compared with values for cells without ouabain within the same experiments. There was no significant difference in inulin space between cells with and without ouabain at any time. For example, at 20 minutes of incubation inulin space with ouabain was 0.017 ±0.001 ml (S.E.M.) and without ouabain was 0.018 ±0.001 ml per 76.3 mg wet wt. cells, when N=4. There was no significant difference in wet weight to dry weight ratio, and concomitantly in cell volume, at any concentration or at any time. The distribution of Na^+ and K^+ was very different in ouabain treated cells and cells without ouabain by 60 minutes of incubation (see tables 3:3 and 3:4). Gain of Na^+ and loss of K^+ should theoretically, according to a double Donnan system, give rise to gain of chloride and cell water. Since no change in cell water was found with ouabain, this suggests that there is an underlying mechanism in addition to the Na-K ATPase which prevents net gain of water and solute.

iii. The Effect of Anaerobiosis on Cell Volume, Dry Weight and Inulin Space in Hepatocytes

Table 4:3 gives the values of wet weight, dry weight, wet weight to dry weight ratio, inulin space and calculated intracellular H_2O in cells incubated in the presence of phosphorus and gassed with 95% N_2 and 5% CO_2. At 20 and 40 minutes wet weight to dry weight ratios had increased in cells incubated anaerobically, and since dry weights were similar in control and anaerobic cells at this time, this increase

Table 4:2. The effect of ouabain on the wet wt. to dry wt. ratio in hepatocytes

Experimental details are described in Chapter 2 and the text. Values are means ± S.E.M. and the number of observations is in parenthesis. Ouabain was added to initial concentrations of 0.5, 1.0 and 2.0 mM as indicated. Values represent wet wt. to dry wt. ratios.

	Incubation (min)		
Ouabain	20	40	60
None	3.65 ± 0.048 (7)	3.80 ± 0.097 (7)	3.66 ± 0.040 (7)
0.5 mM	3.78 ± 0.073 (7)	3.87 ± 0.078 (7)	3.77 ± 0.032 (7)
None	3.62 (2)	3.84 ± 0.13 (4)	3.69 (2)
1.0 mM	3.58 (2)	3.89 ± 0.15 (4)	3.63 (2)
None	-	3.92 ± 0.039 (4)	-
2.0 mM	-	3.87 ± 0.082 (4)	-

Table 4:3. The effect of anaerobiosis on the wet wt. to dry wt. ratio, wet wt., dry wt. and inulin space in hepatocytes

Experimental details are described in Chapter 2 and the text. The values are means ± S.E.M. and the number of observations is in parenthesis. Separation of cells from medium was by Method I. Anaerobiosis was attained by placing yellow phosphorus in the centre well of 50 ml Erlenmeyer flasks and gassing with 95% N_2 and 5% CO_2. Control flasks (50 ml Erlenmeyer flasks without centre wells) contained no phosphorus and were gassed with 95% O_2 and 5% CO_2. Values for anaerobic cells that are significantly different from control cells by Student's *t*-test (paired) are indicated by * = $P < 0.05$

		Incubation (min)			
		20	40	60	80
Wet wt./dry wt. ratio	Control	3.84 (1)	3.82 ± 0.089 (3)	3.88 ± 0.20 (5)	4.11 ± 0.18 (4)
	Anaerobic	4.19 (1)	4.10 ± 0.28 (3)	5.00 ± 0.30* (5)	5.44 ± 0.40* (4)
Total wet wt. mg	Control	81.1 (1)	85.2 ± 1.57 (3)	72.1 ± 3.90 (5)	74.9 ± 4.12 (4)
	Anaerobic	82.2 (1)	90.3 ± 0.90 (3)	87.4 ± 4.16 (5)	88.6 ± 5.39 (4)
Dry wt. mg	Control	21.1 (1)	22.4 ± 0.88 (3)	18.8 ± 1.58 (5)	18.4 ± 1.60 (4)
	Anaerobic	19.6 (1)	22.2 ± 1.24 (3)	17.9 ± 1.74 (5)	16.6 ± 1.82 (4)
H_2O outside µl	Control	21.0 (1)	21.0 ± 0.00 (3)	21.6 ± 0.51 (5)	22.5 ± 0.87 (4)
	Anaerobic	25.0 (1)	26.7 ± 3.28* (3)	29.5 ± 1.96* (5)	37.0 ± 4.53* (4)
H_2O inside µl	Control	39.0 (1)	41.8 ± 0.75 (3)	31.7 ± 2.91 (5)	34.0 ± 3.36 (4)
	Anaerobic	37.6 (1)	40.8 ± 2.23 (3)	40.0 ± 3.94* (5)	35.0 ± 4.85 (4)

was due to increased total wet weight. Increase in wet weight appeared to be due entirely to increase in inulin space while intracellular volume was unchanged. Intracellular H_2O did not appear to increase significantly until 60 minutes ($P<0.05$, paired $\underline{t}$-test) in anaerobic cells. However, by 80 minutes H_2O inside appeared similar in control and anaerobic cells, and at this time the elevated wet weight to dry weight ratio in anaerobic cells appeared to be due to greatly increased inulin space. This raises the whole question of the validity of inulin as a marker of extracellular water, since it is unlikely that at 80 minutes anaerobic cells would have maintained cell volume to similar values as those cells treated with O_2. In these experiments, inulin was not incubated with the cells, but was mixed with cells for about 30 seconds. This suggests that the large apparent inulin space was not due to anaerobic cells taking up inulin by some endocytotic process, but rather that inulin space space in these circumstances represents water space inside damaged and leaky cells together with extracellular H_2O. Consistent with this hypothesis was the observation that when anaerobic cells were separated from medium in conical centrifuge tubes (Method I, see Chapter 2 section 5A) at 80 minutes a pale fluffy layer sedimented above a darker cell layer. This suggests that lysed and damaged cells were indeed present and may account for the large apparent inulin space of anaerobic cells.

Table 4:4 shows that the results of experiments in which anaerobiosis was achieved with cyanide (1.0 mM added as HCN) and 95% N_2 and 5% CO_2 were essentially similar to those in which cells were treated with 95% N_2 and 5% CO_2 and phosphorus (table 4:3). However, intracellular H_2O of cyanide treated cells increased significantly by 40 minutes of incubation ($P<0.01$), whereas that of cells with phosphorus (table 4:3) was similar to that of control cells at 40 minutes. This may be because cells treated with phosphorus contain a residual amount of oxygen which supports aerobic respiration during the

Table 4:4. The effect of cyanide on wet wt. to dry wt. ratio, wet wt., dry wt., inulin space and intracellular H_2O in hepatocytes

Experimental details are described in Chapter 2 and the text. Values are means ± S.E.M. and the number of observations is three. Cyanide was added as HCN to an initial concentration of 1.0 mM. Flasks containing cyanide were gassed with 5% CO_2 and 95% N_2 and flasks with no additions were gassed wtih 5% CO_2 and 95% O_2. Cells were preincubated for 20 min before addition of cyanide. Separation of cells from medium was by Method I. Values that are statistically different from controls by Student's t-test (paired) are indicated by * = $P < 0.05$ and ** = $P < 0.01$.

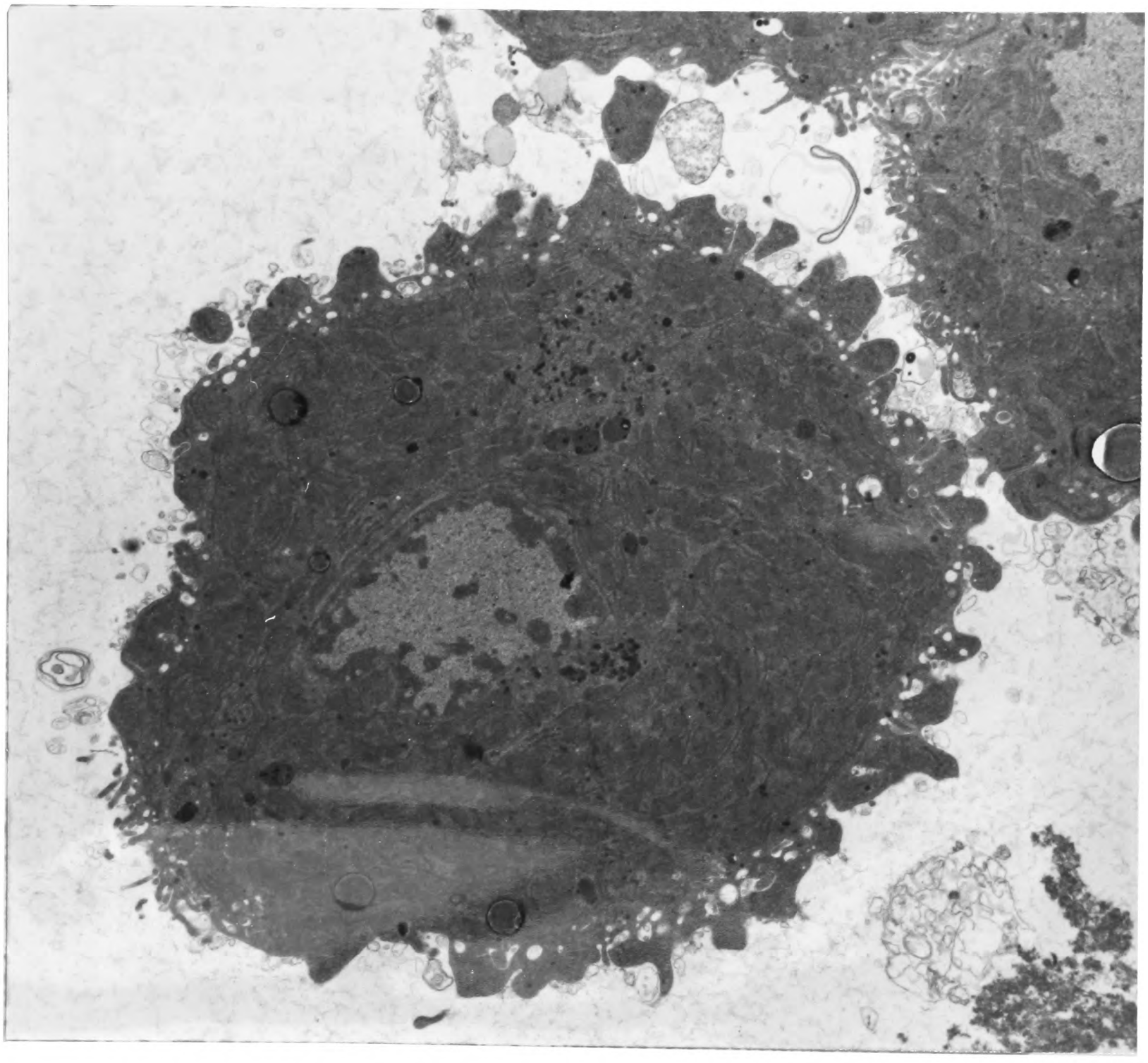
	Cyanide	Incubation (min) 10	20	30	40
Total wet wt. mg	None	73.1 ± 6.5	-	-	70.5 ± 6.1
	1.0 mM	75.1 ± 7.4	76.6 ± 5.8	82.6 ± 9.3	89.1 ± 8.7
Dry wt. mg	None	19.2 ± 1.7	-	-	18.8 ± 1.8
	1.0 mM	19.8 ± 2.0	19.0 ± 1.5	19.1 ± 2.0	18.4 ± 1.7
Wet wt./ dry wt. ratio	None	3.82 ± 0.09	-	-	3.76 ± 0.07
	1.0 mM	3.80 ± 0.02	4.03 ± 0.02	4.31 ± 0.01	4.84 ± 0.03**
Inulin space µl	None	17.7 ± 0.67	16.3 ± 0.67	16.7 ± 0.67	17.0 ± 0.58
	1.0 mM	18.3 ± 1.50	19.7 ± 1.20*	21.7 ± 1.70*	24.0 ± 1.20**
H_2O inside µl	None	36.2 ± 4.7	-	-	34.7 ± 4.9
	1.0 mM	37.0 ± 5.3	37.9 ± 4.1	41.8 ± 6.1	46.7 ± 6.0**

early stage of incubation, whereas cyanide inhibits the electron transport chain very rapidly. As was shown in Chapter 3 section 3, neither addition of cyanide nor treatment with phosphorus and 95% N_2 totally inhibited the Na-K ATPase during the first 20 minutes of incubation. If the Na-K ATPase operates during the initial 20 minute period of incubation, so may other active pumps, and these may help prevent increase in intracellular H_2O in cells treated anaerobically.

Inulin space in cyanide treated cells was significantly elevated by 20 minutes of incubation ($P<0.05$). In order to try to establish a reason for increased inulin space, electron micrographs were taken of cells with cyanide (plates 1a and 2a) and without cyanide (plates 1b and 2b) at 20 minutes of incubation.(details of the techniques used are described in Chapter 2 section 11). The cell membranes of cyanide treated cells had formed blebs in addition to microvilli, but otherwise looked intact. These blebs are similar to those reported by Trump et al. (1974) in rat kidney slices. They may well account for increased inulin space seen in cyanide treated cells at 20 minutes, for not only would carry down of adhering fluid be expected to increase in cells with blebs but also extracellular H_2O could be trapped between blebs after centrifugation.

When cells were treated with cyanide (1.0 mM added as HCN), but were incubated in air rather than with 5% CO_2 (see table 4:1), wet weight to dry weight ratio was increased to 6.01 ±0.14 (S.E.M. when N=6) by 20 minutes of incubation. Inulin space of cyanide and air treated cells was 2.5 times that of control cells at this time. Furthermore, by 40 minutes of incubation dry weight in cells with cyanide had decreased by 20% from control values. This indicates that elevated pH of the incubation medium greatly increased rate of swelling, and probably also of lysis.

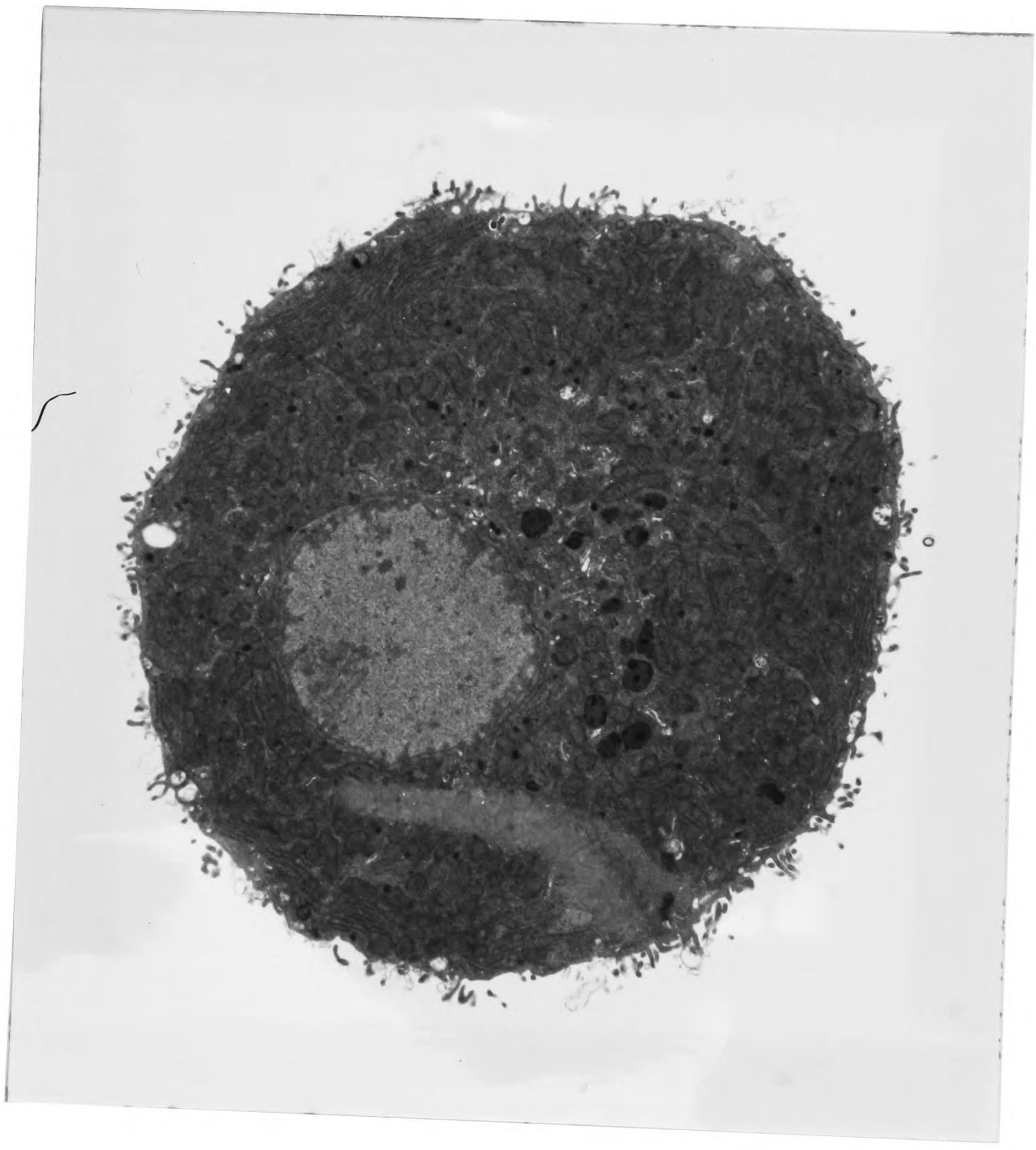
PLATE 1a. Micrograph of an Isolated Hepatocyte Incubated with Cyanide (1.0 mM) in the Presence of 5% CO_2 and 95% N_2



mag: × 7,800

For experimental details see Chapter 4 section iii and Chapter 2 section 11.

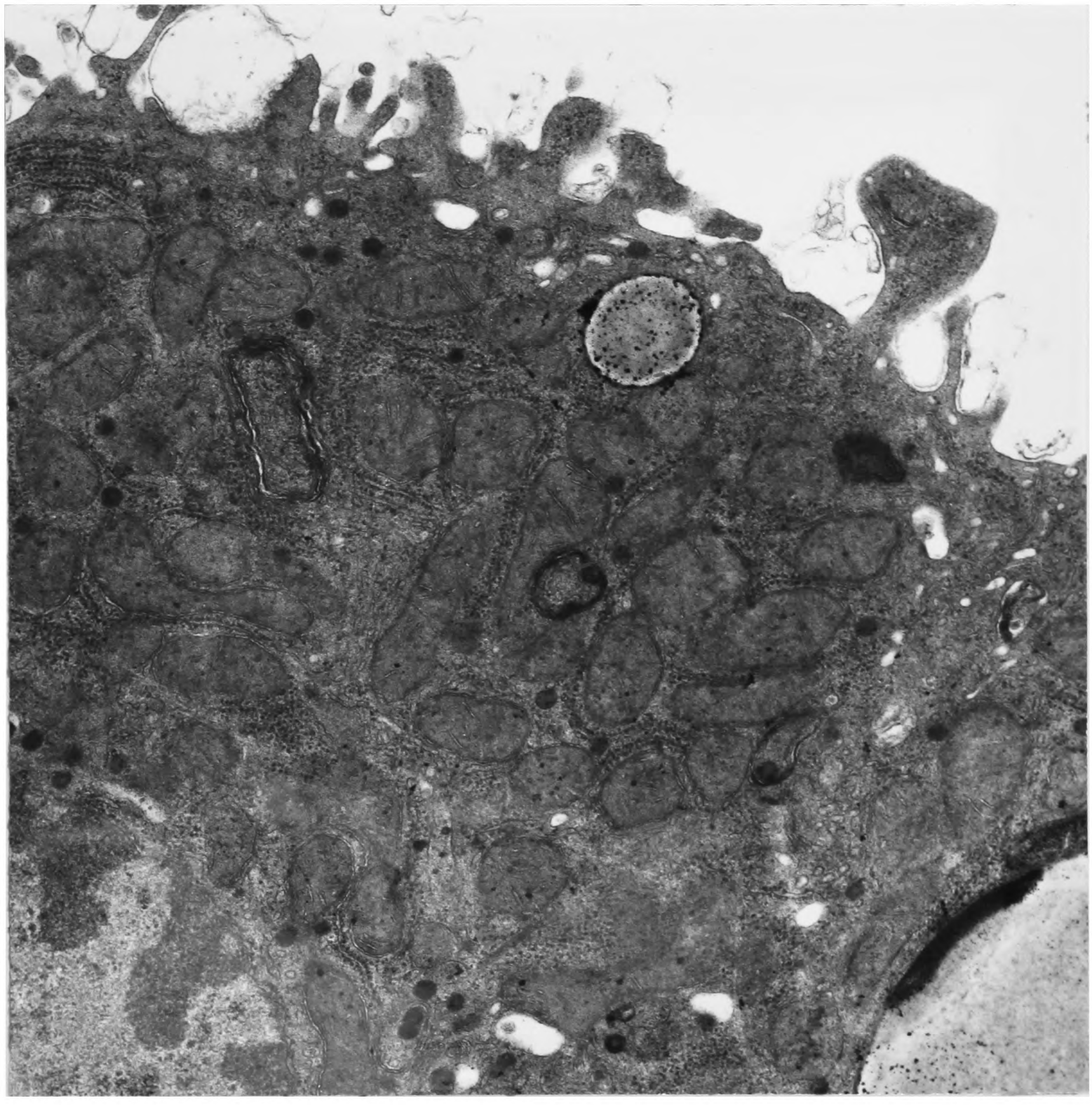
PLATE 1b. Micrograph of an Isolated Hepatocyte Incubated Without Additions in the Presence of 5% CO_2 and 95% O_2



mag: × 7,800

For experimental details see Chapter 4 section iii and Chapter 2 section 11.

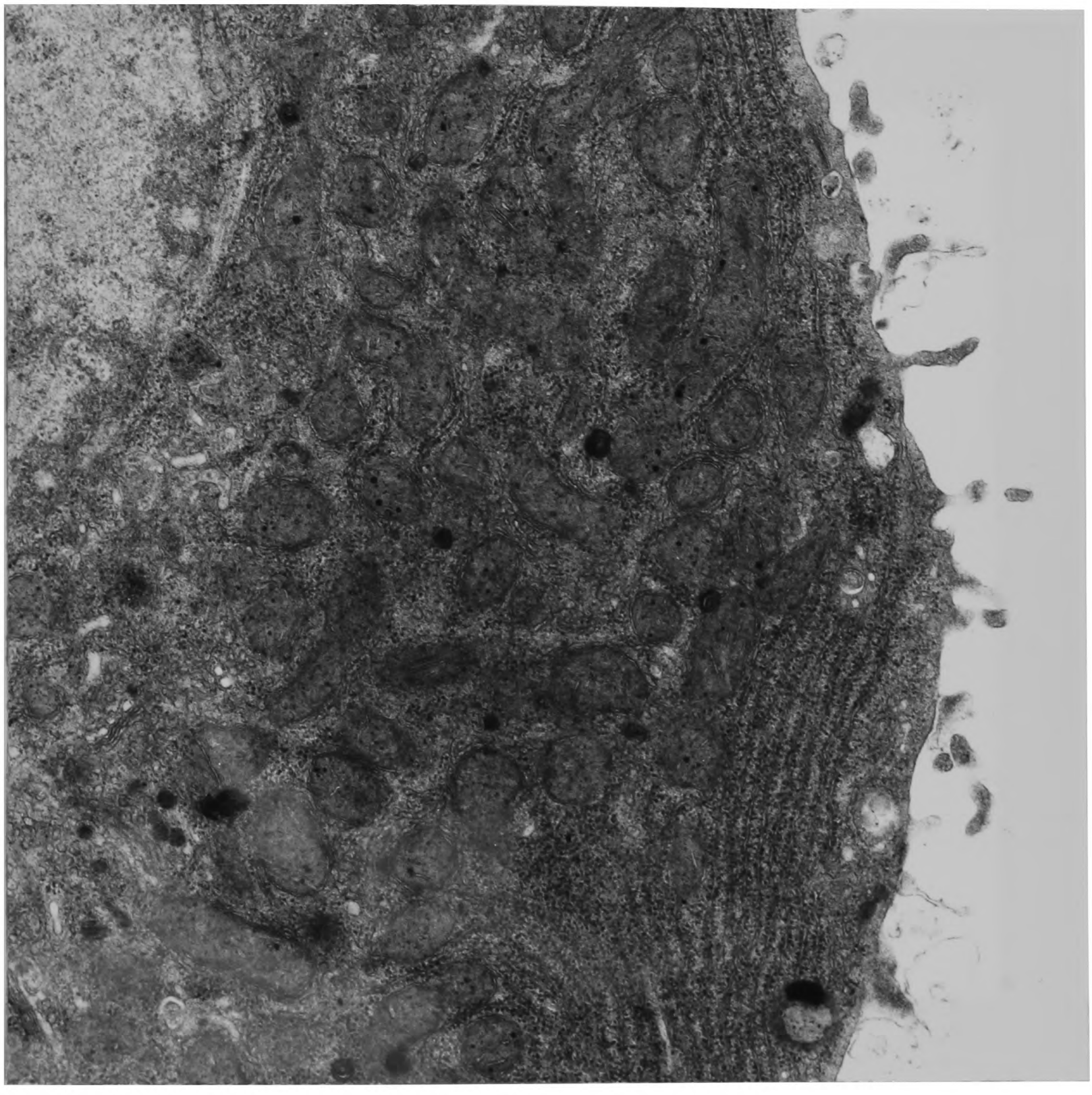
PLATE 2a. Micrograph of an Isolated Hepatocyte Incubated With Cyanide (1.0 mM) in the Presence of 5% CO_2 and 95% N_2



mag: × 33,000

For experimental details see Chapter 4 section iii and Chapter 2 section 11.

PLATE 2b. Micrograph of an Isolated Hepatocyte Incubated Without Additions in the Presence of 5% CO_2 and 95% O_2



mag: × 33,000

For experimental details see Chapter 4 section iii and Chapter 2 section 11.

iv. The Effect of Added NH_4Cl, Lactate and Urea on Cell Volume

NH_4Cl (10 and 20 mM), lactate (10 and 20 mM) and urea (5 mM) had no significant effect on the wet weight to dry weight ratio at any time during incubation (table 4:1). Since inulin space and dry weight were not altered in cells treated with these substances, it can be assumed that cell volume was not altered. When added in these concentrations, neither NH_4Cl, lactate nor urea were concentrated within the cell against a gradient to any great extent: for example, at 20 minutes the NH_4Cl ratio was 1.65 (from table 6:4), and 40 minutes the lactate ratio was 0.96 (from table 2:3) and at 40 minutes the urea ratio was 0.86 (table 2:3).

v. The Effect of Ethanol with NH_4Cl and Lactate, NH_4Cl with Lactate, and Ethanol on Cell Volume in Hepatocytes

The underlying mechanism by which aspartic acid was accumulated in conditions of added NH_4Cl with lactate, and of ethanol with NH_4Cl and lactate, was described in the introduction to this chapter. In this section, tables 4:5, 4:6, 4:7 and 4:8 are discussed. Table 4:5 shows that at 30 minutes a small accumulation of aspartic acid, 7.1 µmol/g dry wt., occurred on addition of NH_4Cl (10 mM) with lactate (7.5 mM), but that this accumulation had decreased to 3.1 µmol/g dry wt. at 80 minutes. Table 4:5 also demonstrates the magnitude of aspartic acid accumulation on addition of ethanol (10 mM), NH_4Cl (10 mM) and lactate (7.5 mM), since at 30 and 80 minutes aspartic acid content was 123 and 155 µmol/g dry wt. respectively, as compared with 0.91 and 1.29 µmol/g dry wt. in cells without additions. Table 4:6 shows that only in conditions of large aspartic acid accumulation (with added ethanol, NH_4Cl and lactate) were there significant increases in wet weight to dry weight ratio at 30, 40 and 60 minutes ($P<0.01$ at all times). Inulin space was similar with and without accumulation of aspartic acid (see table 4:8 for inulin space values from one

Table 4:5. The effect of ethanol, lactate and NH_4Cl, and ethanol with lactate and NH_4Cl on aspartic acid content of hepatocytes

Experimental details are described in Chapter 2 and the text. Values are means ± S.E.M. and the number of observations is in parenthesis. Ethanol and NH_4Cl were added to initial concentrations of 10.0 mM and lactate to 7.5 mM. Values that are statistically different from those with no additions by Student's t-test are indicated by * = $P < 0.05$ and ** = $P < 0.01$.

	Conditions	Incubation (min) 30	40	60	80
Aspartic acid inside umol/g dry wt.	No additions	0.91 (1)	-	-	1.29 ± 0.19 (4)
	NH_4Cl; lactate	7.05 ± 0.36 (3)	5.60 ± 0.62 (3)	5.09 ± 0.16 (3)	3.11* ± 0.11 (3)
	Ethanol	-	-	-	1.45 ± 0.07 (3)
	Ethanol; NH_4Cl; lactate	123 ± 15.3 (4)	150 ± 9.1 (3)	162 ± 7.4 (4)	155** ± 14.8 (4)
[Aspartic acid] inside mM	No additions	0.30 (1)	-	-	0.70 ± 0.10 (4)
	NH_4Cl; lactate	3.85 ± 0.18 (3)	3.05 ± 0.32 (3)	2.78 ± 0.07 (3)	1.70* ± 0.05 (3)
	Ethanol	-	-	-	0.30 ± 0.04 (3)
	Ethanol; NH_4Cl; lactate	54.9 ± 6.58 (4)	66.7 ± 4.2 (3)	72.4 ± 3.2 (4)	68.9** ± 6.2 (4)
[Aspartic acid] outside mM	No additions	-	-	-	< 0.010
	NH_4Cl; lactate	0.010 (2)	0.014 (2)	0.013 (2)	0.018 (2)
	Ethanol	-	-	-	< 0.010
	Ethanol; NH_4Cl; lactate	0.070 ± 0.01 (1)	0.11 ± 0.01 (1)	0.26 ± 0.02 (1)	0.37 ± 0.03 (1)

Table 4:6. The effect of ethanol, lactate and NH_4Cl and ethanol with lactate and NH_4Cl on wet wt. to dry wt. ratio in hepatocytes

Experimental details are described in Chapter 2 and the text. Values are means ± S.E.M. and the number of observations is in parenthesis. Ethanol and NH_4Cl were added to initial concentrations of 10 mM and lactate to 7.5 mM. Values represent wet wt./dry wt. ratios. Values that are statistically significant from those in conditions of no additions by Student's t-test are indicated by ** = $P < 0.01$.

	Incubation (min)			
	30	40	60	80
No additions	3.59 ± 0.076 (4)	3.66 ± 0.068 (4)	3.67 ± 0.093 (3)	3.40 (1)
Lactate; NH_4Cl	3.73 ± 0.076 (4)	3.82 ± 0.065 (4)	3.85 ± 0.074 (4)	3.68 (1)
Ethanol	3.50 ± 0.073 (3)	3.67 ± 0.092 (6)	3.96 ± 0.086 (4)	3.62 (1)
Ethanol; NH_4Cl; lactate	4.38** ± 0.18 (3)	4.56** ± 0.048 (5)	4.62** ± 0.16 (3)	4.07 (1)

experiment), and dry weight increases were insignificant (not shown). Thus in conditions of aspartic acid accumulation there was a direct relationship between increase in wet weight to dry weight ratio and increased intracellular H_2O, such that cell water was 47% and 54% greater than in cells without aspartic acid accumulation at 30 minutes and 60 minutes respectively. Small increases in wet weight to dry weight ratio occurred in conditions of small accumulation of aspartic acid (with NH_4Cl and lactate), whereas on addition of ethanol alone there was neither increased wet weight to dry weight ratio nor increased aspartic acid accumulation. Table 4:8 shows one experiment in which ouabain (1.0 mM) was added in the presence and absence of ethanol (10 mM), NH_4Cl (10 mM) and lactate (10 mM). Intracellular [aspartic acid] in the presence of ouabain was slightly decreased and so was the wet weight to dry weight ratio as compared with when ouabain was absent.

Intracellular accumulation of aspartic acid may affect the ion content of cells in at least two ways:

1. Aspartic acid is chiefly negatively charged at cellular pH (β-carboxyl group pK_a = 3.86, Mahler & Cordes, 1971, p 45), and thus increased [aspartic acid] inside will require movement of stoichiometric quantities of counter ion to maintain intracellular ion balance.
2. If intracellular aspartic acid is stored in an osmotically active form, it will increase intracellular osmotic activity, which will lead to influx of isosmotic extracellular fluid, and hence to increased cell volume.

It therefore seemed important to examine the Na^+ and K^+ content of cells with increased intracellular aspartic acid content. Table 4:7 shows that in conditions of aspartic acid accumulation (with added ethanol, NH_4Cl and lactate), K^+ content (μmol/g dry wt.) increased significantly throughout incubation ($P<0.05$, paired *t*-test), so that

Table 4:7. The effect of ethanol, lactate and NH_4Cl and ethanol with lactate and NH_4Cl on the K^+ content of hepatocytes

Experimental details are described in Chapter 2 and the text. Values are means ± S.E.M. and the number of observations is in parenthesis. Ethanol and NH_4Cl were added to an initial concentration of 10 mM and lactate to 7.5 mM. $^{42}K^+$ was added after 20 min of incubation with substrates for estimation of percent tissue K^+ turned over/min. Values that are statistically different from those with no additions by Student's t-test (paired) are indicated by * = $P < 0.05$ and ** = $P < 0.01$.

	Conditions		Incubation (min) 30		40		60		30	
K^+ inside umol/g dry wt.	No additions	313	± 10.8	(5)	307	± 12.1 (6)	301	± 13.2 (5)	301	± 11.9 (5)
	NH_4Cl; lactate	300	± 9.0	(3)	293	(2)	290	± 8.9 (3)	284	± 9.2 (3)
	Ethanol	311	± 5.7	(3)	305	± 2.3 (3)	303	± 6.3 (3)	302	± 3.3 (3)
	Ethanol; NH_4Cl; lactate	337	± 13.3	(5)	355	± 13.8* (6)	353	± 15.2* (5)	361	± 18.1* (5)
$[K^+]$ inside mM	No additions	170	± 6.2	(5)	167	± 6.6 (6)	163	± 7.0 (5)	163	± 6.3 (5)
	NH_4Cl; lactate	162	± 5.9	(3)	160	(2)	158	± 3.6 (3)	156	± 5.7 (3)
	Ethanol	169	± 4.2	(3)	166	± 1.8 (3)	164	± 3.7 (3)	163	± 2.4 (3)
	Ethanol; NH_4Cl; lactate	149	± 5.1**	(5)	151	± 10.9 (6)	156	± 6.4 (5)	160	± 7.9 (5)
% Tissue K^+ turned over/min	No additions	2.25	± 0.32	(5)	2.24	± 0.21 (6)	2.09	± 0.25 (5)	-	
	NH_4Cl; lactate	2.55		(2)	2.29	(2)	2.36	(2)	-	
	Ethanol	2.60		(2)	2.38	(2)	2.60	(2)	-	
	Ethanol; NH_4Cl; lactate	3.74	± 0.07**	(3)	3.39	± 0.18** (6)	4.07	± 0.1** (5)	-	

Table 4:8. The effect of ethanol, NH_4Cl and lactate in the presence and absence of ouabain on Na^+, K^+, aspartic acid content, wet wt. to dry wt. ratio and inulin space in hepatocytes

Experimental details are described in Chapter 2 and the text. The values are derived from means of pairs of observations in one experiment. Ethanol, NH_4Cl and lactate were added to initial concentrations of 10 mM and ouabain to 1.0 mM. Incubation time was 40 min.

	Conditions			
	No additions	Ethanol; NH_4Cl; lactate	Ethanol; NH_4Cl; lactate; ouabain	Ouabain
Na^+ µmol/g dry wt.	47.1	75.8	198	176
$[Na^+]$	25.7	29.1	84.7	96.1
K^+ µmol/g dry wt.	280	319	121	135
$[K^+]$	154	122	51.9	73.9
$K^+ + Na^+$ µmol/g dry wt.	327	395	319	311
Aspartic acid inside µmol/g dry wt.	-	122	80.5	-
[Aspartic acid] inside	-	46.5	34.5	-
[Aspartic acid] outside	-	0.06	0.09	-
Inulin space µl/15.7 mg dry wt. cells	13.5	14.5	13.5	13.5
Wet wt./dry wt. ratio	3.72	4.56	4.22	3.63
H_2O inside µl/15.7 mg dry wt. cells	29.2	41.4	37.1	27.8

it was 24 and 60 μmol/g dry wt. greater than in cells without additions at 3(and 80 minutes respectively. However, taking increased cell volume into account, $[K^+]$ inside was not increased by aspartic acid accumulation. Indeed, at 30 minutes $[K^+]$ was significantly decreased ($P<0.05$, paired *t*-test) below values from cells with no additions. K^+ content and $[K^+]$ were not significantly altered by addition of NH_4Cl with lactate or with ethanol alone as compared with cells with no additions. K^+ turnover was measured by addition of $^{42}K^+$ to cells which had previously been incubated with ethanol with NH_4Cl and lactate for 20 minutes. Since a steady state system is required for % turnover measurements (see Chapter 2 section 7), the 40 and 60 minute values on table 4:7 are the most accurate. At these times (i.e. 20 and 40 minutes after addition of $^{42}K^+$), the percentage of tissue K^+ turned over per minute was significantly increased ($P<0.01$) in cells with large aspartic acid accumulation, as compared with those with no additions. K^+ turnover was only slightly increased both in conditions of small accumulation of aspartic acid (NH_4Cl with lactate) and with ethanol alone.

Table 4:8 shows that Na^+ content increased in addition to K^+ in cells with accumulation of aspartic acid on addition of ethanol (10 mM) with NH_4Cl (10 mM) and lactate (10 mM). The total Na^+ plus K^+ (μmol/g dry wt.) was increased from 327 μmol/g dry wt. without additions to 395 μmol/g dry wt. with ethanol, NH_4Cl and lactate. The 68 μmol/g dry wt. Na^+ plus K^+ increase in intracellular positive charge in conditions of aspartic acid accumulation did not balance the 122 μmol/g dry wt. aspartic acid negative charge. There are at least two possible reasons for this:

1. The negative charge activity of accumulated intracellular aspartic acid is less than predicted from aspartic acid content values.

2. Intracellular aspartic acid accumulation is accompanied by outward movement of intracellular anion, for example, Cl^- or HCO_3^-.

Since Cl^- and HCO_3^- measurements have yet to be made, it is not possible to pursue this problem further.

The effect of ouabain (1.0 mM) on the Na^+ and K^+ content of cells with and without aspartic acid accumulation is seem on table 4:8. The Na^+ content was increased and the K^+ content decreased in cells with large accumulations of aspartic acid in the presence of ouabain, as compared with cells with ouabain alone. This is consistent with the finding that aspartic acid accumulation is accompanied by an increase in K^+ turnover (increased K^+ influx and efflux). Thus with ouabain, cells with aspartic acid accumulation lost net cell K^+ at a greater rate than those without aspartic acid accumulation. However, in all cells with aspartic acid accumulation and ouabain the Na-K ATPase was inhibited and, as a result, K^+ influx was not concomitantly increased to compensate for increased K^+ loss, as was probably the case when aspartic acid accumulation occurred in cells without ouabain. One surprising finding with ouabain was that the total Na^+ plus K^+ values with and without aspartic acid accumulation were similar (319 and 311 µmol/g dry wt. respectively), whereas without ouabain, total Na^+ plus K^+ values were 395 and 327 µmol/g dry wt. for cells with and without aspartic acid accumulation respectively. Therefore with ouabain, apparently neither K^+ nor Na^+ were involved in balancing the 80 µmol/g dry wt. aspartic acid negative charge accumulated by cells. It may be relevant to note here that, in the presence of ouabain, total Na^+ plus K^+ content in cells without added substrates was lower than in cells without ouabain (Chapter 3 section 2). Hence this is a second instance of increased unidentified cation in the presence of ouabain.

vi. The Effect of Added Alanine on Cell Volume in Hepatocytes

Table 4:9 shows that when alanine (5.0 mM) was added to the suspension medium, alanine was accumulated within the cells to a concentration of 24 mM: concomitantly, there was an increase in wet weight to dry weight ratio as compared with cells without alanine. Inulin space was similar with and without alanine, so that increase in wet weight to dry weight ratio can be attributed to increase in intracellular water content. At 60 minutes of incubation, wet weight to dry weight ratio in cells with alanine had decreased from the 40 minute value of 4.35 to 4.05. At the same time, alanine content of the cells had decreased from 61.6 µmol/g dry wt. at 40 minutes to 50.8 µmol/g dry wt. at 60 minutes. Change in cell volume meant that [alanine] inside remained relatively constant throughout incubation, despite change in alanine content (µmol/g dry wt.). Alanine is converted into glucose and urea during incubation, and neither of these compounds is accumulated within the cell: hence intracellular alanine is likely to be the chief osmotically active substance in these experiments. The rate of conversion of alanine to glucose and urea equalled the rate of entry of alanine into the cell at 20 and 40 minutes, but exceeded it by 60 minutes. Table 4:1 shows that on addition of glutamine (10 mM) to cells, there was an increase in wet weight to dry weight ratio throughout incubation. The difference between the effects of glutamine and alanine on wet weight to dry weight ratio may lie in the fact that some of the glutamine is converted into glutamate, which is in turn accumulated by cells (see Lund & Watford, 1976), whereas the products of alanine metabolism are not accumulated. Hence on addition of glutamine, intracellular osmotic activity continues to increase during incubation, and cell volume increases to counteract this stress.

Alanine, unlike aspartic acid, is a neutral amino acid, and so its

Table 4:9. The effect of alanine on wet wt. to dry wt. ratios, inulin space and Na^+ and K^+ content of hepatocytes

Experimental details are described in Chapter 2 and the text. Values are means ± S.E.M. and the number of observations is in parenthesis. The initial concentration of alanine was 5.0 mM. Values with alanine that are statistically different from those without alanine by Student's t-test (paired) are indicated by * = $P < 0.05$ and ** = $P < 0.01$.

	Alanine	Incubation (min) 20	40	60
Wet wt./dry wt. ratio	None	3.80 ± 0.040 (5)	3.67 ± 0.042 (5)	3.78 (2)
	5.0 mM	4.18 ± 0.076** (5)	4.35 ± 0.038** (5)	4.05 (2)
Inulin space % of dry wt.	None	96% (3)	98% (3)	104% (2)
	5.0 mM	94% (3)	94% (3)	102% (2)
Alanine inside µmol/g dry wt.	5.0 mM	59.4 ± 3.1 (5)	61.6 ± 1.08 (5)	50.8 (2)
[Alanine] inside mM	5.0 mM	23.9 ± 1.75 (5)	24.9 ± 1.06 (5)	23.20 (2)
K^+ inside µmol/g dry wt.	None	351 ± 12.8 (5)	303 ± 10.9 (5)	270 (2)
	5.0 mM	330 ± 14.2* (5)	335 ± 11.8* (5)	305 (2)
$[K^+]$ inside mM	None	159 ± 7.1 (5)	155 ± 7.8 (5)	164 (2)
	5.0 mM	132 ± 5.1* (5)	138 ± 6.7* (5)	140 (2)
Na^+ inside µmol/g dry wt.	None	61.0 (1)	55.8 (1)	-
	5.0 mM	59.7 (1)	70.0 (1)	-
$[Na^+]$ inside mM	None	28.7 (1)	26.9 (1)	-
	5.0 mM	22.2 (1)	26.1 (1)	-
$Na^+ + K^+$ µmol/g dry wt.	None	376	359	-
	5.0 mM	390	405	-

accumulation will not affect the ion balance of the cell directly. However, as will be discussed in detail in Chapter 5, the transport machanism of alanine is associated with movements of Na^+ and K^+. Thus both transport and osmotic changes will affect ion content in hepatocytes incubated with alanine. Table 4:9 shows that K^+ content was slightly elevated in the presence of alanine at 20 minutes as compared with cells with no additions. Na^+ content was similar with and without alanine. By 40 minutes, Na^+ and K^+ content were increased in cells with alanine such that Na^+ plus K^+ content was 46 µmol/g dry wt. above control values. It is of interest to note that increase of Na^+ influx into cells with alanine occurs not only as a result of influx of extracellular fluid in response to osmotic effects, but also as part of the alanine transport mechanism (see Chapter 5). Thus in order to maintain $[Na^+]$ values similar to control values, as was seen with alanine (table 4:9), increased Na^+ efflux must also occur.

Discussion

i. The Effect of Anaerobiosis on Cell Volume

Experiments with cyanide and anaerobiosis raise two main points. First, the validity of the inulin method of extracellular fluid volume estimation in isolated hepatocytes is questionable in conditions where aerobic respiration is limited. Estimation of wet and dry weights include weights of lysed cells. Thus measurement of wet and dry weight and inulin space does not give an accurate indication of the intracellular volume of individual cells in cases where cells vary in intactness. In experiments reported here, use of the above technique for estimation of cell volume may well be justified for the first 40 minutes of incubation with cyanide or with phosphorus and N_2 in the presence of 5% CO_2, but after this time another method will have to be sought. Morphometric methods of estimation of cell volume are notoriously difficult. The implication of this problem of cell water estimation

in anaerobic cells is that calculation of intracellular concentrations of solutes is inaccurate in these circumstances.

The second main problem raised by the experiments with cyanide is that despite inhibition of aerobic respiration cells maintained cell volume for at least 20 minutes, despite substantial increase in intracellular Na^+ content at this time. This indicates that ion pumps can operate initially in cells from starved animals. The energy sources are probably residual stores of ATP, and ATP from anaerobic respiration. Since gluconeogenesis and urea synthesis in cells treated with cyanide are grossly inhibited at 20 minutes of incubation, it appears that plasma membrane ion pumps are preferentially using ATP. Steps of gluconeogenesis and urea synthesis which require ATP occur chiefly in the mitochondria. Thus the regulatory factor controlling ATP supply to cell membrane transport systems, rather than gluconeogenesis or urea synthesis, may be the compartmentation of ATP. ATP from anaerobic sources is generated in the cytosolic compartment. Furthermore, there is increasing evidence to suggest that glycolytic enzymes may be associated with cell membranes (see review by Clarke & Masters, 1976), and thus ATP from glycolysis may be directly supplied to cell membrane pumps.

ii. The Effects of Ouabain on Cell Volume

The experiments reported here establish that a mechanism for control of cell volume distinct from the Na-K ATPase exists in isolated hepatocytes. Chiefly on the basis of electronmicroscopy, Russo et al. (1977) suggest that an Na-K ATPase independent volume control mechanism operates by formation of small vesicles in the Golgi region and subsequent extrusion of the contents of these vesicles by exocytosis into the canalicular lumen. This bulk transport mechanism for the removal of Na^+, Cl^- and H_2O would require energy, and so would be inhibited in conditions of anaerobiosis. Isolated hepatocytes do not appear to have specialized borders, such as those adjacent to the canaliculi and

those opposite the space of Disse in whole liver. Nevertheless, they appear able to regulate cell volume in the presence of ouabain. The next question to answer, with electron microscope techniques, is whether isolated hepatocytes also form secretory vesicles as do those in whole liver, and, if so, whether extrusion of the content of these vesicles takes place in any particular region of the cell membrane.

When sufficient energy supplies are available, hepatocytes appear to control cell volume in conditions where there is an increase in intracellular Na^+ (with Cl^-), i.e. in conditions of added ouabain. However, normally functioning hepatocytes can undergo cell volume increases, as can be seen from the experiments in which amino acids accumulated.

iii. The Effect of Intracellular Accumulations of Amino Acids on Cell Volume

In all experiments in which amino acids were accumulated against a concentration gradient there was an increase in cell volume. In conditions where the amino acid accumulation increased and then decreased during incubation, cell volume also increased and then decreased, as was the case on addition of 5.0 mM alanine to hepatocytes. From all the evidence presented, it is likely that volume increase, in cells with accumulation of amino acids, is in direct response to osmotic activity of the intracellular fluid.

Cell volume changes in hepatocytes treated with alanine or ethanol, with lactate and NH_4Cl, have two distinct components: the initial stage in which additional fluid is taken up by the cells; and the steady state stage in which cell volume is neither increasing nor descreasing but remains at an elevated level.

During the initial stage, influx of extracellular fluid containing high $[Na^+]$ and low $[K^+]$ will tend to decrease intracellular $[K^+]$ and elevate intracellular $[Na^+]$, which in turn will stimulate the Na-K ATPase. Intracellular Na^+ will be exchanged for extracellular K^+.

The net effect of increase in cell water will therefore be to increase the rate of K^+ influx and K^+ content. The importance of the Na-K ATPase in maintaining low intracellular Na^+ values was demonstrated by experiments with ouabain in conditions of aspartic acid accumulation, when intracellular Na^+ values were elevated above those with ouabain alone.

In the steady state stage of cell volume change in cells with aspartic acid accumulation, $^{42}K^+$ experiments showed that K^+ turnover was elevated. This suggests that the permeability of the cell to K^+ was increased, and K^+ loss was compensated by increased K^+ uptake. Since in this case increased K^+ uptake was not related to net influx of extracellular fluid (water movement was in a steady state), some other mechanism must exist. Lambotte (1977) suggests that, in liver cells with increased volume, permeability of the membrane to ions such as K^+ increases as a consequence of stretching of the cell membrane by increased volume. Conditions other than those of accumulated amino acids have been reported in which K^+ permeability and cell volume increases occur concomitantly. For example, Mazet et al. (1974) found that cells incubated in 56 mM or 112 mM K^+ swelled and K^+ permeability increased. If the prediction that increase in cell volume is always accompanied by increase in K^+ permeability holds, then cells from fed animals should show increased rate of K^+ turnover. This has not yet been investigated. It would also be of interest to see if decrease in cell volume is accompanied by decrease in K^+ permeability and decrease in Na-K ATPase activity. Addition of an osmotically active but non-permeant substance such as polyethylene glycol (mol wt. 6,000) to the cell medium in conditions of amino acid accumulation should lead to decrease in cell volume but would still allow accumulation of intracellular amino acids, since increased osmotic activity occuring as a result of amino acid accumulation would be balanced by increased osmotic activity of the medium. Measurement of cell volume in

conjunction with estimation of ion content of cells and rate of turnover of K^+ should answer this question. The use of aspartic acid accumulation on addition of ethanol with lactate and NH_4Cl is of value for such experiments, since transport of amino acids across the plasma membrane is not involved. Furthermore, during the 40 to 80 minute period of incubation in these conditions, K^+ and cell water are in a relatively steady state, thus allowing investigation of K^+ and Na^+ isotope movements. In further investigations of the hepatocyte response to volume changes during accumulation of amino acids, chloride estimations will be of considerable value in ascertaining the ionic balance sheet.

iv. General Consideration Concerning Change in Hepatocyte Volume

Results presented in this chapter show the need to consider cell volume when investigating changes in concentrations of intracellular solutes. This point has often been ignored. For example, Williamson et al. (1967) based calculations of the Redox state in rat liver on the explicit assumption that µmol/g wet wt. of tissue was equal to µmol/ml or mM. This assumption would be valid only if cell water volume was constant in all conditions used in the experiments.

CHAPTER 5. FACTORS AFFECTING THE ACCUMULATION OF ALANINE BY HEPATOCYTES

Introduction

A considerable amount of work has been done on the mechanism by which neutral amino acids are actively accumulated by such tissues as gut, kidney cortex, erythrocytes, brain and ascites tumours in mice (for reviews, see Schultz & Curran, 1970; Heinz, 1972). Less is known about the accumulation of neutral amino acids by liver, because, as will be discussed shortly, some liver preparations, such as slices and perfusions, are less suitable for such studies. However, indications that liver also accumulates amino acids come from Riggs & Walker (1963) and Sanders & Riggs (1967), who showed that the neutral amino acid analogue α-aminoisobutyric acid (AIB) was accumulated *in vivo*. Later, Tews & Harper (1969a) showed that AIB was concentrated by rat liver slices and by weanling rat liver slices (1969b). These findings were in contrast to those of Neame (1962), who found that there was little or no active transport of amino acids into liver. Isolated hepatocytes are potentially a more suitable preparation for measuring intracellular and extracellular content of amino acids and ions in the investigation into factors affecting amino acid accumulation and transport, for at least three reasons: first, hepatocytes maintain a K^+ and Na^+ content similar to that found *in vivo*; secondly, the preparation is composed almost entirely of parenchymal cells, unlike the slice preparation which also contains Kuppfer and blood cells; thirdly, hepatocytes can be separated relatively easily from the incubation medium to allow estimation of intracellular and extracellular content of metabolites and ions. Workers who have used isolated hepatocytes for study of amino acid uptake have reported conflicting findings. For example, Schreiber & Schreiber (1972, 1973), Ayala and Canonico (1975) and Kletzien et al. (1976) have stated that freshly prepared isolated hepatocytes do not accumulate amino acids. By contrast,

Jefferson (1976) and Le Cam & Freychet (1977) found that isolated hepatocytes can accumulate such amino acids as AIB. Owing to the disparity between reports, it was of interest to attempt a further investigation of the problem. Previous work in this laboratory indicated that isolated hepatocytes concentrated the neutral amino acid glutamine (Lund & Watford, 1976).

Preliminary work on factors affecting rate of K^+ turnover in hepatocytes (see Chapter 3 section 4) indicated that alanine, when added to the medium, led to an increase in K^+ turnover and in cell volume. Both these findings suggested that alanine was accumulated by hepatocytes in these circumstances, since increase in K^+ turnover may well have been related to influx of alanine by an Na^+ dependent mechanism as was defined by Oxender & Christensen (1963), whilst increase in volume may have been related to an accumulation of osmotically active amino acid as reported by Christensen et al. (1952).

Alanine is metabolized at a considerable rate in liver, and is thought to be a major gluconeogenic substrate during starvation (see Ishikawa, 1972). A rapidly metabolized amino acid is not suitable for kinetic analysis of amino acid transport in liver. For this reason, I do not attempt a kinetic study of alanine transport, but confine the work to factors affecting intracellular content of alanine

Le Cam & Freychet (1977), on the other hand, have attempted a kinetic analysis using AIB in place of alanine. Since they suggest that AIB and alanine are thought to use either a similar or the same transport system in liver, their findings are of interest here. They demonstrated that rat liver has an 'A' type neutral amino acid system for AIB, as defined by Oxender & Christensen (1963) in Ehrlich cells. This system is dependent on extracellular $[Na^+]$ and is sensitive to pH in such a way that inhibition occurs when pH decreases. In addition, metabolic inhibitors decrease accumulation of AIB. They also suggest that ouabain

has little or no effect on this transport system, which is surprising since numerous workers have reported inhibition of neutral amino acid accumulation in the presence of ouabain (for discussion, see review by Schultz & Curran, 1970, pp 677-681).

In this chapter, I intend to show not only that alanine accumulation occurs, but also that this accumulation is modified by ouabain and insulin. Furthermore, I examine the underlying Na^+ and K^+ distribution changes which occur on incubation of alanine with and without insulin, and relate these changes to the hypothesis that energization of alanine transport by the 'A' system is linked to the activity of the Na-K ATPase. The reader is referred to the definition of 'X ratio' in the table of Definitions and Abbreviations.

Results

i. The Accumulation of Alanine by Hepatocytes

Alanine, in concentrations ranging from 0.63 to 10.0 mM, was added to the suspension medium of cells which had been preincubated for 20 minutes (table 5:1).

On addition of 10.0 mM alanine, intracellular [alanine] was 27.5 mM after 10 minutes of incubation. At this time, extracellular [alanine] was 9.44 mM. Thus at 10 minutes the alanine ratio was 2.91. During the subsequent 50 minutes of incubation, intracellular [alanine] increased whilst extracellular [alanine] decreased, so giving rise to an increasing alanine ratio. The alanine removed from the medium is accounted for not only by alanine accumulated as such by the cells, but also by alanine metabolism, whose products are chiefly glucose and urea. The rate of removal of alanine during the 10 to 60 minute period was in the order of 8.0 µmol/min/g dry wt.

On addition of 5.0 mM alanine to the medium, the results were substantially the same as with 10 mM alanine, with the following differences. Intracellular [alanine] increased for the first 40 minutes, but by 60 minutes was decreasing. However, [alanine] in the medium was decreasing

Table 5:1. The effect of different concentrations of alanine in the suspension medium on the alanine content of hepatocytes

Experimental details are described in Chapter 2 and the text. Values, derived from three similar experiments, are means. The number of observations is in parenthesis. Cells were preincubated for 20 min before the addition of alanine. Alanine was added to the medium to give initial concentrations of 0.63 to 10.0 mM. Intracellular alanine concentrations are corrected for tissue water. The ratio is the intracellular concentration (mM) divided by the extracellular concentration (mM).

Incubation (min)	Alanine mM	Alanine added to the medium (mM) 10.00	5.00	2.50	1.25	0.63
10	Inside	27.5 (1)	17.4 (1)	5.07 (1)	2.00 (1)	0.77 (1)
	Outside	9.44	4.76	2.29	1.26	0.57
	Ratio	2.91	3.66	2.21	1.59	1.35
20	Inside	46.5 (2)	19.65 (3)	6.98 (2)	1.56 (1)	0.73 (1)
	Outside	9.68	4.58	1.79	0.96	0.47
	Ratio	4.80	4.29	3.90	1.63	1.55
40	Inside	59.9 (2)	26.2 (2)	9.69 (1)	-	-
	Outside	8.30	3.71	1.36	-	-
	Ratio	7.21	7.06	7.13	-	-
60	Inside	63.9 (2)	23.2 (2)	4.37 (1)	-	-
	Outside	7.14	3.10	0.92	-	-
	Ratio	8.95	7.48	4.75	-	-

at a greater rate than in the cells, so that the alanine ratio increased throughout incubation, and was similar to that found on addition of 10 mM alanine. The rate of removal of alanine from the medium was in the order of 6.4 µmol/min/g dry wt. during the 10 to 60 minute period.

On addition of 2.5 mM alanine, intracellular [alanine] reached a maximum at 40 minutes and decreased markedly by 60 minutes, so that despite decreased extracellular [alanine] the alanine ratio had fallen by 60 minutes. Thus 2.5 mM alanine maintained similar ratios to those found with 10.0 and 5.0 mM alanine for the first 40 minutes. The rate of removal of alanine (2.5 mM) was 8.61 µmol/min/g dry wt. between 10 and 20 minutes.

On addition of lower [alanine], large alanine ratios were not established but intracellular [alanine] was maintained, so that alanine ratios increased slightly despite decreases in extracellular [alanine]. The rate of removal of alanine from the medium in the 10 to 20 minute period was 1.92 and 5.76 µmol/min/g dry wt. for 0.63 and 1.25 mM alanine respectively.

Increasing extracellular [alanine] from 1.25 mM to 5.0 mM did not affect the rate of glucose formation which was in the order of 2.0 µmol/min/g dry wt. in all cases.

ii. The Effect of Alanine on the K^+ and Na^+ Content of Hepatocytes

This section includes discussion of results from tables 5:2, 5:3, 5:4 and 5:5. Table 5:2 shows that the K^+ content in hepatocytes (µmol/g dry wt.) increased with increasing [alanine] in the medium. However, intracellular water was increased on incubation with alanine, so that intracellular [K^+] (mM) was lower in all conditions of added alanine than in cells without alanine. A similar pattern of increased K^+ content and decreased [K^+] was seen in the experiments reported in table 5:4, when cells incubated with alanine (5.0 mM) were compared with cells without alanine. (The experiment of table 5:2 gave higher [K^+] values because in this case the cells contained less intracellular

Table 5:2. The effect of alanine on the K^+ content of hepatocytes

Experimental details are described in Chapter 2 and the text. Values are from one experiment. Cells were preincubated for 20 min before the addition of alanine. Alanine was added to initial concentrations of 5.0, 2.50, 1.25 and 0.63 as appropriate. The values of H_2O inside were derived from measurements of wet and dry wt. and inulin space, as described in Chapter 2. Cells were incubated with alanine for 10 min in all cases. Dry wt. was 16.8 mg/flask.

Alanine mM	K^+ inside µmol/g dry wt.	$[K^+]$ inside mM	H_2O inside µl/flask
5.0	322	153	35.4
2.5	314	157	33.7
1.25	300	157	32.1
0.63	302	159	32.1
None	295	173	28.7

Table 5:3. The effect of insulin on the alanine content of hepatocytes

Experimental details are described in Chapter 2 and the text. Values are means ± S.E.M. The number of observations is five. Cells were preincubated for 20 min before the addition of alanine and insulin. Alanine was added to an initial concentration of 5.0 mM to all flasks and 0.25 units of insulin were added to 4 ml suspension where appropriate. Values with insulin that are statistically different from those without by Student's t-test (paired) are indicated by * = $P < 0.05$ and ** = $P < 0.01$.

	Addition	Incubation (min) 20	30	40
Alanine inside µmol/g dry wt.	None	59.4 ± 3.10	61.6 ± 3.03	61.6 ± 1.08
	Insulin	66.7 ± 3.99*	72.3 ± 2.57**	70.1 ± 2.22**
[Alanine] inside mM	None	23.9 ± 1.75	24.6 ± 0.97	24.9 ± 1.06
	Insulin	27.7 ± 0.79*	30.3 ± 1.22**	29:5 ± 1.59**
[Alanine] outside mM	None	4.14 ± 0.16	3.79 ± 0.13	3.43 ± 0.13
	Insulin	4.10 ± 0.15	3.82 ± 0.16	3.45 ± 0.17
Ratio [Alanine] inside/ [Alanine] outside	None	5.85 ± 0.63	6.54 ± 0.38	7.27 ± 0.51
	Insulin	6.80 ± 0.31*	7.83 ± 0.21**	8.59 ± 0.57**
Glucose µmol/g dry wt.	None	41.5 ± 2.4	59.5 ± 5.6	74.1 ± 4.8
	Insulin	42.0 ± 2.2	57.7 ± 5.2	73.5 ± 4.4
Urea µmol/g dry wt.	None	52.3 ± 8.0	73.5 ± 8.3	112 ± 12.7
	Insulin	48.0 ± 5.1	72.4 ± 7.7	110 ± 14.5

Table 5:4. The effect of insulin on the potassium and sodium content of hepatocytes in the presence of alanine

Experimental details are described in Chapter 2 and the text. Values are means ± S.E.M. The number of observations for K^+ values is five and for Na^+ is two. Cells were preincubated for 20 min before addition of alanine and insulin. Alanine was added to all flasks to an initial concentration of 5.0 mM and 0.25 units of insulin were added to 4.0 ml suspension where appropriate. Values with insulin and alanine which are different from those with alanine alone by Student's t-test (paired) are indicated * = $P < 0.05$

	Additions	Incubation (min) 20	30	40
K^+ inside µmol/g dry wt.	None	315 ± 12.8	308 ± 9.1	303 ± 10.9
	Alanine	330 ± 14.2	336 ± 9.3	335 ± 11.8
	Alanine + insulin	327 ± 10.7	341 ± 12.2	343 ± 12.4*
$[K^+]$ inside mM	None	159 ± 7.1	158 ± 6.6	155 ± 7.8
	Alanine	132 ± 8.6	135 ± 7.3	134 ± 6.3
	Alanine + insulin	131 ± 5.1	137 ± 5.4	138 ± 6.7*
$[K^+]$ outside mM	None	6.3 ± 0.16	6.3 ± 0.21	6.3 ± 0.12
	Alanine	6.2 ± 0.10	6.2 ± 0.08	6.2 ± 0.07
	Alanine + insulin	6.2 ± 0.08	6.2 ± 0.10	6.2 ± 0.11
Na^+ inside µmol/g dry wt.	None	61.0	63.2	55.8
	Alanine	59.7	68.0	70.0
	Alanine + insulin	66.3	58.2	56.7
$[Na^+]$ inside mM	None	28.7	29.8	26.9
	Alanine	22.2	25.3	26.1
	Alanine + insulin	24.7	21.7	21.1
$K^+ + Na^+$ µmol/g dry wt.	None	376	371	359
	Alanine	390	404	405
	Alanine + insulin	393	399	400

H_2O than they did in other experiments.) Table 5:4 also shows that Na^+ content (μmol/g dry wt.) was similar in conditions of added alanine at 20 minutes, but that during the following 20 minutes of incubation Na^+ content increased when alanine was present. Thus at 20 minutes $[Na^+]$ was decreased in conditions of added alanine, but by 40 minutes $[Na^+]$ was similar with and without alanine. In these conditions, Na^+ and K^+ content increases do not occur simultaneously.

Table 5:5 shows that in the presence of alanine (5.0 mM) the % of tissue K^+ turned over per minute was greatly increased as compared with that observed when no additions were made (4.8% as compared with 2.2% after 10 minutes of incubation with $^{42}K^+$). In these experiments, cells had been preincubated without substrates for 20 minutes before addition of alanine. Cells were incubated for a further 10 minutes with alanine to achieve a relatively steady state of cell volume, ion content and alanine content before addition of $^{42}K^+$ at zero time. At 10 minutes after zero time, K^+ content in cells with alanine was relatively constant but was elevated above control values (see the 20 minute K^+ μmol/g dry wt. value in table 5:4, and compare with the 10 minute value on table 5:5). Thus $^{42}K^+$ experiments show that both efflux and influx of K^+ were markedly increased above the values found in cells without alanine.

Having established both the magnitude of the alanine ratios across hepatocyte membranes, and the disturbance in K^+ and Na^+ distributions, it was of interest to attempt to ascertain the mechanisms involved in maintaining the alanine ratios. It was not possible to use physiological concentrations of alanine in these experiments for the following reasons. Plasma alanine concentrations are estimated by Ishikawa (1976) to range from 0.18 mM in fasted rat arterial plasma to 0.73 mM in fed rat arterial plasma and 1.30 mM in casein-fed rat portal vein plasma. The alanine ratios approached 1.0 at added alanine concentrations in this range (table 5:1), and thus approached

Table 5:5. The effect of alanine, alanine with insulin and alanine with ouabain on the exchange of K^+ in hepatocytes

Experimental details are described in Chapter 2 and the text. Values are means ± S.E.M. and the number of observations is in parenthesis. Cells were preincubated for 20 min (with ouabain where appropriate) before the addition of alanine and insulin. Cells were incubated for an additional 10 min with alanine and insulin before the addition of $^{42}K^+$, thus the incubation time refers to incubation after the addition of $^{42}K^+$ in this table. Alanine was added to an initial concentration of 5.0 mM, ouabain to 0.5 mM and 0.25 units of insulin were added to 4.0 ml suspension. Values with alanine that are statistically different from those without by Student's t-test (paired) are indicated ** = $P < 0.01$. Values with alanine plus insulin that are statistically different from those with alanine alone by Student's t-test (paired) are indicated † = $P < 0.05$ and †† = $P < 0.0125$.

Conditions	% Tissue K turned over/min at incubation time (min)		
	10	20	30
No additions	2.20 ± 0.14 (5)	2.10 ± 0.16 (5)	2.01 ± 0.14 (5)
Alanine	4.80 ± 0.53** (5)	4.57 ± 0.56** (5)	4.56 ± 0.65** (5)
Alanine + insulin	4.96 ± 0.52†† (4)	4.72 ± 0.49† (4)	4.33 ± 0.26 (4)
Insulin	2.16 (2)	2.14 (2)	2.08 (2)
Alanine + ouabain	2.60 ± 0.24 (3)	2.66 ± 0.32 (3)	3.12 ± 0.69 (3)
Ouabain	1.44 ± 0.16 (4)	1.31 ± 0.11 (4)	1.35 ± 0.12 (4)

a situation in which the phenomenon to be investigated, namely the accumulation of alanine against a gradient, was absent. In addition, metabolism of added alanine altered extracellular concentrations in such a way that on addition of 1.25 or 0.63 mM alanine removal was almost complete by 20 minutes of incubation. Thus in subsequent experiments on the effects of ouabain and insulin on hepatocytes, 5.0 mM alanine was added.

iii. The Effect of Insulin on the Alanine Content of Hepatocytes

Table 5:3 shows the effects of insulin on the intracellular and extracellular content of alanine on the addition of 5.0 mM alanine to the medium. At all times, the intracellular content of alanine was greater in the cells incubated with insulin. The presence of insulin made no difference to the content of cell water in cells incubated with alanine, and this meant that intracellular [alanine] in the presence of insulin was significantly greater ($P<0.05$, 0.01 and 0.01 for 20, 30 and 40 minutes of incubation respectively, paired *t*-test). Insulin made no difference to extracellular [alanine] at any time (table 5:3). This was reflected in the values of glucose and urea, which were also not significantly affected by insulin (table 5:3). The alanine ratio was significantly greater in the presence of insulin throughout ($P<0.05$, 0.01 and 0.01 for 20, 30 and 40 minutes respectively, paired *t*-test). Thus insulin stimulated an increase in inward transport of alanine across the membrane, but in these conditions this did not result in an increase in the rate of metabolism of alanine.

iv. The Effect of Insulin on the K^+ and Na^+ Content of Hepatocytes in the Presence of Alanine

When insulin was added with alanine (5.0 mM), at 20 and 30 minutes there was no significant difference between intracellular K^+ content or $[K^+]$ as compared to conditions with alanine alone (table 5:4). However, at 40 minutes the difference became significant ($P<0.05$, paired *t*-test). (There was no difference in content of cell

water between conditions of alanine alone and conditions of alanine with insulin.) Table 5:5 shows that at 10 and 20 minutes insulin, in the presence of alanine, significantly increased the % turnover of tissue K^+, as compared with cells with alanine alone.($P<0.0125$ and 0.05, paired t-test, at 10 and 20 minutes respectively). At 30 minutes, the effect of insulin with alanine was not significant.

Table 5:4 shows that Na^+ content (µmol/g dry wt.) in cells incubated with alanine with insulin, decreased slightly throughout incubation, whereas when alanine alone was present Na^+ content increased. The Na^+ plus K^+ content (µmol/g dry wt.) in the presence and absence of insulin with alanine were similar throughout incubation (table 5:4). In the two experiments where intracellular Na^+ was measured and alanine and insulin were present, the decrease in Na^+ was balanced by an increase in K^+. Although the effects on Na^+ plus K^+ of insulin in the presence of alanine were not large, both the finding that K^+ turnover was increased and the finding that intracellular Na^+ was decreased by insulin are consistent with the hypothesis that insulin increases Na-K ATPase activity. Blatt et al. (1972) have already given evidence that the action of insulin was mediated via the Na-K ATPase in liver (tadpole) in another context. Since my results showed that insulin affected alanine accumulation, the hypothesis that it did so via an effect on the Na-K ATPase was investigated. The hypothesis requires that alanine accumulation be ouabain sensitive, so the next step was to determine whether or not this was the case.

v. The Effect of Ouabain on the Alanine Content of Hepatocytes

In the experiments of table 5:6, cells were preincubated with and without ouabain for 20 minutes before the addition of alanine. This ensured that cells had recovered their ion content after preparation, and that ouabain inhibition was almost maximal (see diagram 3:1). Table 5:6 shows that when cells were incubated with alanine (5.0 mM) in the presence of ouabain (0.5 mM) intracellular alanine content

Table 5:6. The effect of ouabain on the alanine content of hepatocytes

Experimental details are described in Chapter 2 and the text. Values are means ± S.E.M. The number of observations is in parenthesis. Cells were preincubated with ouabain for 20 min before addition of alanine at zero time. Alanine was added to an initial concentration of 5.0 mM to all flasks. Values with ouabain that are statistically different from those without by Student's t-test are indicated by ** = $P < 0.01$.

	Ouabain	Incubation (min) 20	30	40
Alanine inside umol/g dry wt.	None	53.5 ± 4.47 (7)	61.6 ± 3.03 (5)	59.0 ± 2.79 (6)
	0.5 mM	16.9 ± 2.33** (7)	14.2 ± 2.14** (5)	12.8 ± 1.59** (6)
[Alanine] inside mM	None	22.4 ± 1.58	24.6 ± 0.97	24.4 ± 0.95
	0.5 mM	7.27 ± 0.69**	5.99 ± 0.69**	5.52 ± 0.44**
[Alanine] outside mM	None	4.22 ± 0.12	3.79 ± 0.13	3.47 ± 0.11
	0.5 mM	4.67 ± 0.13	4.32 ± 0.093	4.41 ± 0.12
Ratio inside/ outside	None	5.37 ± 0.53	6.54 ± 0.38	7.08 ± 0.46
	0.5 mM	1.56 ± 0.14**	1.40 ± 0.18**	1.25 ± 0.099**
Urea µmol/g dry wt.	None	52.3 ± 4.40 (5)	73.5 ± 8.34 (3)	110 ± 9.15 (4)
	0.5 mM	39.0 ± 3.21** (5)	54.1 ± 5.83** (3)	74.4 ± 7.29** (4)
Glucose µmol/g dry wt.	None	42.2 ± (7)	59.5 ± 5.57 (3)	73.9 ± 3.91 (6)
	0.5 mM	43.2 ± (7)	61.0 ± 5.89 (3)	77.9 ± 4.81 (6)

(μmol/g dry wt.) was significantly decreased as compared with cells incubated with alanine alone ($P<0.01$ at all times). Furthermore, the intracellular alanine content decreased throughout incubation, in the presence of ouabain, so that by 40 minutes the alanine ratio approached 1.0. By contrast, intracellular [alanine] and the alanine ratio increased throughout incubation in cells without ouabain. Extracellular [alanine] was more slowly removed when ouabain was present. There are three possible reasons for this. First, cells incubated with ouabain accumulated less intracellular alanine. Secondly, though glucose formation from alanine was not affected, ouabain significantly inhibited urea synthesis ($P<0.01$ at all times, paired *t*-test). Thirdly, 'new' alanine may have been reformed from the ammonia released from alanine during glucose synthesis: for one thing, this released ammonia could not be accounted for by measured urea and ammonia (not shown), and, for another, extracellular [alanine] was only slightly decreased in the presence of ouabain despite considerable removal of alanine by glucose formation. The problem with the last hypothesis is to identify the carbon source for the 'new' alanine. This has yet to be investigated.

At lower [alanine] outside (0.63, 1.25 and 2.5 mM), ouabain also decreased alanine ratios to approximately 1.0. For example, at 20 minutes, on addition of 1.25 mM alanine, the alanine ratio was decreased from 1.25 in cells without ouabain to 0.96 in those with ouabain. The effects of ouabain were less marked under these circumstances because of the lower alanine ratios with low added [alanine] outside. However, these experiments indicate that even at low extracellular [alanine] a concentrating mechanism for alanine exists.

vi. The Effect of Ouabain on the K^+ and Na^+ Content of Hepatocytes Incubated With and Without Alanine

Table 5:7 shows the K^+ and Na^+ content of cells incubated with ouabain (0.5 mM) in the presence of, and without, alanine (5.0 mM). The K^+ content (μmol/g dry wt.) of cells incubated with alanine and

Table 5:7. The effect of ouabain on the potassium and sodium content of hepatocytes in the presence of alanine

Experimental details are described in Chapter 2 and the text. Values are means ± S.E.M. The number of observations is in parenthesis. Cells were preincubated with ouabain for 20 min before addition of alanine at zero time. Ouabain was added to all flasks to an initial concentration of 0.5 mM. Alanine was added to an initial concentration of 5.0 mM.

	Alanine	Incubation (min) 20	30	40
K^+ inside µmol/g dry wt.	None	190 ± 10.3 (7)	191 ± 6.41 (5)	178 ± 9.3 (6)
	5.0 mM	137 ± 7.72 (7)	126 ± 6.35 (5)	119 ± 7.02 (6)
$[K^+]$ inside mM	None	98.8 ± 3.80	95.0 ± 2.89	91.0 ± 2.79
	5.0 mM	60.8 ± 4.28	54.0 ± 3.01	52.1 ± 3.30
$[K^+]$ outside mM	None	6.69 ± 0.093	6.78 ± 0.079	6.76 ± 0.064
	5.0 mM	6.88 ± 0.11	7.20 ± 0.066	7.22 ± 0.097
Na^+ inside µmol/g dry wt.	None	119 (2)	134 (2)	148 (2)
	5.0 mM	181 (2)	215 (2)	220 (2)
$[Na^+]$ inside mM	None	55.9	63.0	69.7
	5.0 mM	67.5	80.2	82.0
$Na^+ + K^+$ inside µmol/g dry wt.	None	309	325	326
	5.0 mM	318	341	339

ouabain was significantly lower than when ouabain alone was present at 20, 30 and 40 minutes of incubation with alanine ($P<0.01$ at all times). Indeed, K^+ content was 53 µmols less during the first 20 minutes of incubation with alanine as compared with cells without alanine. This initial loss of K^+ content found with alanine and ouabain was followed by a much more gradual loss during the next 20 minutes. Thus the effect of ouabain with alanine reversed the effect of alanine alone on K^+ content: as is shown in table 5:2, K^+ content with alanine alone was 332 µmol/g dry wt., as compared with a K^+ content of 295 µmol/g dry wt. when no additions were made. The K^+ content loss in cells with alanine and ouabain as compared with ouabain alone was compensated by increased Na^+ content (181 as compared with 119 µmol/g dry wt. at 20 minutes of incubation). Hence throughout incubation the total Na^+ plus K^+ content of cells was elevated when alanine was present (for example, at 40 minutes Na^+ plus K^+ was 326 µmol/g dry wt. with ouabain, and 339 µmol/g dry wt. with ouabain and alanine).

Experiments with $^{42}K^+$ showed that K^+ content changes found in the presence of alanine with ouabain were not due merely to increased efflux of K^+ from the cells. Table 5:5 shows that the % tissue K^+ turnover per minute was significantly increased at all times when cells incubated with ouabain had alanine (5.0 mM) present. Furthermore, the % turnover of K^+ increased throughout incubation of cells with alanine and ouabain, whereas with ouabain alone the turnover was steady. This indicates that in the presence of alanine and ouabain, K^+ influx and efflux were both significantly increased, efflux being slightly greater than influx, as compared with cells incubated with ouabain alone. (The 20-30 minute value for % tissue K^+ turned over per minute (table 5:5) was the most accurate, since this corresponds to the 30-40 minute period of incubation of alanine and ouabain (table 5:7) in which Na^+ and K^+ content were in near steady state conditions which, as explained in Chapter 2 section 7(c), are required for the calculation

of % tissue K^+ .)

Discussion

The experiments reported in this chapter show that correctly prepared hepatocytes are suitable for the study of accumulation of amino acids by liver. This is in agreement with the findings of Le Cam & Freychet (1977), and contrasts with the suggestion of Schreiber & Schreiber (1972). Alanine is accumulated by hepatocytes such that on addition of high [alanine] (2.0 - 10.0 mM) alanine ratios of 7.0 - 8.0 are established over a 60 minute incubation period. One notable property of alanine accumulation by hepatocytes is that when extracellular [alanine] is greater than 1.5 mM, the alanine ratio is independent of extracellular [alanine]. For example, there are cases in which intracellular [alanine] is as high as 60 mM or as low as 10 mM, yet the alanine ratio in both cases is 7.0. Given the supposed similarities (noted in the introduction to this chapter) between alanine and AIB accumulation, it is interesting to discover that these alanine ratios are in the same order as those reported by Le Cam & Freychet (1977) for AIB in isolated hepatocytes (4.0 - 7.0) and by Tews & Harper (1969a) in rat liver slices (6.0 - 7.0).

The results reported in this chapter establish that ouabain decreases the alanine ratio to about 1.0. This suggests the hypothesis that the Na-K ATPase is involved in alanine accumulation by liver. The finding that the Na-K ATPase is involved in alanine accumulation harmonizes with findings concerning uptake of neutral amino acids such as glycine, alanine and AIB in some other tissues. For example, in mouse ascites tumour cells, alanine and glycine accumulation were inhibited by ouabain (Eddy et al., 1967). However, there is one unexplained piece of contrary evidence: Le Cam & Freychet report that ouabin did not affect AIB accumulation in isolated hepatocytes.

The hypothesis under discussion is given further support by the result, reported in this chapter, that insulin increases alanine

accumulation. This harmonizes with other work: Riggs (1968) reported that insulin affects the 'A' system of amino acid transport; similarly, Kletzien et al. (1976) found higher initial rates of AIB transport in primary cultures of adult rat isolated hepatocytes in the presence of insulin.

The findings concerning the effect both of insulin and ouabain on alanine accumulation are consistent with the hypothesis that with high [alanine] in the medium alanine is transported principally by the Na^+-dependent 'A' system as characterized by Oxender & Christensen (1963) in Ehrlich cells.

For the sake of the subsequent discussion, a brief account of the mechanism by which, it is widely thought, the 'A' system operates is called for. This Na^+-dependent system is thought to be energized in the way specified by the gradient hypothesis, a hypothesis which arose from the work of Riggs et al. (1958), but which was first explicitly proposed by Crane (see 1960 and 1965) for sugar transport. The hypothesis states that the inward movement of Na^+, down the electrochemical gradient for Na^+, supplies energy for uphill transport of solutes. The site at which the Na^+ gradient is maintained is not necessarily the same as the solute transport site. Indeed, it is thought that the Na-K ATPase may maintain the Na^+ gradient whilst Na^+ and the transported solute enter by a different route. Evidence for this came from experiments with the small intestine. Ouabain, added to the serosal surface of the tissue, markedly inhibited Na^+, amino acid and sugar transport, but when added to the mucosal surface it had no effect. The Na^+ extrusion mechanism for intestinal epithelial cells is thought to reside on the serosal and lateral surfaces, whereas the Na^+-dependent solute transport processes take place on the mucosal surface (see Schultz & Zalusky, 1964; Field et al., 1967).

Even if the 'A' system in part explains alanine accumulation, my results, together with those of other workers (for example, Le

Cam & Freychet, 1977), show that this is not the only mechanism by which alanine enters cells. Underlying the Na^+-dependent system is an Na^+-independent system mediated by passive diffusion (Le Cam & Freychet, 1977). The operation of this system is manifest when alanine is incubated in the presence of ouabain. In these circumstances, there is no decrease in the rate of glucose formation from alanine, which indicates that there is a continuous influx of alanine down a concentration gradient to give an alanine ratio of 1.0. Utilization of alanine for glucose formation in the cell provides a 'sink' to allow further influx of alanine. However, the quantitative contribution of the Na^+-independent system cannot be fully ascertained from ouabain experiments, since the Na^+ ratio is much greater than 1.0 and thus, according to the Na-gradient hypothesis, would allow some transport inward by the Na^+-dependent system. Indeed, some residual Na^+-dependent transport is probably occurring in these experiments (see this discussion, below, on concentration of ouabain, and also Chapter 3 section 2, discussion i). The quantitative contribution of the Na^+-independent system can only be determined in conditions of complete removal of Na^+ from the medium and replacement with K^+, Rb^+ or choline.. It would be of interest to see if, when $[Na^+]_{outside}$ is replaced by [choline], alanine utilization for glucose formation is decreased because of decreased transport of alanine, for rate of alanine transport would then be entirely dependent on rate of passive diffusion of alanine via the Na^+-independent system. At physiological concentrations of alanine in the medium (e.g. 0.63 mM), the alanine ratio is merely 1.35 after 10 minutes of incubation. This means that the Na^+-independent system contributes a large percentage of the total entry of alanine in physiological conditions, providing that the liver <u>in vivo</u> behaves as do hepatocytes. This raises the question of why two alanine transport systems exist. The answer may lie in the Na^+-dependent system's property of being regulated by such factors as hormones (insulin) and ion

concentrations. The rate of glucose formation in starved rat hepatocytes does not seem to be increased by high $[alanine]_{inside}$ over the range 0.63 - 5.0 mM added [alanine]. Thus accumulation of alanine by the Na^+-dependent system is unlikely to be related to modulation of glucose formation. Protein synthesis is more likely to be sensitive to variations in $[alanine]_{inside}$, over the physiological range. Indeed, Tews & Harper (1969b) show that transport of AIB by rat liver slices is high in newborn rats, and declines thereafter, in such a way that accumulation of amino acids is related to the growing period of the rat liver. Likewise, higher concentrations of amino acids in vivo have been found in regenerating liver (Christensen et al., 1948) and in foetal liver (Ryan & Carver, 1966) than in non-regenerating adult liver. The functional role of the Na^+-dependent system may thus be related to maturation.

The Na^+ and K^+ data given in this chapter indicate that during the first 20 minutes of incubation with alanine, K^+ content and K^+ turnover are increased, whilst Na^+ content is unaltered. It is likely that one Na^+ enters with each alanine molecule entering the cell (Le Cam & Freychet, 1977). Thus to maintain constant Na^+ content, one Na^+ must move out for each alanine molecule transported in by the Na^+-dependent system. The ouabain experiments indicate that removal of Na^+ is by the Na-K ATPase, since on addition of ouabain Na^+ content increased rapidly. Thus in the presence of alanine alone, every Na^+ entering the cell with an alanine molecule is immediately exchanged for a K^+ from the medium. During the 20 to 40 minute period of incubation, it seems that the Na-K ATPase may not be able to keep pace with the influx of Na^+ since Na^+ content does increase to a small extent in this period. The rapid exchange of the Na^+ entering with alanine for a K^+ from the medium should result in an increase in K^+ content of the cells which is stoichiometrically related to alanine influx by the Na^+-dependent mechanism. (The fate of the alanine once inside the cell is not relevant here.) For example, from my data, at 40 minutes of incubation

of 75 mgs of hepatocytes in 5.0 mM alanine, 311 µmol/g dry wt. alanine was moved into the cells, yet the net increase in K^+ plus Na^+ was merely 46 µmol/g dry wt. (The value of 311 µmol/g dry wt. assumes that there was no loss of alanine from the cell after it had been taken up, which is unlikely since passive diffusion down the concentration gradient probably occurs; the value is thus an underestimation of the true influx of alanine into the cells.) The deficit of K^+ content of the cells can be explained by the results of the $^{42}K^+$ experiments on rate of turnover of K^+. These indicate that not only K^+ influx but also K^+ efflux is greatly increased in the presence of alanine. Thus increased permeability of the cell to K^+ offsets the continual accumulation of net cation which accompanies alanine. Hempling & Hare (1961) also noted an increase in both efflux and influx of K^+ in Ehrlich mouse ascites tumour cells in the presence of glycine. Increase in permeability of the cell to K^+ would also fit in with the findings of Dambach & Friedmann (1974b) that rat liver membranes were hyperpolarized in the presence of added alanine. The membrane potential should increase as the P_{Na}/P_K ratio is moved further from 1.0. The increased permeability of the membrane to K^+ could be related to the increase in volume of the cells on addition of alanine (for discussion, see Chapter 4).

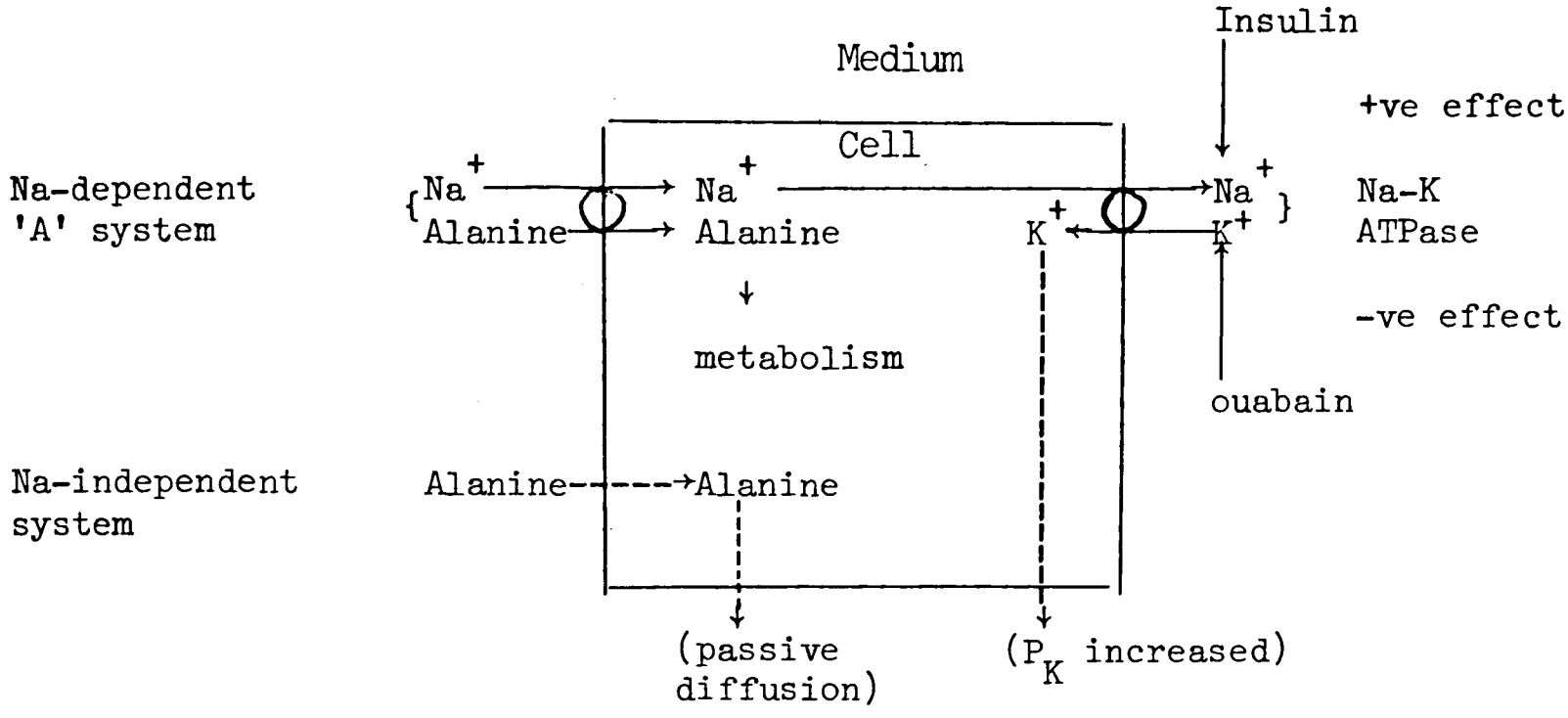
However, the ouabain experiments reported in this chapter seem not to be entirely consistent with this hypothesis, since, on the basis of $^{42}K^+$ data, K^+ permeability increased when cells were incubated with alanine and ouabain, yet in these conditions volume increases were small. It seems more likely, therefore, that K^+ permeability increase is in some way related to transport of alanine, though this does not rule out the superimposition on this effect of increased K^+ permeability as a result of cell volume change.

The effect of insulin in the presence of alanine is consistent with the hypothesis that the influx of Na^+ with alanine is accompanied by an increase in the rate of the Na-K ATPase. It is well established

in other tissues that insulin can stimulate activity of the Na-K ATPase (Gavryck et al., 1975, in frog skeletal muscle and rat brain; Jarett & Smith, 1974, in rat adipocytes; Blatt et al., 1972, in tadpole liver). As early as 1968, Riggs suggested that the 'A' transport system responded to insulin, though he did not elucidate the mechanism by which insulin acted. In the experiments reported here, insulin effects on Na^+ and K^+ content suggest that insulin may act via the Na-K ATPase in rat liver. The increase in K^+ in the presence of insulin was stoichiometrically related to the decrease in Na^+ by one to one, so that the total Na^+ plus K^+ content was the same with and without insulin. This ion redistribution in the presence of insulin is consistent with an increased rate of Na-K ATPase activity. No effect of insulin was seen on the rate of metabolism of alanine even though the alanine ratio and $[\text{alanine}]_{\text{inside}}$ was increased. Thus the action of insulin on the uptake of alanine by hepatocytes can be explained in terms of the Na-gradient hypothesis, since insulin has the effect of lowering $[Na^+]_{\text{inside}}$, and thus creates an increased Na^+ electrochemical gradient which, according to the Na-gradient hypothesis, is the driving force for the active influx of alanine.

In the above discussion I have tried to show that ion balance in the presence of alanine is maintained partly by increase in the rate of the Na-K ATPase, and partly by increase in K^+ permeability. This explains why, in these circumstances, ouabain tends to abolish the Na^+ electrochemical gradient by inhibiting the exchange of Na^+ with K^+ from the medium. According to the Na-gradient hypothesis, it is this decrease in driving force on inward Na^+ movement that leads to the failure of cells treated with ouabain to accumulate amino acids such as alanine. Likewise, insulin stimulates removal of Na^+ from the cell, increases the driving force on inward Na^+ movement, which in turn leads to an increase in alanine flux by the cell (see diagram 5:1).

DIAGRAM 5:1. PROPOSED ACCOUNT OF ALANINE ACCUMULATION AND ITS RELATIONSHIP TO THE Na-K ATPase IN HEPATOCYTES



There are two points in this chapter which need clarification. First, the $^{42}K^+$ turnover experiments in the presence of ouabain (0.5 mM) show that K^+ turnover is increased when alanine is present with ouabain, as compared with when ouabain is incubated alone. This means that K^+ influx is increased in these circumstances, yet in the presence of ouabain the Na-K ATPase should be inhibited. This apparent anomaly can be explained by the finding, reported in Chapter 3, that there was a small residual pumping activity of the Na-K ATPase in the presence of 0.5 mM ouabain. Further experiments are thus required, in which 2.0 mM ouabain is incubated with and without alanine, in order to distinguish whether the K^+ influx increase in the presence of alanine is mediated entirely by the Na-K ATPase, or whether a separate inward K^+ channel is also involved. The problem here is that $^{42}K^+$ turnover experiments require that the cells be in a steady state. With 0.5 mM ouabain this was achieved by preincubating cells for 20 minutes with ouabain, so that Na^+ and K^+ content of the cells were stable. With 2.0 mM ouabain, an 80 minute preincubation would be required to reach the same state. In our hands, the hepatocytes function less well after 80 minutes of incubation, so experiments with 2.0 mM ouabain and $^{42}K^+$ would not be reliable. For such experiments, a primary culture of hepatocytes, as used by Dickson & Pogson (1977), would be more suitable.

Secondly, the concentration of insulin used in these experiments was greatly in excess of physiological values. The calculated concentration added was 63 mUnits/ml medium (which, assuming a mol. wt. of insulin of 30,000 and 25 Units/mg insulin, is equivalent to 83.2 nM insulin). By contrast, physiological concentrations of insulin in human serum in the fasting state are in the order of 25 μUnits/ml. One of the problems of the use of insulin <u>in vitro</u> is that it is difficult to estimate the actual concentration of insulin present. Cecil & Robinson (1975) have shown that insulin binds to glass at 37°C, so that after 40 minutes of incubation less than half of the original 10^{-9} M insulin

was left in the incubation medium. Furthermore, insulin may degrade during storage and incubation. Only by assay of the incubation medium at the end of incubation would the actual concentration of insulin present be known. Kletzien (1975), working with cultured hepatocytes, used 80 nM insulin, a concentration comparable to that used in my experiments. He found that this concentration gave a maximal response and that higher concentrations gave a smaller response. He did, however, find a significant response of AIB transport to insulin in cultured hepatocytes with 50 pM insulin.

Future work on the accumulation of amino acids by rat liver could include a comparison of the alanine ratios with those of glutamine. Lund & Watford (1976) found that glutamine ratios were high in isolated hepatocytes. For example, with 0.25 - 2.0 mM added glutamine, the glutamine ratio at 60 minutes was in the order of 16, but at higher added [glutamine] the ratio was lower. Thus the pattern of ratios of glutamine and alanine are remarkably different. This may be related to difference in rate of utilization of these amino acids, or to difference in transport systems. Examination of underlying ionic mechanisms may distinguish these systems. It would also be of interest to compare alanine ratios with those of AIB in hepatocytes, since from the findings of Le Cam & Freychet (1977) and those reported here, alanine and AIB ratios are similar, yet alanine undergoes metabolism and AIB does not.

CHAPTER 6. A GRADIENT OF AMMONIA IN RAT HEPATOCYTES?

Introduction

In this chapter the ammonium ion will be referred to as NH_4^+, the undissociated form as NH_3 and the total $\{NH_4^+ + NH_3\}$ as ammonia. The term ammonia ratio will be used to denote the following ratio:

$$\frac{[\text{total ammonia}]_{\text{inside}}}{[\text{total ammonia}]_{\text{outside}}}$$

where [total ammonia] is determined by all the ammonia measured in tissue and in supernatant samples. This should be distinguished from the term ammonia gradient, which will be used to denote the following ratio:

$$\frac{[\text{freely diffusible ammonia}]_{\text{inside}}}{[\text{freely diffusible ammonia}]_{\text{outside}}}.$$

The ammonia ratio may differ from the ammonia gradient if the total ammonia includes a proportion of ammonia which is bound *in vivo*. With the technique for measuring ammonia described in Chapter 2 section 9, it is not possible to tell whether or not part of the intracellular or extracellular ammonia content is in bound form.

Analysis of intracellular [ammonia] of freeze clamp rat liver and of [ammonia] of arterial blood gave values of 1.42 mM and 0.02 mM respectively (Brosnan, 1976). Brosnan suggests that there is a large ammonia gradient between cells and medium. High ratios of ammonia have been reported between other tissues and blood. For example, Brosnan (1976) found that abdominal muscle had an ammonia content of 0.867 µmol/g wet wt., brain 0.34 µmol/g wet wt., and thigh muscle 0.255 µmol/g wet wt. According to Bromberg et al. (1960), Caesar (1962) and Seligson & Hirahara (1957), red blood cells contained between 1.4 and 2.8 times as much ammonia as was found in the surrounding plasma. The distribution between cerebrospinal fluid and plasma was found by

Hindfelt (1975) to be in the opposite direction, with 1.25 times as much ammonia in the plasma as in the cerebrospinal fluid. The mechanism of maintenance of a large ammonia gradient (if one exists) would be obscure, since 'ammonium ions probably penetrate without any limitation through cellular and mitochondrial membranes' (Zahlten et al., 1974), and the free base, NH_3, penetrates membranes considerably more readily than NH_4^+ (Jacobs, 1940). Brosnan (1976) discusses the alternative mechanisms for maintaining an ammonia gradient. They include: distribution of ammonia across the plasma membrane according to a pH gradient; active pumping of ammonium ions by, for example, the Na-K ATPase; and distribution of NH_4^+ according to the cell membrane potential. However, it is possible that a large gradient of ammonia does not exist, but that some of the measured intracellular ammonia is in bound form, for example, $NH_4MgPO_4 \cdot 6H_2O$, and is thus not freely diffusible (Hoek, 1972; Chow & Pond, 1972). In short, it may be that an ammonia ratio is being incorrectly interpreted as an ammonia gradient.

In this chapter, I attempt to shed light on the mechanism by which the high ammonia ratio is maintained. Ammonia ratios in hepatocytes have been measured under various conditions: with no additions, anaerobiosis, added ouabain, various concentrations of NH_4Cl, added ornithine and lactate, and added fructose.

The addition of ornithine and lactate was motivated by the fact that, as Krebs et al. (1976) have shown, ornithine can increase the rate of endogenous urea synthesis in hepatocytes, and ornithine and lactate have long been known to increase urea synthesis from added NH_4Cl in liver slices (Krebs, 1934). Stimulating endogenous urea synthesis would tend to decrease intracellular [ammonia], and it was hoped that this would throw light on the question of whether intracellular ammonia was freely available for urea synthesis.

The addition of fructose was designed to explore the possibility that ammonia is stored in hepatocytes as $NH_4MgPO_4.6H_2O$. Fructose

is rapidly phosphorylated to fructose-1-phosphate, and this leads to a decrease in cellular P_i (Woods et al., 1970; for further explanation, see Chapter 7). If ammonia were stored as the complex salt $NH_4MgPO_4.6H_2O$, and if this salt is in equilibrium with ammonia and phosphate (see diagram 6:1), then depletion of intracellular phosphate might be expected to lead to release of ammonia, magnesium and phosphate from the complex. Free ammonia would then be available for urea synthesis. The net effect would be that fructose would lead to decrease in measurable intracellular [ammonia].

Calculations of intracellular [ammonia] assume that ammonia is equally distributed throughout the cell interior. However, Robin et al. (1960) and Harris & Basset (1971) report that mitochondria contained more ammonia than the suspension medium. This is surprising, because the experiments of Chappell & Crofts (1966) and Chappell & Haarhoff (1967) demonstrate clearly that all mitochondrial membranes are readily permeable to ammonia. It was therefore of interest to ascertain the distribution of ammonia within the liver cell by separation of the cytosol from the mitochondria and membrane fraction by the digitonin method (Zuurendonk & Tager, 1974).

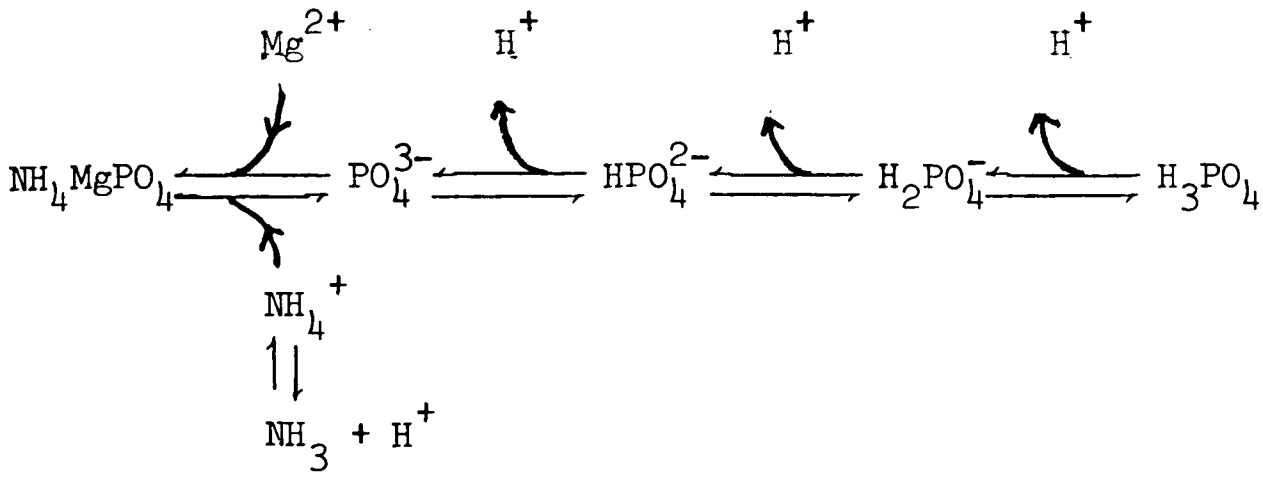
Results

i. The Distribution of Ammonia Between Cells and Medium During Incubation of Hepatocytes Without Added Substrates

Table 6:1 shows that, in conditions where no substrates were added to the medium, endogenous [ammonia] remained nearly constant throughout 80 minutes of incubation. The intracellular isolated hepatocyte [ammonia] values of 1.77 mM at zero time and 1.37 mM at 80 minutes were similar to those found by Brosnan (1976) in freeze clamp rat liver (1.34 mM). Hepatocyte medium [ammonia] increased significantly ($P<0.01$) during the 80 minutes of incubation. At zero time, medium [ammonia] of 0.027 mM was similar to that found by Brosnan (1976) in rat plasma (0.020 mM). The ammonia ratio in isolated

DIAGRAM 6:1. Some Properties of Ammonium Magnesium Phosphate: $NH_4MgPO_4.6H_2O$

Equation (1):



$NH_4MgPO_4.6H_2O$ (guanite, struvite) has a molecular wt, of 245.4 and a density of 1.711. It is insoluble in alkali and very soluble in dilute acid, with a solubility of 0.036 g/100 ml H_2O at 40°C and pH 7.0.

Table 6:1. The distribution of ammonia between cells and medium during incubation without added substrates

Experimental details are described in Chapter 2 and the text. Values are means ± S.E.M. and the number of observations is in parenthesis. Intracellular concentration terms are corrected for cell water; extracellular concentration terms refer to µmol/ml medium.

Incubation (min)	Ammonia inside µmol/g dry wt.	[Ammonia] inside mM	[Ammonia] outside mM	Ratio inside/ outside
0	3.25 ± 0.37 (10)	1.77 ± 0.23 (10)	0.027 ± 0.002 (10)	65.6
10	3.16 ± 0.24 (5)	1.60 ± 0.12 (5)	0.031 ± 0.003 (5)	51.6
20	2.80 ± 0.19 (19)	1.51 ± 0.11 (19)	0.033 ± 0.003 (19)	45.8
40	2.53 ± 0.20 (19)	1.36 ± 0.11 (19)	0.036 ± 0.002 (19)	37.8
60	2.70 ± 0.17 (11)	1.39 ± 0.07 (11)	0.047 ± 0.001 (11)	29.6
80	2.59 ± 0.23 (8)	1.37 ± 0.12 (8)	0.057 ± 0.002 (8)	24.0

hepatocytes was 65.6 at zero time, but fell to 24.0 by 80 minutes. Most of this fall is accounted for by the two-fold increase in [ammonia] in the medium.

ii. The Effects of Various Concentrations of Added NH_4Cl on the Distribution of Ammonia Between Cells and Medium

Table 6:2 shows that the high ammonia ratios observed in the absence of added substrates were abolished on addition of high concentrations of NH_4Cl, so that with 20 mM NH_4Cl the ammonia ratio approached 1.0. In these experiments, cells were incubated for 20 minutes, and urease was added to prevent the accumulation of urea. A five-fold increase in extracellular [ammonia] from 0.033 to 0.17 mM only gave rise to a 1.10-fold increase in intracellular [ammonia]. Thus high ammonia ratios appear to occur only when ammonia is present in the physiological range (i.e. 0.02 - 0.15 mM). The ratios found on addition of large amounts of ammonia differ from ratios of some other molecules accumulated by hepatocytes, such as alanine and glutamine. The alanine ratio (see table 5:1) was in the order of 7.0 whether there was 2.5 or 10.0 mM [alanine] in the medium, and a similar pattern was seen with glutamine (Lund & Watford, 1976). In other words, the alanine ratio was independent of $[\text{alanine}]_{\text{outside}}$. The results in table 6:2 can be explained by the hypothesis that most of the endogenous ammonia inside the cell is bound, and that [free intracellular ammonia] is similar to [ammonia in the medium], i.e. 0.033 mM. However, a small pH gradient between cell and medium would lead to a small ammonia gradient, which can be calculated from equation (2) (Milne et al., 1958):

Equation (2)

$$\frac{[\text{ammonia}]_{\text{inside}}}{[\text{ammonia}]_{\text{outside}}} = \frac{1 + 10^{(pK_a - pH_{in})}}{1 + 10^{(pK_a - pH_{out})}}$$

(Distribution of weak acid and weak base according to this equation makes the assumption that the dissociation constant is the same in

Table 6:2. The effect of various concentrations of added NH_4Cl on the distribution of ammonia between cells and medium.

Experimental details are described in Chapter 2 and the text. Values are means ± S.E.M. and the number of observations is in parenthesis. Cells were incubated for 20 min. Urease was added as 1 mg/4ml suspension in order to prevent accumulation of urea.

Additions NH_4Cl mM	[Ammonia] inside mM	[Ammonia] outside mM	Ratio inside/ outside mM
None	1.51 ± 0.11 (19)	0.033 ± 0.003 (19)	45.8
None + urease	1.66 ± 0.062 (6)	0.17 ± 0.02 (6)	9.8
0.5	1.84 ± 0.19 (8)	0.29 ± 0.02 (8)	6.3
0.5 + urease	2.97 ± 0.19 (7)	0.62 ± 0.026 (7)	4.8
1.0 + urease	3.42 ± 0.25 (4)	1.15 ± 0.038 (4)	3.0
2.0 + urease	5.97 ± 0.27 (9)	2.06 ± 0.071 (9)	2.9
5.0 + urease	9.76 ± 0.34 (5)	4.84 ± 0.088 (5)	2.0
10.0 + urease	16.52 ± 0.51 (5)	9.84 ± 0.19 (5)	1.7
20.0 + urease	25.24 ± 1.30	19.96 ± 0.59	1.3

extracellular and intracellular fluids.) Even in the unlikely event of a pH gradient of 0.5 pH units between cell and medium, the ammonia gradient would be only 3.0. From table 6:2, about 0.033 mM of the 1.55 mM [endogenous ammonia] will be free, and about 1.48 mM will be bound. Assuming that the contribution of bound ammonia is constant (i.e. that it always contributes 1.48 mM to intracellular [ammonia]), then by subtracting it from the intracellular [ammonia] in circumstances where ammonia was added to the medium, ammonia gradients can be calculated. Table 6:3 shows the result of modifying the values given in table 6:2 by the subtraction of 1.48 from the $[ammonia]_{inside}$ values (column B). The ammonia gradients calculated after subtraction of the contribution of bound ammonia range only between 1.1 and 2.4 (column D). Ratios calculated before the subtraction of 1.48, representing the contribution of bound ammonia, are shown in parentheses in table 6:3, and, unlike the gradients of column D, range from 1.3 to 45.8. The gradients in D can be explained in terms of small pH gradients between cell and medium. For example, a ratio of 2.4 would represent a pH gradient of 0.28 pH units, lower on the inside. Such a pH gradient would be unsurprising, in the light of Masoro & Siegel's estimation (1971) that intracellular liver pH is 7.23 and extracellular pH in the order of 7.45.

Table 6:2 gives values of intracellular [ammonia] at 20 minutes of incubation. The question arose of whether added ammonia entered the cells at a great enough rate for the maximum ammonia ratio to be achieved at this time. Table 6:4 shows an 80 minute incubation in which NH_4Cl (10 mM) was added (without urease) after a preincubation period of 10 minutes. Intracellular [ammonia] increased rapidly for the first 2.5 minutes. During this time, ammonia was moving inwards down an electrochemical gradient. Ammonia inside then increased more slowly, reaching a steady state at 40 minutes (see diagram 6:2). Thus 20 minute values of intracellular [ammonia] shown in table 6:2 may slightly underestimate the maximum possible ammonia ratios, but

Table 6:3. Calculation of the ammonia ratio after subtraction of bound ammonia

Values in column A are taken from Table 2. Values in column B are derived from those in column A, minus a constant value of 1.48 for bound ammonia. Values in column C are taken from Table 2. Values in parenthesis are ratios from Table 2.

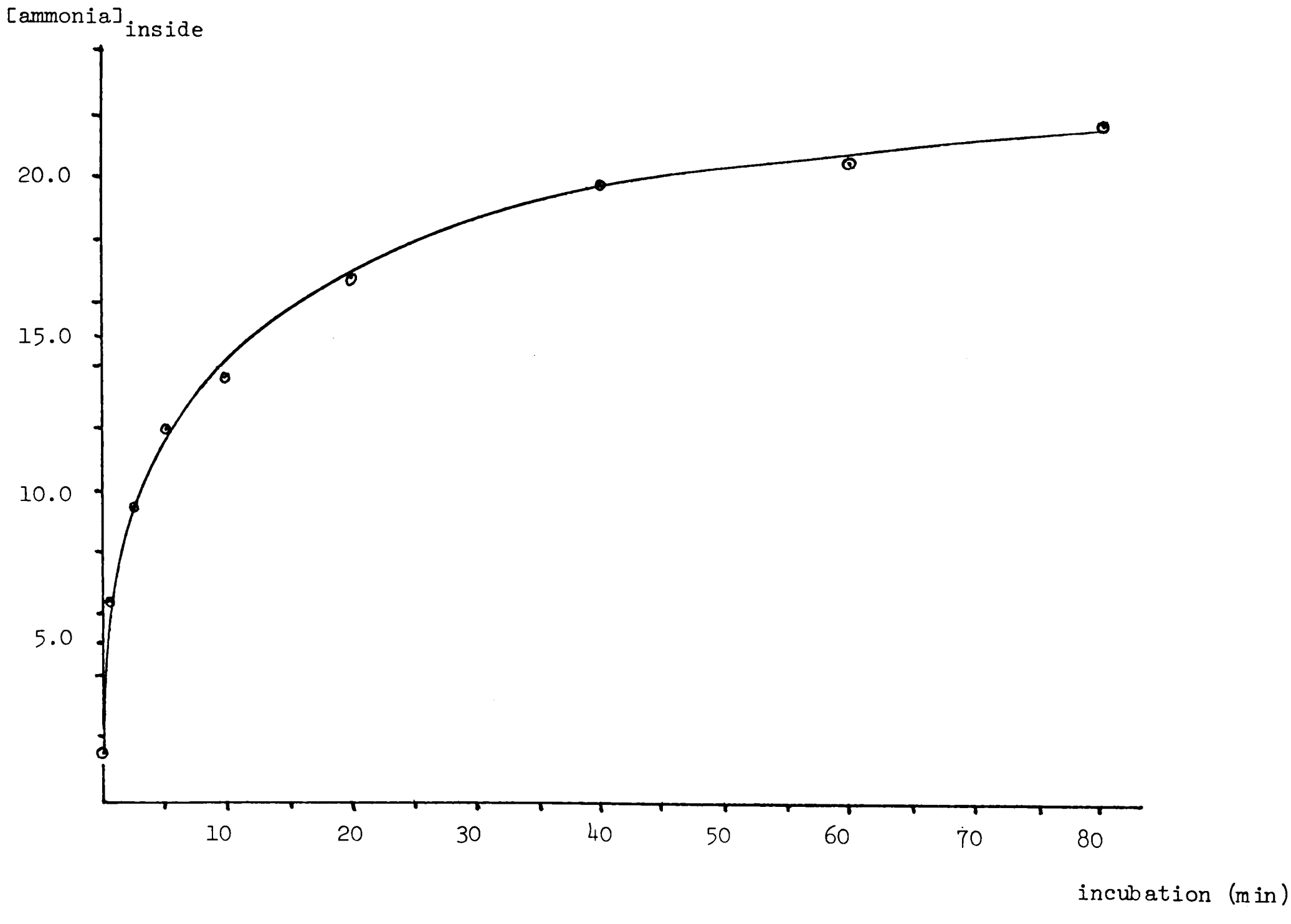
A	B	C	D	
[Ammonia] inside	[Ammonia] inside -1.48 mM	[Ammonia] outside	Ammonia gradient B/C	Ammonia ratio A/C
1.51	0.033	0.033	1.0	(45.8)
1.66	0.18	0.17	1.1	(9.8)
1.84	0.36	0.29	1.2	(6.3)
2.97	1.49	0.62	2.4	(4.8)
3.42	1.94	1.15	1.7	(3.0)
5.97	4.49	2.06	2.2	(2.9)
9.76	8.28	4.84	1.7	(2.0)
16.52	15.04	9.84	1.5	(1.7)
25.24	23.76	19.96	1.2	(1.30)

Table 6:4. The effect of incubation of hepatocytes with NH_4Cl on the distribution of ammonia and K^+ between cells and medium

The experimental details are described in Chapter 2 and the text. Values are means ± S.E.M. and the number of observations is in parenthesis. Cells were preincubated for 10 min before the addition of NH_4Cl which was added to an initial concentration of 10 mM.

Incubation (min)	[Ammonia] inside	[Ammonia] outside	Ammonia ratio	A No additions $[K^+]$ inside	B NH_4Cl $[K^+]$ inside	A - B mM
0.5	6.44 ± 0.80 (4)	10.22 ± 0.55 (4)	0.63	165 ± 5.8 (4)	160 ± 2.1 (4)	5
2.5	9.62 ± 0.62 (5)	10.26 ± 0.30 (5)	0.91	160 ± 4.3 (5)	156 ± 3.8 (5)	4
5.0	12.20 ± 0.35 (4)	10.38 ± 0.18 (3)	1.18	163 ± 5.1 (4)	154 ± 6.1 (4)	9
10.0	13.57 ± 0.68 (4)	10.43 ± 0.26 (4)	1.30	162 ± 4.8 (4)	145 ± 3.2 (4)	17
20.0	16.89 ± 0.62 (4)	10.25 ± 0.42 (4)	1.65	160 ± 2.9 (4)	145 ± 4.1 (4)	15
40.0	19.93 ± 0.51 (4)	9.84 ± 0.20 (4)	2.03	155 ± 5.9 (4)	140 ± 7.2 (4)	15
60.0	20.11 ± 1.31 (3)	9.51 ± 0.53 (3)	2.33	150 ± 8.0 (3)	136 ± 9.3 (3)	14
80.0	21.8 (2)	9.9 (2)	2.20	157 (2)	136 (2)	21

DIAGRAM 6:2. Intracellular [ammonia] in condition of added NH_4Cl (10.0 mM) (from table 6:4)



nevertheless ratios with high concentrations of added NH_4Cl are still much less than those found at low extracellular [ammonia]. Table 6:4 also shows that ammonia can penetrate cell membranes rapidly, for within 0.5 minutes intracellular [ammonia] had increased by 5.0 mM, which represents a rate of influx of approximately 18 μmol/min/g dry wt.

iii. The Effect of Added NH_4Cl on K^+ and Na^+ Content of Hepatocytes

Table 6:4 also shows $[K^+]_{inside}$ in the presence and absence of NH_4Cl (10 mM). Ammonia exists in solution in two forms according to the equation:

Equation (3)

$$NH_4^+ \rightleftharpoons NH_3 + H^+ \quad \text{(at 37°C)}.$$

In human plasma the pKa of this reaction is 9.49 (Jacquez et al., 1959), and though cytosolic pK_a has not been established, there is no reason to think that it is likely to be markedly different. Hence one can infer that, at physiological pH, 99% of ammonia exists as the cation NH_4^+. It was of interest to attempt to ascertain whether increase in intracellular NH_4^+ was balanced ionically by a concomitant decrease in another cation such as Na^+ or K^+, or by an increase in an anion such as Cl^-. Table 6:4 shows that decreases in $[K^+]$ in the presence of NH_4Cl were similar to increases in intracellular [ammonia], which suggests that K^+ could be balancing the NH_4^+ charge. The finding that intracellular K^+ decreases in conditions of added NH_4Cl is similar to that of Hawkins et al. (1973), who gave rats an intravenous load of ammonium acetate and measured a rapid increase in plasma K^+.

In two experiments where satisfactory measurements of Na^+ were made, there was a decrease in $[Na^+]_{inside}$ from 24.4 to 16.5 mM at 40 minutes.

Turnover of K^+, measured with $^{42}K^+$, was decreased from 2.25 %/min without additions to 1.53 %/min in the presence of NH_4Cl (10 mM) (see table 3:12). This suggests that NH_4^+ was competing with K^+ for entry into the cells. NH_4^+ was shown by Post & Jolly (1957) to compete with K^+ for inward transport into erythrocytes, and also

was found to replace K^+ in the Na-K ATPase (Skou, 1960; Rendi & Uhr, 1964). The K^+ turnover experiments indicate that NH_4^+ transport is likely to occur in hepatocytes.

A fuller discussion of the results reported in table 6:4 is given in the final discussion at the end of this chapter.

iv. The Effects of Anaerobiosis and Added Cyanide on the Distribution of Ammonia Between Cells and Medium

These experiments were conducted in order to test the hypothesis that energy is required to maintain the high ammonia ratio found in cells with little or no added NH_4Cl. If metabolic energy is required for this purpose then cyanide (added as 1.0 mM HCN), which inhibits cytochrome oxidase, should decrease the ammonia ratio towards 1.0. Table 6:5 shows the effect of cyanide on the distribution of ammonia between cells and medium in conditions of NH_4Cl (0.5 mM) and urease. NH_4Cl (0.5 mM) was added at this concentration because, though high, it is nonetheless within the physiological range, being similar to that found in portal blood *in vivo*: for example, rat portal venous blood contain 0.26 µmol/ml ammonia (Brosnan, 1976), and, on a high protein diet, guinea pig portal blood was found by Warren & Newton (1959) to be as high as 1.20 mM. Urease was added to prevent accumulation of urea. Flasks containing cyanide were incubated in air, and those without cyanide were gassed with 95% O_2 and 5% CO_2. The ammonia content (µmol/g dry wt.) of cells incubated with cyanide was significantly decreased ($P<0.01$) as compared with cells without cyanide at 20 and 40 minutes. Thus cells do appear to require energy to maintain high $[ammonia]_{inside}$.

Cells incubated with cyanide increased in cell volume (see Chapter 4 section iii). When corrections were made for tissue water, [ammonia] in cells with cyanide was decreased, with the result that the ammonia ratio was 1.1 and 0.80 at 20 and 40 minutes respectively. At 40 minutes cell lysis may have occurred in cells treated with cyanide,

Table 6:5. The effect of cyanide on the distribution of ammonia between cells and medium in hepatocytes

Experimental details are described in Chapter 2 and the text. All flasks contained NH_4Cl (0.5 mM) and urease to an initial content of 1.0 mg/4 ml cell suspension. Cyanide was added as HCN to an initial concentration of 1.0 mM. Flasks containing cyanide were incubated in air and control flasks were gassed with 95% O_2 and 5% CO_2. Values are means ± S.E.M. of five or six observations.

Incubation (min)	Additions cyanide mM	Ammonia inside µmol/g dry wt.	[Ammonia] inside mM	[Ammonia] outside mM	Ratio inside/ outside mM
20	None	6.56 ± 0.70	3.60 ± 0.40	0.65 ± 0.015	5.5
	1.0	2.04 ± 0.12	0.75 ± 0.06	0.70 ± 0.023	1.1
40	None	5.98 ± 0.32	3.31 ± 0.22	0.75 ± 0.037	4.4
	1.0	1.65 ± 0.12	0.59 ± 0.048	0.74 ± 0.018	0.8

and this would account for an ammonia ratio below 1.0. As reported in Chapter 3 (section 3, ii), in conditions of cyanide incubated in air, intracellular $[K^+]$ was decreased, and the K^+ ratio was 0.89 at 40 minutes: this is a further indication that cell lysis occurs under these conditions. Furthermore, by 40 minutes, dry weight of cells with cyanide was significantly decreased (see Chapter 4 section iii).
As was discussed in Chapter 4, calculation of intracellular concentrations from inulin space and wet and dry weight data is not satisfactory in these circumstances.

Because of the deleterious effects of HCN on cells incubated in air, it was decided to investigate the effects of limiting O_2 supply by anaerobiosis. In these experiments, all cells were gassed with a mixture containing 5% CO_2. This avoided the additional variable of increased pH which occurred in experiments with cyanide treated cells incubated in air (which contains only 0.03% CO_2).

Table 6:6 shows the ammonia content of cells incubated with and without O_2. Anaerobiosis was achieved by gassing flasks for 30 seconds with 95% N_2 and 5% CO_2 with yellow phosphorus in the flask centre well to remove residual O_2. Ammonia content (µmol/g dry wt.) in anaerobic cells descreased throughout the incubation despite greatly increased $[ammonia]_{outside}$, which occurred as a result of decreased urea synthesis from ammonia released by proteolysis. Ammonia content decreased more slowly in anaerobic conditions that in cyanide treated cells, but this was also true of decrease in K^+ content (see Chapter 3). Anaerobiosis greatly decreased the ammonia ratio, partly because $[ammonia]_{outside}$ increased, and partly because $[ammonia]_{inside}$ decreased. (Anaerobic cells increase in volume (see Chapter 4 section iii), so corrections for cell water were made in calculating $[ammonia]_{inside}$.)

Experiments with anaerobiosis indicate that high ammonia ratio in hepatocytes is energy dependent. The next hypothesis to be investigated was that there was a relationship between the energy source of high

Table 6:6. The effect of anaerobiosis on the distribution of ammonia between cells and medium

Experimental details are described in Chapter 2 and the text. The values are means ± S.E.M. and the number of observations was six. Anaerobiosis was attained by the addition of phosphorus placed in the centre well of 50 ml Erlenmeyer flasks and gassed with 95% N_2 and 5% CO_2. Control flasks contained no phosphorus and were gassed with 95% O_2 and 5% CO_2. Intracellular concentration values are corrected for cell water.

Incubation (min)	Conditions	Ammonia inside µmol/g dry wt.	[Ammonia] inside mM	[Ammonia] outside mM	Ratio inside/ outside
40	Control	3.22 ± 0.17	1.66 ± 0.10	0.035 ± 0.003	47.4
	Anaerobic	2.87 ± 0.06	1.18 ± 0.09	0.11 ± 0.010	10.7
60	Control	2.71 ± 0.07	1.49 ± 0.05	0.044 ± 0.002	33.9
	Anaerobic	2.09 ± 0.08	0.84 ± 0.082	0.15 ± 0.018	5.6
80	Control	2.63 ± 0.15	1.46 ± 0.069	0.055 ± 0.003	26.6
	Anaerobic	1.83 ± 0.12	0.76 ± 0.080	0.19 ± 0.024	4.0

ammonia ratios and activity of the Na-K ATPase.

v. The Effect of Ouabain on the Ammonia Content of Hepatocytes

Lund et al. (1970) suggested that a gradient of ammonia might be maintained by active pumping of NH_4^+ into the cell. The fact that NH_4^+ has a similar diameter (1.43 Å) to that of K^+ (1.33 Å) is consistent with the hypothesis that NH_4^+ is pumped inwards by the Na-K ATPase. As has been mentioned, NH_4^+ has been found to replace K^+ in the Na-K ATPase *in vitro* (Skou, 1960; Kinsölving & Post, 1960; Rendi & Uhr, 1964), and Post & Jolly (1957) have shown that NH_4^+ can compete with K^+ in inward transport. Ouabain, which inhibits the Na-K ATPase, should lower the ammonia ratio and intracellular ammonia concentration if the hypothesis in question is correct.

Table 6:7 shows the results of experiments in which hepatocytes were incubated with 0.5 mM NH_4Cl and urease for 20 and 40 minutes in the presence and absence of ouabain (1.0 mM). Urease was added to keep the total ammonia content the same in all flasks. The intracellular ammonia content of ouabain treated cells was significantly higher than in those without ouabain at 20 and 40 minutes ($P<0.05$ and 0.01 respectively, paired *t*-test). In two similar experiments in which 2.5 and 5.0 mM NH_4Cl was added to the medium in the presence and absence of 1.0 mM ouabain, intracellular ammonia was slightly higher in flasks with ouabain, which indicates that ammonia entered the cells rapidly despite inhibition of the Na-K ATPase.

The conclusion to be drawn from these experiments with ouabain is that it is not the case that high $[ammonia]_{inside}$ is maintained by the active pumping of the Na-K ATPase. For when the Na-K ATPase was inhibited with ouabain, not only did intracellular ammonia content increase, extracellular [ammonia] tended to decrease.

vi. The Effect of Ornithine, in the Absence and Presence of Lactate, on the Distribution of Ammonia Between Cells and Medium

The experiments reported in this section were designed to test

Table 6:7. The effect of ouabain on the distribution of ammonia between cells and medium in the presence of NH_4Cl and urease

Experimental details are described in Chapter 2 and the text. Values are means ± S.E.M. and the number of observations is six or seven. All flasks contained NH_4Cl added to an initial concentration of 0.5 mM. Urease was added as 1 mg/4 ml suspension. Ouabain was added to an initial concentration of 1.0 mM. Values in the presence of ouabain that are statistically significant from those without ouabain by Student's t-test are indicated by * = $P < 0.05$ and ** = $P < 0.01$.

Incubation (min)	Additions ouabain mM	Ammonia µmol/g dry wt.	[Ammonia] inside	[Ammonia] outside	Ratio inside/ outside
20	None	5.52 ± 0.34	2.97 ± 0.19	0.62 ± 0.026	4.8
	1.0	6.27* ± 0.47	3.37* ± 0.20	0.63 ± 0.021	5.4
40	None	5.48 ± 0.29	3.21 ± 0.16	0.78 ± 0.048	4.1
	1.0	7.33** ± 0.51	3.93** ± 0.27	0.72 ± 0.025	5.5

the hypothesis that the energy required to maintain high [ammonia]$_{inside}$ does not pump free ammonia against a concentration gradient, but rather maintains other intracellular ions in the appropriate concentrations for the formation of a complex ammonium salt such as $NH_4MgPO_4.6H_2O$. If ammonia is stored intracellularly in the form of a salt, it would not be freely available for urea synthesis. In the following experiments, endogenous ammonia was stimulated maximally with added ornithine and lactate in order to ascertain whether endogenous [ammonia]$_{inside}$ was freely available to urea cycle enzymes. If so, stimulation of urea synthesis would be correlated with decrease of [ammonia]$_{inside}$.

Table 6:8 shows that cells incubated in conditions of stimulated urea synthesis, that is, with added ornithine (1.0 mM) in the presence and absence of lactate (2.0 mM), contained similar intracellular [ammonia] to those incubated with no additions, despite the fact that extracellular [ammonia] was increased in the presence of ornithine. At 40 minutes, urea content was increased from 7.7 µmol/g wet wt. in cells with no additions to 11.6 and 9.9 µmol/g wet wt. in cells with ornithine, and ornithine plus lactate, respectively. Therefore, although urea formation was increased in the presence of ornithine, intracellular [ammonia] was not altered. This suggests that some intracellular ammonia is not available for urea synthesis.

Additional ammonia found in the medium and as urea on addition of ornithine, and ornithine plus lactate, was produced from metabolism of ornithine. Ornithine is transaminated with α-oxoglutarate to yield glutamate and glutamate γ-semialdehyde. Glutamate γ-semialdehyde is then converted to glutamate. Both glutamate molecules derived from ornithine and α-oxoglutarate can undergo deamination by glutamate dehydrogenase to yield α-oxoglutarate and ammonia. In the experiments reported above, glutamate content was elevated above contol values in the presence both of ornithine and of ornithine plus lactate.

Table 6:8. The effect of ornithine in the presence and absence of lactate on the distribution of ammonia between cells and medium in hepatocytes

Experimental details are described in Chapter 2 and the text. Values are means ± S.E.M. and the number of observations is five or six. Ornithine and lactate were added to initial concentrations of 1.0 and 2.0 mM respectively.

Incubation (min)	Additions	Ammonia inside µmol/g dry wt.	[Ammonia] inside mM	[Ammonia] outside mM	Ratio inside/ outside
20	None	2.57 ± 0.38	1.42 ± 0.21	0.027 ± 0.002	52.6
	Ornithine	2.63 ± 0.24	1.45 ± 0.13	0.037 ± 0.003	39.2
	Ornithine + lactate	2.34 ± 0.23	1.30 ± 0.11	0.027 ± 0.001	48.2
40	None	1.92 ± 0.10	1.06 ± 0.06	0.035 ± 0.002	30.3
	Ornithine	2.32 ± 0.25	1.27 ± 0.12	0.046 ± 0.008	27.6
	Ornithine + lactate	2.06 ± 0.25	1.15 ± 0.12	0.044 ± 0.004	26.1

Thus the experiments with ornithine failed to falsify the hypothesis that part of intracellular ammonia is stored in a form which makes it unavailable to enzymes of urea synthesis. The results are therefore consistent with the hypothesis that ammonia is sequestered in the form $NH_4MgPO_4.6H_2O$, and this hypothesis was further probed in the experiments reported in the next section.

vii. The Effect of Fructose on Intracellular Ammonia Content of Hepatocytes

Theoretically, if fructose (10 mM) is added to cells in the presence of urease the resulting decrease in intracellular P_i should lead to alterations in the phosphate - $NH_4MgPO_4.6H_2O$ equilibrium, so that P_i, NH_4^+ and Mg^{2+} would be released from NH_4MgPO_4 into solution. Initially, this would lead to an increase in intracellular ammonia content, but since it is likely that NH_3 permeates membranes very rapidly ammonia would ultimately be released into the medium. Urease prevents the accumulation of urea, so the net effect of incubation with fructose and urease, if the hypothesis that some intracellular ammonia is sequestered in the form $NH_4MgPO_4.6H_2O$ is correct, would be decrease in intracellular ammonia and increase in extracellular ammonia.

Table 6:9 shows that with added fructose (10 mM) in the presence of urease, both intracellular P_i and ammonia contents were significantly decreased at 20 and 60 minutes (for $[ammonia]_{inside}$, $P<0.05$ and 0.01 respectively, paired *t*-test). The 60 minute ammonia values tend to decrease further below control values, whereas the 60 minute P_i values increase slightly towards control values. These results are consistent with the hypothesis under investigation.

However, in the presence of fructose and urease, extracellular [ammonia] did not increase as predicted: instead, at 60 minutes it was significantly decreased. This was because, in conditions of added fructose, alanine formation was increased from 0.52 and 0.60 µmol/g wet wt. without fructose to 2.71 and 3.60 µmol/g wet wt. with fructose, at 20 and 60 minutes respectively. Thus in the presence of fructose

Table 6:9. The effect of fructose on the distribution of ammonia between cells and medium

Experimental details are described in Chapter 2 and the text. Values are means ± S.E.M. and the number of observations is in parenthesis. Fructose was added to an initial concentration of 10 mM and 1 mg/4 ml cell suspension urease was added to all flasks. Values in the presence of fructose that are statistically significant from those without fructose by Student's t-test (paired) are indicated by * = $P < 0.05$ and ** = $P < 0.01$.

	Incubation (min)			
	20		60	
Additions:	None	Fructose	None	Fructose
Ammonia inside µmol/g dry wt.	4.36 ± 1.35 (3)	2.43 ± 0.26* (3)	3.35 ± 0.26 (3)	2.25 ± 0.27** (3)
[Ammonia] inside	2.36 ± 0.73 (3)	1.40 ± 0.069* (3)	1.81 ± 0.14 (3)	1.22 ± 0.15** (3)
[Ammonia] outside	0.16 ± 0.013 (3)	0.11 ± 0.009 (3)	0.33 ± 0.014 (3)	0.10 ± 0.004 (3)
Ammonia ratio	11.1	12.7	5.4	11.0
$[P_i]$ inside	3.21 ± 0.41 (4)	1.06 ± 0.10 (4)	2.70 ± 0.25 (4)	1.82 ± 0.44 (4)
[ATP] inside	1.93 ± 0.074 (5)	0.67 ± 0.049 (5)	1.94 ± 0.060 (3)	1.04 ± 0.17 (3)

cellular ammonia was removed by alanine formation, and did not appear in the medium.

Ammonia nitrogen was probably incorporated into alanine by the following reactions:

1. α-oxoglutarate + NH_4^+ + NAD(P)H $\rightleftharpoons$ glutamate + NAD(P) + H_2O
2. glutamate + pyruvate $\rightleftharpoons$ α-oxoglutarate + alanine

The sum of these reactions is an alanine dehydrogenase with an equilibrium constant favouring alanine formation:

$$K = \frac{[\text{pyruvate}]\,[NH_4^+]\,[\text{NADH}]}{[\text{alanine}]\,[\text{NAD}]} = 2.61 \times 10^{-6} M \quad (38^\circ C;\ I: 0.25;\ pH: 7.0)$$

(See Brosnan et al., 1970.) On addition of fructose, lactate and pyruvate concentrations are greatly elevated (see table 7:7). Increased [pyruvate] and $[NH_4^+]$ will thus lead to alanine formation. Since endogenous $[\text{ammonia}]_{\text{inside}}$ released on addition of fructose cannot account for the total amount of nitrogen in the alanine formed, it is likely that ammonia was also generated from adenine nucleotide breakdown. Table 7:7 shows that total adenine nucleotides in the presence of fructose were 2.46 µmol/g wet wt., as compared with 2.91 µmol/g wet wt. without fructose.

It is possible that intracellar and extracellular [ammonia] were decreased on addition of fructose solely because alanine formation was stimulated. To exclude this possibility, cells were incubated in the presence and absence of pyruvate (10 mM), which also gave rise to increased alanine formation. Both intracellular and extracellular [ammonia] were found to be similar with or without pyruvate, which suggests that the effect of fructose on $[\text{ammonia}]_{\text{inside}}$ was not due to the stimulation of alanine synthesis.

Interpretation of the effects of fructose on the ammonia content of cells is complicated by the fact that incubation with fructose leads to a large decrease in cell [ATP] (see table 6:9). Thus decreased

intracellular ammonia could be directly related to decrease in metabolic energy supply as was seen in experiments with anaerobiosis (section iv above), rather than to a specific effect of fructose on the phosphate equilibrium. This does not exclude the possibility that metabolic energy is required indirectly for the maintainance of cell NH_4MgPO_4 content. It is relevant here that $[K^+]_{inside}$ in conditions of added fructose was maintained at a significantly higher value than in cells without fructose (see Chapter 7 section i). Thus concentration ratios of certain ions can be maintained despite decreased [ATP] in the presence of fructose.

Since it was not possible to find a situation in which intracellular $[P_i]$ was sufficiently decreased yet [ATP] maintained, it was not possible to distinguish the general effects of energy supply from the specific effects of $[P_i]$ on the ammonia distribution. Thus although the results of this section do not conflict with the hypothesis that some intracellular ammonia is stored as $NH_4MgPO_4.6H_2O$, they did not yield the degree of corroboration that might have been hoped. The problem was accordingly approached from another angle.

viii. The Intracellular Location of Ammonia in Hepatocytes

Compartmentation of ammonia in intracellular organelles, resulting in little or no gradient of ammonia between cell and medium, would mean that the actual ammonia content of the organelles would be high. A mechanism for maintaining this high organelle content would have to be postulated. The first step was to attempt to measure [ammonia] in cell cytosol and in organelles. In the experiments described in this section, hepatocyte cytosol was separated from the particulate matter, namely mitochondria, and membranes. The method of separation, using digitonin, is described in Chapter 2 section 5B (e). The resulting mitochondrial fraction (the mitochondrial pellet) includes cell, nuclear and endoplasmic reticular membranes whilst the cytosolic fraction contains cell and nuclear cytosol. Before discussing the

distribution of ammonia found in the mitochondrial and cytosolic compartments, it is necessary to consider the validity of the digitonin method. After separation, the mitochondrial pellet contained 97.4% ±0.42 (S.E.M.) of the total cell glutamate dehydrogenase activity, whilst the cytosolic and medium fraction contained only 2.6% glutamate dehydrogenase activity. Since glutamate dehydrogenase is a mitochondrial matrix enzyme, this implies that almost all the mitochondrial matrices were intact. Activity of adenylate kinase, which is localized between the two mitochondrial membranes, was 91.9% (± 0.61% S.E.M.) in the mitochondrial pellet fraction, which suggests that the more outer mitochondrial membranes were only slightly more leaky than the matrix membranes. Enzymes are large molecules. It is therefore possible that some leakage of small molecules from the mitochondrial cytosol and matrix occurred without loss of large enzyme molecules. Activity of enzymes may not be an ideal marker for integrity of mitochondrial membranes. Siess et al. (1977) showed that values of mitochondrial metabolites such as aspartate, glutamate, citrate and malate obtained by the digitonin method compared favourably with values of the same metabolites obtained by another method (Soboll et al., 1976), and concluded that the digitonin method was satisfactory for measurement of mitochondrial metabolites. However, in the experiments described in this section, mitochondrial K^+ content was low. The mitochondrial pellet contained 16.86 µmol/g dry wt., which, corrected for mitochondrial water on the assumption that mitochondrial volume is 24% of total cell volume (Bolender & Weibel, 1973), is equivalent to 41.6 mM $[K^+]$ (±1.59 S.E.M.). The potassium content of the whole cell was 282 µmol/g dry wt., which, corrected for tissue water, is equivalent to 166 mM $[K^+]$ (±6.4 S.E.M.). The calculated K^+ content expected in the mitochondrial fraction was therefore 67.7 µmol/g dry wt., assuming that K^+ is equally distributed throughout the cell interior, whereas the actual K^+ content found was 16.9 µmol/g dry wt. Even if mitochondrial $[K^+]$ is as low as 107 mM,

as has been suggested by Diwan & Tedeschi (1975), the K^+ content found in digitonin separated mitochondria was 60% too low. The validity of the digitonin method is fairly well established for large molecules and intrachondrial metabolites (Siess et al., 1977), but some caution should be exercised over values for ions. Whether or not the ammonia values reported below are correct will probably depend on the degree (possibly zero) to which ammonia is bound. If all the ammonia is free in solution, the mitochondrial values are likely to be an underestimation. The greater the degree to which the ammonia is bound, the greater the likely accuracy of the values.

Table 6:10 shows that when the mitochondrial fraction of hepatocytes was separated from the cell cytosol, it contained a higher content of ammonia (1.35 µmol/g dry wt.) than predicted if ammonia were equally distributed throughout the cell interior (0.50 µmol/g dry wt.). [Ammonia] in the mitochondrial pellet was 3.31 mM (table 6:10,(c)), as compared with the expected 1.24 mM found in the whole cells (table 6:10 (a)). This is similar to the value of 3.0 mM obtained for isolated mitochondria by Hoek (1972). Mitochondrial [ammonia] is calculated on the assumption that the mitochondrial volume is 24% of the total cell volume (Bolender & Weibel, 1973). The ammonia content of the cell cytosol can be calculated by subtraction of the medium ammonia content (table 6:10,(b)) from the ammonia content of the supernatant of the mitochondrial pellet (table 6:10,(d)). Cytosolic [ammonia] was 0.49, which was less than the 1.24 mM predicated if ammonia were equally distributed thoughout the cell. Thus the ratio of cell cytosol ammonia to medium ammonia was 13.6, and the ratio of mitochondrial to cell cytosol ammonia was 6.8. In short, there were large ammonia ratios both between cell cytosol and medium and between mitochondria and cell cytosol.

Table 6:10. Distribution of ammonia between cell and nuclear cytosol, the mitochondrial and cell membrane fraction and the cell medium

Experimental details are described in Chapter 2 and the text, (Chapter 2, section 5B, e) values are means ± S.E.M. and the number of observations was three. Cells were incubated without additions for 20 min before separation of cells from medium and mitochondria and membrane fraction from cell cytosol fraction. SN refers to supernatant. Mean dry wt. of cells = 67.6 mg/flask; mean wet wt. of cells = 263 mg/flask; mean inulin space = 81 µl/flask.

	Ammonia		
	µmol/flask	µmol/g dry wt.	mM
Whole cell pellet fraction (a)	0.142 ± 0.024	2.10 ± 0.35	1.24 ± 0.21
Medium (b) (SN from (a))	0.144 ± 0.020	-	0.036 ± 0.005
Mitochondrial and cell membrane fraction (c)	0.091 ± 0.015	1.35 ± 0.22	3.31 ± 0.54
Cell cytosol with cell medium (d) (SN from (c))	0.187 ± 0.006	-	-
Cell cytosol ((d) - (b))	0.043	0.64	0.49

Discussion

i. Ammonia Ratios Between Cells and Medium and Within Hepatocytes

The experiments reported in this chapter establish that the endogenous ammonia content of isolated hepatocytes is similar to that of rat liver *in vivo*. Endogenous intracellular [ammonia] appeared to be as much as 60 times greater than extracellular [ammonia]. However, calculation of $[ammonia]_{inside}$ makes the assumption that ammonia is equally distributed throughout the cell. Separation of cell cytosol from mitochondria in cells incubated without added substrates showed that this assumption is not justified, since there was a seven-fold ratio of ammonia between mitochondria and cell cytosol and a 14-fold ratio of ammonia between cytosol and medium. Mechanisms for maintenance of gradients are dependent on the permeability properties of membranes. In the next section, theories concerning the possible mechanisms for maintaining ammonia gradients are discussed.

ii. Permeability of Membranes to Ammonia

Since NH_3 and NH_4^+ exist in solution at physiological pH (7.4) in proportions of approximately 1:100, both species must be taken into account in considering mechanisms for maintaining an ammonia gradient. The large hydration diameter and charge of NH_4^+ are thought to render this ion relatively impermeable. For example, Binstock & Lecar (1969) suggest that it is five times less permeable than K^+ in squid axon. NH_4^+ may permeate the membrane slowly via the Na-K ATPase (for example, in kidney, Kinsölving & Post, 1960). However, NH_3 is generally thought to be the main permeating species of ammonia, despite the small proportion of it which exists at physiological pH (Jacobs, 1940). The high lipid and water solubility of NH_3 means that it can move down concentration gradients across membranes faster than O_2 and CO_2. Once across the membrane, the following equilibrium will have to be restored:

$$NH_4^+ \rightleftharpoons NH_3 + H^+$$

The result is that for every 100 molecules of NH_3 entering a cell, about 99 will be converted to NH_4^+ at the expense of H^+ from inside. The following factors could limit the net entry of NH_3:

1. Lack of a concentration gradient of NH_3 between inside and outside.
2. Unavailability of H^+ for generation of NH_4^+.
3. Limited movement of counterions to balance the NH_4^+ generated from NH_3.

Different mechanisms which have been proposed for the generation of ammonia gradients require incompatible permeability properties of membranes. For exmaple, Hindfelt (1975), Mossberg (1967) and Mossberg & Ross (1967) propose that ammonia gradients are created by active pumping of NH_4^+ from cerebrospinal fluid to brain (Hindfelt), and from mucosal to serosal sides of ileal sacs (Mossberg & Ross). This would require either that NH_3 is non-existent or that the membrane is totally impermeable to NH_3, since otherwise, in a steady state, any pumping of NH_4^+ would be immediately counteracted by back diffusion of NH_3. The second mechanism proposed for maintainance of an ammonia gradient also requires that the membrane is impermeable to NH_3. The proposal is that NH_4^+ distributes passivly according to the membrane potential, on the assumption that the ammonia gradient can be calculated from the Nernst equation:

Equation (4)

$$mV = -61 \log \frac{[NH_4^+]_{inside}}{[NH_4^+]_{outside}} \qquad \text{(at 37°C)}.$$

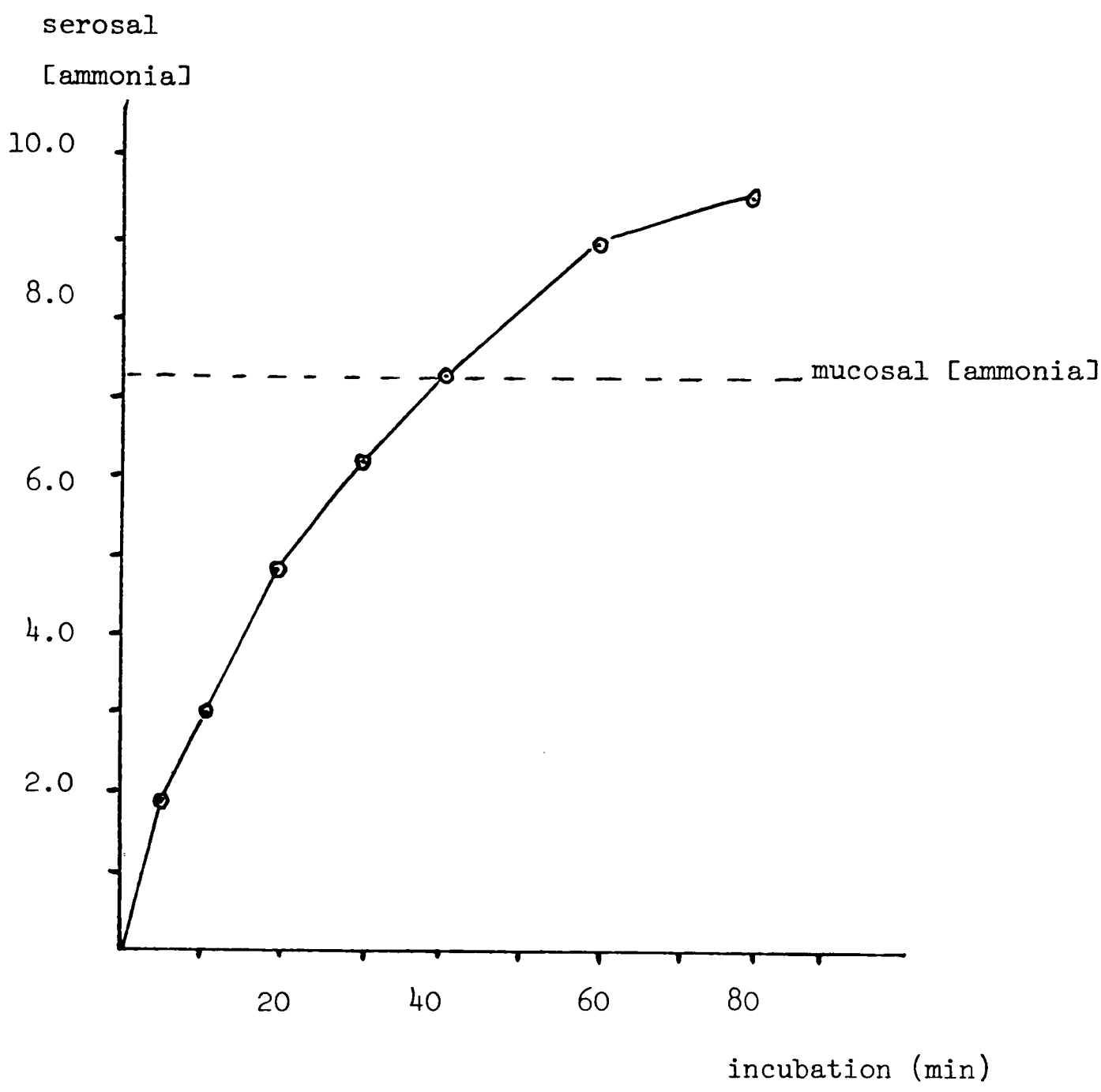
Since the membrane potential of rat liver is in the order of 36 mV (Baur et al., 1975), the ammonia gradient should on this hypothesis be 3.9, which is considerably less than the 66-fold ratio found in hepatocytes. The third proposal is by Stabenau et al. (1959). They suggest that the ammonia gradient between blood and cerebrospinal fluid is entirely consistent with distribution of ammonia according to a

pH gradient (see Equation (2)). This mechanism requires that the membrane is highly permeable to NH_3 and relatively impermeable to NH_4^+.

Bearing these three proposed mechanisms in mind, it is of interest to discuss the rate of entry of added ammonia seen in diagram 6:2 and table 6:4, and to compare it with entry of ammonia into ileal sacs (Mossberg & Ross, 1967).

Jacobs (1940) proposed that NH_3 permeates membranes extremely rapidly whereas NH_4^+ moves through membranes relatively slowly. If this is correct, then during the first two minutes after addition of NH_4Cl (10 mM), NH_3 diffuses inwards down a concentration gradient, and, once inside, the $NH_4^+ \rightleftharpoons NH_3 + H^+$ equilibrium is restored, chiefly at the expense of H^+ from the intracellular H_2CO_3 buffering system. Cell pH would tend to increase and extracellular pH to fall. (Aickin & Thomas (1977) measured with intracellular microelectrodes a similar pattern of pH changes when they added $(NH_4)_2SO_4$ (10 mM) to muscle fibres.) At two minutes, according to my data.(diagram 6:2 and table 6:4), the ammonia and pH gradients are decreased to 1.0. During the next 78 minutes, ion pumps restore cell pH, either by exchanging H^+ from the medium with intracellular Na^+, or by $HCO_3^- - Cl^-$ exchange. Both these mechanisms have been proposed for snail neurones by Thomas (1976, 1977). In addition there will be slower entry of NH_4^+ from the medium, and H^+ will be generated intracellularly during urea synthesis. The final steady state distribution of ammonia in these circumstances would reflect a pH gradient of about 0.3 pH units, lower on the inside. Since intracellular pH has been estimated to be about 7.22 (Krebs, 1967) and that of the medium is about 7.45, the hypothesis is not implausible. The curve of diagram 6:2 is similar to that of entry of ammonia from mucosal to serosal sides of inverted ileal sacs (diagram 6:3, from Mossberg & Ross, 1967). However, Mossberg & Ross's findings are not consistent with the above hypothesis. In their experiments the sac interior (serosal side) and medium (mucosal side) contained Krebs-Ringer

DIAGRAM 6:3. Entry of Ammonia into the Ilial Sac (after Mossberg & Ross, 1967)



(7.1 mM NH_4Cl was added to the Krebs Ringer Bicarbonate of the mucosal side of the sac at zero time. Incubation was aerobic and at 37°C.)

bicarbonate; NH_4Cl (7.1 mM) was added to the mucosal side, and the concentration of ammonia on the serosal side was measured over an 80 minute time course. The striking difference between Mossberg & Ross's results and those reported in diagram 6:2 is that ammonia entered the serosal compartment down a chemical gradient at a considerably slower rate, so that the gradient was decreased to 1.0 only after 35 Minutes, as compared with two minutes. This suggests that ammonia entry was much slower than would be expected if NH_3 permeates membranes rapidly, for NH_3 entry was not limited by H^+ availability. After 80 minutes of incubation, Mossberg & Ross found a final ammonia ratio of about 1.3, greater on the serosal side, and a pH gradient with pH lower on the serosal side. This is consistent with the hypothesis that ammonia distributes according to a pH gradient. However, in other experiments they replaced bicarbonate buffer with phosphate buffer and this abolished the ammonia ratio; yet the pH gradient was similar to that found in the experiments with bicarbonate buffer. This suggests that HCO_3^- was necessary for uphill transport of NH_4^+, and is inconsistent with the hypothesis that ammonia distributes according to a pH gradient.
In other experiments they added HCl in the presence of bicarbonate buffer to the mucosal compartment, and there was a pH gradient of 0.6, higher on the inside. The ammonia ratio was the same as that found when the pH gradient was in the other direction. This indicates that NH_3 did not penetrate membranes rapidly, and Mossberg & Ross were forced to postulate an active transport mechanism for maintainance of the ammonia ratio in ileal sacs. These findings are extremely puzzling in the light of the view that NH_3 migrates rapidly across membranes.

Taking into consideration Mossberg & Ross's findings, it is conceivable, but I think unlikely, that NH_4^+ is the major permeating species. If this were true, the curve of diagram 6:2 could be explained in the following way. During the first two minutes, NH_4^+ enters down an electrochemical gradient and the intracellular $NH_4^+ \rightleftharpoons NH_3 + H^+$

equilibrium would be restored, with the result that H^+ is generated in the cell. Since only 1.0 mM H^+ would be generated for every 10 mM NH_4^+ entering, intracellular buffers would probably prevent any pH change. Extracellular pH would tend to increase. An ammonia gradient could then be established over the following 78 minutes, either passively according to the membrane potential (equation (4)), or by active pumping of NH_4^+ inwards. The pH changes occurring as a result of this mechanism of ammonia transport are not consistent with those measured by Aickin & Thomas (1977) in muscle fibres with pH electrodes, for on addition of $(NH_4)_2SO_4$ they measured an increase in cell pH and a decrease in medium pH.

At present, it is not possible to determine which of these explanations, if either, is correct. The first hypothesis, which assumes that NH_3 permeates membranes rapidly, would seem more likely in the light of the experiments of Aickin & Thomas (1977). Direct measurement of intracellular pH should shed light on the mode in which ammonia enters liver cells: on addition of NH_4Cl to the medium, a rapid increase in intracellular pH should be followed by a slow decrease, if NH_3 is the permeating species. Furthermore, increase in rate of restoration of cell pH by increasing HCO_3^- - Cl^- exchange or H^+ - Na^+ exchange should, in this case, increase the rate of ammonia uptake by hepatocytes.

iii. Is Part of Intracellular Ammonia in Bound Form?

The question of what mechanisms maintain ammonia ratios greater than about 3.0, and of what modes of transport are related to such mechanisms, fade away if the major part of ammonia within hepatocytes is in bound form: for then large ratios may be associated with only small gradients.

In the experiments reported in table 6:2, when varying amounts of ammonia were added to the medium, an explanation of the resulting ammonia ratios was offered in terms of the hypothesis that part of the endogenous intracellular ammonia is bound. To the extent that

there were differences between concentration of free intracellular ammonia and concentration of ammonia in the medium, these were explainable in terms of distribution of ammonia according to a pH gradient between cell and medium. There are further reasons for probing this hypothesis more deeply.

The six-fold mitochondrial/cytosolic ratio of ammonia is hard to explain in the light of Chappell's experiments, which demonstrated a rapid rate of ammonia entry into the mitochondria (Chappell & Crofts, 1966). As has been discussed, high NH_3 permeability would lead to distribution of ammonia according to a pH gradient. With a cytosolic pH of 7.22 (Krebs, 1977), and the pK_a of ammonia 9.49 (Jacquez et al., 1959), the intramitochondrial pH required to obtain an ammonia ratio of 7.0 would be 6.36. So far, attempts to estimate the intramitochondrial pH have indicated that it is higher than the cytosol (Mitchell & Moyle, 1967, 1969, by the detergent method; Nicholls, 1974, by distribution of weak acid and weak base method). Thus the pH explanation of the high ammonia ratio across mitochondrial membranes can be eliminated. High permeability of the mitochondrial membrane to NH_3 renders the membrane potential explanation void, since backflux of NH_3 would dissipate the NH_4^+ gradient. So far, the most convincing explanation of the high ammonia ratio between mitochondria and cytosol is that part of measured ammonia is bound (Harris & Bassett, 1971; Hoek, 1972).

In the present state of knowledge, the truth or falsehood of the bound ammonia hypothesis cannot be finally settled. However, in the remainder of this discussion I shall consider some of the evidence for and against it.

An interesting piece of supposed evidence against the bound ammonia hypothesis cited by Brosnan (1976) has arisen from work on the glutamate dehydrogenase (GLDH) reaction which takes place in the mitochondrial matrix (see Equation (5)).

Equation (5)

$$\frac{[\alpha\text{-oxoglutarate}] \times [NH_4^+] \times [H^+] \times [NADH]}{[\text{glutamate}] \times [NAD] \times [H_2O]} = K$$

($K = 2.95 \times 10^{-14} M^2$, 25°C, I=0.47, Engel & Dalziel, 1967.)

Values for K (in equation (5)) from experiments with purified enzymes *in vitro* indicated that GLDH is in equilibrium or near equilibrium. Calculation of a theoretical $[NH_4^+]$ value from K and and the measured values of the other reactants was found to be in the order of 0.7 mM. Direct estimation of $[NH_4^+]$ in liver tissue also gave a value in the order of 0.7 mM (though it must be emphasised that this latter value is based on the assumption that ammonia is distributed equally throughout the cell). Other evidence that the GLDH reaction was indeed near equilibrium came from experiments in which metabolic conditions were varied greatly (Williamson et al., 1967; Brosnan et al., 1970). This reinforced the idea that K values for the GLDH reaction derived from experiments *in vitro* could be extrapolated to those for the mitochondrial matrix *in vivo*. This in turn suggested that $[NH_4^+]$ values in equation (5) were correct. Greatly lower $[NH_4^+]$ values would indicate that GLDH was not an equilibrium enzyme. Equations such as (5) above require that all reactants be in aqueous solution. Thus it was thought to follow that all the measured $[NH_4^+]$ must represent ammonia free in solution, and not bound. Following this line of argument, Brosnan proposed that, since GLDH was an equilibrium enzyme, measured cell ammonia content was in solution.

However, results given in this thesis show that the assumption that ammonia is equally distributed throughout the cell is not correct. This means that the fact that the value of 0.7 mM $[NH_4^+]$ was similar by both methods of estimation (see above) was fortuitous. My value of total intramitochondrial ammonia was 3.3 mM. Furthermore, if the assumption is made that there is no ammonia gradient across the outer mitochondrial membrane, an ammonia value of 12.0 mM can be calculated

for the mitochondrial matrix. At present, there is no way of knowing how much of this ammonia is free or bound, though it is my belief that the greater part is bound and that the matrix free [ammonia] is in the order of 0.03 mM. The suggestion that the value for free mitochondrial matrix [ammonia] could lie anywhere between 0.03 and 12.0 mM casts doubt on the validity of the K value for the GLDH reaction in vivo. Furthermore, if intramitochondrial matrix [ammonia] was as low as 0.03 mM the GLDH reaction might no longer be in near equilibrium. From this it follows that the GLDH equilibrium argument cannot be used decisively to establish the free ammonia hypothesis.

Other pieces of evidence are consistent with the bound ammonia hypothesis. The experiments in which urea synthesis was stimulated by addition of ornithine showed that a pool of endogenous ammonia was not accessible to urea cycle enzymes. These experiments were similar to those of Hoek (1972), who added NH_4Cl (5.0 mM) to isolated mitochondria and measured intracellular and extracellular [ammonia]. A rapid removal of added ammonia by citrulline synthesis decreased $[ammonia]_{outside}$ to 0.1 mM. The initially high intramitochondrial ammonia decreased from 9.0 mM at 10 minutes to 3.0 mM at 25 minutes, after which citrulline synthesis ceased almost completely. Hoek concluded that since the K_m of carbamyl phosphate synthetase for ammonia is about 0.7 mM (Charles et al., 1967; 0.25 mM, Elliott, 1976), the residual 3.0 mM ammonia was not free to react in citrulline synthesis.

That the maintenance of a high ammonia ratio requires energy is consistent with the bound ammonia hypothesis. In the absence of metabolic energy, both cytosolic and mitochondrial compartments increase in volume (see Russo et al., 1977). This would tend to dilute intracellular constituents which could change the amount of ammonia, phosphate and magnesium bound in NH_4MgPO_4. Absence of metabolic energy might also lead to decrease in cell pH, which would tend to bring NH_4MgPO_4 into solution (see diagram 6:1). Furthermore, decrease

of availability of metabolic energy leads to decrease in ion gradients across membranes. This might result in loss of free Mg^{2+} and P_i from the matrix, which would lead to release of ammonia, Mg^{2+} and P_i from NH_4MgPO_4. Both Mossberg & Ross and Brosnan suggest that inhibition of energy supply abolishes the ammonia gradient because energy is directly required for pumping ammonia. However, energy may be required indirectly for maintaining the ammonia ratio.

On addition of ouabain, increased intracellular [ammonia] was found (section iv). So far, there is no explanation of this, though the observation of Russo et al. (1977) that ouabain markedly increased the number of dense granules in mitochondria may have some bearing on the problem. Lehninger (1970) showed a micrograph of rat liver mitochondria after respiration-coupled accumulation of Ca^{2+} and P_i. He believes that the mass of dark electron dense bodies correpond to granular deposits having the composition of hydroxyapatite. It is not unreasonable to suggest that NH_4MgPO_4 may also be electron dense and could thus be seen as dense granules in ouabain treated cells. Electron micrographs of hepatocytes subjected to ammonia loads in high phosphate and magnesium medium should also contain additional dense granules. This experiment has not yet been carried out.

A further observation which is compatible with the bound ammonia hypothesis is that, during separation of cells from medium in the bulb separation devices, there was no apparant loss of intracellular ammonia, yet intracellular water, Na^+ and urea were greatly decreased during this process. This suggests that ammonia is not in aqueous solution.

The experiments in which cytosol was separated from mitochondria raise one problem. Formation of NH_4MgPO_4 in the mitochondrial matrix seems plausible, since the matrix contains high contents of both P_i (Thiers & Vallee, 1957; Wilson et al., 1974) and of Mg^{2+} (Thiers & Vallee, 1957; Lehninger et al., 1967; Veloso et al., 1973), and the pH

is higher in the matrix than in the cytosol. Deposits of NH_4MgPO_4 in the cell cytosol are far less likely because cytosolic pH is more acid and cytosolic Mg^{2+} and P_i are lower than in the matrix. However, the separation experiments showed that the [ammonia] of the cytosol was much higher than in the medium (0.43 mM and 0.033 for the cytosol and medium respectively). It is possible that ammonia was lost from the matrix to the cytosol during separation, as was mitochondrial K^+. Part of the explanation of the high cytosolic [ammonia] could be that lysozomes have a pH as low as 5.0 (Mego, 1971) and would tend to accumulate ammonia to a concentration of about 30 times that in the cytosol. However, lysosomal volume is merely 0.4% of the total hepatocyte volume (Bolender & Weibel, 1973), so this cannot account for all the extra ammonia of the cytosol. Formation of $NH_4Mg\ PO_4$ is unlikely to occur in lysozomes since at lysosomal pH this salt is in solution. A further possibility is that ammonia is bound in some way to plasma membranes, and on digitonin treatment is liberated into the cytosol. So far, there is no adequate solution to this problem.

iv. The Implications of the Bound Ammonia Hypothesis

If most of the intracellular ammonia is bound, and free ammonia is distributed according to pH gradients, then mitochondrial matrix 'free' ammonia will be in the order of 0.03 mM, as is found in the cell medium. This concentration is an order of magnitude less than that required for GLDH to be an equilibrium enzyme. The whole question of whether or not glutamate dehydrogenase is a near equilibrium enzyme is in dispute (for example, see McGivan & Chappell, 1975).

Another question is that of the role of NH_4MgPO_4, if it does indeed exist. The possibility arises that this complex could act as an ammonia 'buffer', which ensures that the liver traps all surplus ammonia entering the liver from the portal vessels. For example, temporary storage of ammonia as NH_4MgPO_4 following a high portal load of ammonia after a high protein intake would avoid overloading urea

cycle enzymes and elevation of liver arterial ammonia above 0.02 mM. The toxicity of high blood [ammonia] is well established (see Katanuma et al., 1966). Uptake of ammonia into the NH_4MgPO_4 complex could have more subtle implications, since free matrix [Mg^{2+}] and [P_i] would be concomitantly decreased, and H^+ would be generated (see diagram 6:1). Alterations in concentrations of these ions could, for example, have a regulatory role in oxidative phosphorylation.

Three main questions arise from work on the distribution of ammonia across membranes. The first lies in determining the permeability of the membrane to NH_3 and NH_4^+. Related to this is the question of the transport mechanisms of NH_4^+ and NH_3, especially that of an active transport mechanism, apart from the Na-K ATPase, for NH_4^+. The second question is to determine the species of ammonia which predominantly migrates through hepatocyte membranes. The third question is that of the existence of bound forms of ammonia in cells. If the answer to the first two questions is that NH_4^+ can be actively transported against a concentration gradient and that NH_3 is not the major permeating species, then the bound ammonia hypothesis is unnecessary. At the present, however, evidence for storage of ammonia in a non-diffusible form, for rapid transport of NH_3 through membranes and for low permeability of membranes to NH_4^+, begins to mount. The most convincing counter evidence comes from Mossberg & Ross (1967). However, it is possible that the serosal compartment of ileal sacs in their experiments accumulated Mg^{2+} and P_i from the medium, in which case Mossberg and Ross may have been misled in treating the measured ammonia as free ammonia. The ileal sac preparation may be of use in further investigations of this interesting problem.

CHAPTER 7. THE EFFECTS OF FRUCTOSE ON THE DISTRIBUTION OF K^+ AND P_i BETWEEN HEPATOCYTES AND SUSPENSION MEDIUM

Introduction

The work presented in this chapter was initiated by the finding that on addition of fructose to hepatocytes intracellular $[K^+]$ was significantly increased (see Chapter 6 section vii). In order to discuss the effects of fructose on intracellular ion content, it is first necessary to describe the metabolic events associated with fructose loading.

i. The Metabolism of Fructose in Liver

Following a fructose loading of liver, fructose is rapidly phosphorylated to fructose-1-phosphate (F-1-P) by the ketohexokinase (EC 2.7.1.3.) reaction:

(1) Fructose + ATP $\rightleftharpoons$ F-1-P + ADP

There is a concomitant depletion of adenine necleotides (Mäenpää et al., 1968). The removal of F-1-P is brought about by the ketose-1-phosphate aldolase (EC 4.1.2.7.) reaction:

(2) F-1-P $\rightleftharpoons$ dihydroxyacetone phosphate + D-glyceraldehyde

However, during the metabolism of fructose, reaction (2) above is partially inhibited by the increase in inosine monophosphate concentration which follows the adenine nucleotide depletion (Woods et al., 1970). Phosphorylation and subsequent accumulation of F-1-P depletes the cell of ATP, the resynthesis of which ultimately depletes the cell of inorganic phosphate (P_i). P_i is taken up from the extracellular medium (Sestoft, 1974). The extent to which the F-1-P acts as a phosphate sink can be seen from the data given by Woods (1970). In perfused rat liver, after 10 minutes of incubation, F-1-P was elevated from a basal value of 0.23 µmol/g wet wt. to 8.72 µmol/g wet wt. In addition, inosine monophosphate and α-glycerophosphate were increased by about 1.0 µmol/g wet wt. each. This means that approximately 10.5 µmol/g wet wt. phosphate was required to synthesise F-1-P, inosine monophosphate and α-glycerophosphate in these conditions. Adenine nucleotides could

provide about 1.89 µmol/g wet wt. phosphate, and the intracellular store of phosphate a further 2.58 µmol/g wet wt. The deficit of at least 6.25 µmol/g wet wt. phosphate is likely to have been derived from the extracellular medium during the first 10 minutes of incubation.

ii. Relationships Between Fructose Metabolism and Ionic Movements

At least four factors in fructose loading may be related to the disturbance of ions such as K^+ and Na^+ in hepatocytes:

1. Depletion of ATP may deprive active membrane transport systems of energy supply (Folke, 1974). In this case, decreased rate of the Na-K ATPase would lead to an increase in intracellular $[Na^+]$ and a decrease in intracellular $[K^+]$.

2. Movement of P_i across the membrane may:

(a) indirectly require movement of a counter ion either in the same direction (+ve) or in the opposite direction (-ve) to maintain electrical balance between cell and medium.

Phosphate in physiological conditions is distributed according to the following equilibrium:

$$H_3PO_4 \rightleftharpoons H_2PO_4^- + H^+ \rightleftharpoons HPO_4^{2-} + H^+ \rightleftharpoons PO_4^{3-} + H^+$$

(pK_a 1.959) (pK_a 6.7) pK_a 12.44)

(Umbreit 1964.) Thus most phosphate in physiological conditions will either be in the form $H_2PO_4^-$ or HPO_4^{2-}. It is not known in which form the P_i crosses plasma membranes and therefore how many negative charges will enter the cell with each molecule of P_i;

(b) be directly linked with movement of a co-ion or counter ion in a specific membrane carrier system. A stoichiometric movement of the co-ion or counter ion would then be expected;

(c) involve a specific electrogenic mechanism which requires energy. This will lead to a direct increase in membrane potential.

3. Potassium movement may be related to the marked changes in pH which occur during fructose metabolism (Folke, 1974).

4. Increase in intracellular lactate which occurs during fructose metabolism may lead to an increase in intracellular cation or decrease in intracellular anion (see Chapter 3 section 4).

The aims of the experiments in this chapter are:

(a) To establish that movement of P_i from the incubation medium into isolated hepatocytes occurs in conditions of fructose metabolism;

(b) to investigate the relationship between changes in intracellular K^+ and Na^+ with movement of P_i;

(c) to ascertain whether increase in K^+ content is due to an increase in rate of K^+ influx or to a change in the permeability of the membrane to K^+ by means of $^{42}K^+$ experiments;

(d) to establish the role of the Na-K ATPase in transport of phosphate.

iii. The Clinical Relevance of the Understanding of the Effects of Fructose Metabolism in Liver

Elucidation of the mechanism underlying the ionic and metabolic disturbances caused by fructose loads is of interest to clinicians. As early as 1910, Van Noorden undertook trials of oral fructose in diabetic patients to ascertain its value as a replacement for glucose. Since then, fructose has been utilized by clinicians in the following ways:

1. As a source of parenteral nutrition (Allen & Lee, 1969).
2. As an antiketogenic agent (Rosecan & Daughaday, 1954).
3. As a sweetening agent for patients with diabetes mellitus (Marks, 1971).
4. In treatment of acute alcohol intoxication (Brown et al., 1972).

The dangers to patients in the use of fructose infusions have been pointed out by Woods & Alberti (1972).

In addition to the use of fructose in clinical medicine, a syndrome, Hereditary Fructose Intolerance (H.F.I.) was recognised and

described in 1965 by Chambers & Pratt. It is characterized by severe hypoglycaemia, vomiting and decreased serum P_i after injection of fructose. The biochemical findings are absence of hepatic fructose-1-phosphate aldolase and an accumulation of F-1-P.

Results

i. The Effect of Fructose on Intracellular K^+, Na^+, P_i and ATP Content of Hepatocytes

Table 7:1 shows the effect of fructose (10 mM) on the K^+ distribution in hepatocytes. $[K^+]$ was not changed at 10 minutes of incubation. However, at both 20 and 60 minutes of incubation the $[K^+]$ was significantly increased in the presence of fructose ($P<0.01$, paired *t*-test). (Cell volume was not significantly altered by fructose at 20 or 60 minutes (see table 4:1).)

Table 7:2 shows the effects of fructose on the Na^+ and K^+ distribution in hepatocytes in one experiment. After incubation with fructose for 10 minutes, intracellular K^+ content was not elevated, but Na^+ content was elevated by 18 μmol/g dry wt. Thus intracellular Na^+ was elevated initially whilst K^+ was not, but by 60 minutes K^+ was elevated and Na^+ was not.

Table 7:3 shows the effects of fructose on $[P_i]$ and [ATP] in hepatocytes. P_i was measured separately in cells and medium, and ATP was measured in the whole perchloric extract of cells together with medium in parallel flasks. In the presence of fructose, intracellular P_i content was decreased by 31% after 10 minutes of incubation. By 20 minutes of incubation intracellular $[P_i]$ was decreased by 65% as compared with $[P_i]$ in cells incubated with fructose at 20 minutes (the difference was significant: $P<0.01$, paired *t*-test). At 60 minutes, intracellular $[P_i]$ had increased in fructose treated cells, but was still significantly less than in cells without fructose

Table 7:1. The effect of fructose on the distribution of K^+ between hepatocytes and medium

Experimental details are described in Chapter 2 and the text. Values are means ± S.E.M. and the number of observations is in parenthesis. Fructose was added to an initial concentration of 10 mM. Values with fructose which are statistically different from those without by Student's t-test are indicated by * = $P < 0.05$ and ** = $P < 0.01$.

		Incubation (min)		
	Fructose	10	20	60
K^+ inside µmol/g dry wt.	None	310 ± 18.0 (3)	290 ± 8.4 (7)	278 ± 4.7 (6)
	10.0 mM	314 ± 14.1 (3)	318 ± 5.1** (7)	319 ± 5.3** (6)
$[K^+]$ inside mM	None	163 ± 10.5 (3)	157 ± 4.3 (7)	151 ± 2.5 (6)
	10.0 mM	166 ± 7.6 (3)	169 ± 2.73** (7)	170 ± 2.7** (6)
$[K^+]$ outside mM	None	5.75 ± 0.09 (3)	6.06 ± 0.11 (7)	6.14 ± 0.12 (6)
	10.0 mM	5.75 ± 0.09 (3)	5.81 ± 0.05 (7)	5.80 ± 0.07 (6)

Table 7:1. The effect of fructose on the distribution of K^+ between hepatocytes and medium

Experimental details are described in Chapter 2 and the text. Values are means ± S.E.M. and the number of observations is in parenthesis. Fructose was added to an initial concentration of 10 mM. Values with fructose which are statistically different from those without by Student's t-test are indicated by * = $P < 0.05$ and ** = $P < 0.01$.

		Incubation (min)					
	Fructose	10		20		60	
K^+ inside µmol/g dry wt.	None	310	± 18.0	290	± 8.4	278	± 4.7
	10.0 mM	314 (3)	± 14.1	318 (7)	± 5.1**	319 (6)	± 5.3**
$[K^+]$ inside mM	None	163	± 10.5	157	± 4.3	151	± 2.5
	10.0 mM	166 (3)	± 7.6	169 (7)	± 2.73**	170 (6)	± 2.7**
$[K^+]$ outside mM	None	5.75 ± 0.09		6.06 ± 0.11		6.14 ± 0.12	
	10.0 mM	5.75 ± 0.09 (3)		5.81 ± 0.05 (7)		5.80 ± 0.07 (6)	

Table 7:2. The effect of fructose on the distribution of Na^+ and K^+ between cells and medium in hepatocytes

Experimental details are described in Chapter 2 and the text. The values are from one experiment. Fructose was added to an initial concentration of 10 mM. Cells were preincubated without fructose for 20 min. Fructose was added at zero time.

	Fructose	Incubation (min) 10	20	60
$[K^+]$ inside mM	None	160	158	152
	10 mM	160	168	168
$[K^+]$ outside mM	None	5.67	5.61	5.72
	10 mM	5.56	5.56	5.56
K^+ inside µmol/g dry wt.	None	302	298	288
	10 mM	302	317	317
$[Na^+]$ inside mM	None	25.4	21.6	32.5
	10 mM	34.8	36.6	32.2
$[Na^+]$ outside mM	None	147	147	147
	10 mM	147	147	147
Na^+ inside µmol/g dry wt.	None	48.0	40.8	61.5
	10 mM	65.8	69.2	61.0
$Na^+ + K^+$ µmol/g dry wt.	None	350	339	350
	10 mM	368	386	378

Table 7:3. The effect of fructose on phosphate and ATP content in hepatocytes

Experimental details are described in Chapter 2 and the text. The values are means ± S.E.M. and the number of observations is in parenthesis. Fructose was added to an initial concentration of 10 mM. Phosphate was measured in cells and medium separately. ATP was measured in parallel flasks in the same experiment on the whole perchloric acid extract of cells together with medium. Values with fructose which are statistically different from those without by Student's t-test (paired) are indicated by * = $P < 0.05$ ** = $P < 0.01$.

		Incubation (min)		
	Fructose	10	20	60
P_i inside µmol/g dry wt.	None	7.38 (1)	6.22 ± 0.68 (5)	4.97 ± 0.46 (4)
	10 mM	5.11	2.20 ± 0.27** (5)	3.42 ± 0.82* (4)
$[P_i]$ inside mM	None	3.82 (1)	3.36 ± 0.35 (5)	2.70 ± 0.25 (4)
	10 mM	2.65 (1)	1.17 ± 0.13** (5)	1.82 ± 0.44* (4)
$[P_i]$ outside mM	None	1.02 (1)	1.11 ± 0.09 (5)	1.15 ± 0.12 (4)
	10 mM	1.01 (1)	0.96 ± 0.07** (5)	1.00 ± 0.11** (4)
ATP µmol/g wet wt.	None	2.06 (2)	1.96 ± 0.09 (5)	1.94 ± 0.06 (3)
	10 mM	0.72 (2)	0.67 ± 0.06** (5)	1.04 ± 0.17** (3)

($P<0.05$, paired t-test).

After 10 minutes of incubation of hepatocytes with fructose extracellular $[P_i]$ was unchanged, but by 20 minutes $[P_i]$ in the medium of fructose treated cells was 0.96 mM as compared with 1.11 mM in cells without fructose; thus the 4 ml of medium had lost 0.60 µmoles to the cells at 20 minutes. This was equivalent to an uptake of 7.10 µmol/g wet wt. (26.20 µmol/g dry wt.) P_i by the hepatocyte. At 60 minutes of incubation, extracellular P_i showed a slight tendency to increase towards control values. The difference in P_i in the suspension medium between fructose and control cells was significant at 20 and 60 minutes ($P<0.01$, paired t-test). Thus at 20 and 60 minutes of incubation $[P_i]$ was significantly decreased in cells and medium.

[ATP] was significantly lower in the presence of fructose at 20 and 60 minutes of incubation ($P<0.01$, paired t-test).

ii. The Effect of Fructose on the Rate of Turnover of K^+ and on the Uptake of $^{42}K^+$ into Hepatocytes

The results of two experiments in which $^{42}K^+$ and fructose were added simultaneously to preincubated cells is given in table 7:4. The rate of turnover of K^+ (for calculation, see Chapter 2 section 7) was similar in both conditions, though it was slightly lower in fructose treated cells. The difference between fructose treated and control cell K^+ content increased throughout the incubation (fructose treated cells contained an additional 8, 11, 21 and 39 µmol/g dry wt. at 10, 20, 30 and 40 minutes respectively). This difference increased because fructose treated cells gained 6 µmol/g dry wt. K^+ from 10 to 60 minutes, whereas control cells lost 25 µmol/g dry wt. during the same period.

In fructose treated cells, the lowered rate of K^+ turnover together with increased K^+ content suggests that the permeability of the cell to K^+ may have been decreased. The evidence should be

Table 7:4. The effect of fructose on $^{42}K^+$ content, K^+ turnover and intracellular K^+ content of hepatocytes

Experimental details are described in Chapter 2 and the text. Values are means of two experiments. Fructose was added to an initial concentration of 10.0 mM. Cells were preincubated for 20 min without $^{42}K^+$ and fructose. Fructose and $^{42}K^+$ were added at zero time. The ratio is: $\frac{\text{Intracellular } ^{42}K^+ \text{ (cpm/g dry wt.)}}{\text{Extracellular } ^{42}K^+ \text{ (cpm/ml)}}$

		Incubation (min)			
Fructose		10	20	30	40
K^+ inside µmol/g dry wt.	None	316	310	304	291
	10 mM	324	321	325	330
% tissue K^+ turned over/min	None	1.72	1.55	1.57	1.52
	10 mM	1.52	1.41	1.42	1.50
Ratio	None	8.5	14.4	20.4	23.8
	10 mM	8.6	14.1	20.5	27.5

treated with caution in virtue of the small number of experiments.

iii. The Effect of Ouabain on the Entry of P_i into Hepatocytes, and on the Distribution of K^+ inside and outside, in the Presence of Fructose

Table 7:5 shows that ouabain had no significant effect on intracellular P_i content in fructose loaded cells as compared with those incubated with fructose alone. For example, at 20 minutes 7.75 µmol/g wet wt. P_i had entered the fructose loaded cells treated with ouabain, as compared with 8.36 µmol/g wet wt. with fructose alone. This indicates that inhibition of the Na-K ATPase did not affect entry of P_i into hepatocytes either directly or indirectly. At 60 minutes, extracellular P_i was further decreased in fructose with ouabain treated cells, whereas in cells with fructose alone some recovery of the P_i content of the medium had occurred.

The ATP content of fructose loaded cells treated with ouabain was not different from cells incubated with fructose alone at 20 minutes, but was significantly different at 60 minutes ($P<0.01$, paired t-test); thus the cells treated with ouabain and fructose recovered their ATP content more slowly than cells treated with fructose alone (table 7:5).

Table 7:6 shows the effects of ouabain on the intracellular $[K^+]$ in the presence of fructose (10 mM). Intracellular K^+ content (µmol/g dry wt.) was significantly higher with fructose together with ouabain treated cells than with ouabain alone at 20 and 60 minutes ($P<0.05$, paired t-test). However, intracellular $[K^+]$ (mM) was not significanly different at these times. This was because cell volume increased when cells incubated with fructose were treated with ouabain, whereas cell volume did not increase with fructose alone. The significant increase in intracellular $[K^+]$ found with fructose loaded cells was not found when fructose with ouabain treated cells were compared with ouabain treated cells. In one experiment in which Na^+ and K^+ were

Table 7:5. The effect of ouabain on P_i and ATP content of hepatocytes incubated with fructose

Experimental details are described in Chapter 2 and the text. Values are means ± S.E.M. and the number of observations is three. Ouabain was incubated with hepatocytes for 20 min of preincubation before addition of fructose at zero time. Fructose was added to an initial concentration of 10 mM and ouabain to an initial concentration of 0.5 mM. Values with ouabain and fructose which are statistically different from those with fructose alone by Student's t-test (paired) are indicated by ** = $P < 0.01$

	Additions	Incubation (min) 20	60
P_i inside µmol/g dry wt.	Fructose	1.98 ± 0.19	3.42 ± 0.82
	Fructose + ouabain	1.36 ± 0.34	2.49 ± 0.39
$[P_i]$ inside mM	Fructose	1.06 ± 0.10	1.82 ± 0.44
	Fructose + ouabain	0.70 ± 0.18	1.25 ± 0.19
$[P_i]$ outside mM	Fructose	0.95 ± 0.09	1.00 ± 0.11
	Fructose + ouabain	0.92 ± 0.02	0.89 ± 0.05
ATP µmol/g wet wt.	Fructose	0.68 ± 0.05	1.04 ± 0.17
	Fructose + ouabain	0.59 ± 0.12	0.78 ± 0.17**

Table 7:6. The effect of fructose on the distribution of K^+ between cells and medium in hepatocytes treated with ouabain

Experimental details are described in Chapter 2 and the text. Values are means ± S.E.M. and the number of observations is three. Cells were preincubated with ouabain (0.5 mM) before addition of fructose to an initial concentration of 10 mM at zero time. Values with ouabain and fructose which are statistically different from those with fructose alone by Student's t-test (paired) are indicated by * = $P < 0.05$.

	Additions	Incubation (min) 20		Incubation (min) 60	
K^+ inside µmol/g dry wt.	Fructose + ouabain	188	± 15.1*	185	± 15.1*
	Ouabain	170	± 19.3	164	± 20.5
$[K^+]$ inside mM	Fructose + ouabain	97.8	± 7.1	92.7	± 7.7
	Ouabain	89.7	± 10.4	87.9	± 11.0
$[K^+]$ outside mM	Fructose + ouabain	6.48	± 0.09	6.59	± 0.13
	Ouabain	6.61	± 0.11	6.70	± 0.10

both measured, Na^+ values were similar both in conditions with fructose with ouabain and in conditions of ouabain alone.

iv. The Effect of Fructose on Contents of Some Intermediary Metabolites in Hepatocytes

Table 7:7 shows the effects of fructose on some intermediary metabolites at 20 and 60 minutes of incubation. Although the total content of adenine nucleotides was not significantly altered by fructose, the content of each adenine nucleotide was considerably different. ATP was significantly decreased whilst ADP and AMP were increased both at 20 and 60 minutes in fructose loaded cells.

Fructose loading decreased endogenous urea formation and increased formation of alanine. It is interesting to note that if this decrease in urea formation was caused by decrease in availability of ATP, then other ATP requiring processes such as the Na-K ATPase could also be inhibited. Table 7:1 shows that K^+ content was not only maintained but increased in fructose loading. Na^+ content was increased at 10 minutes (table 7:2), which might indicate that the Na-K ATPase was slightly inhibited at 10 minutes, but this increase of Na^+ did not continue during the subsequent incubation period. This implies that at 20 minutes the Na-K ATPase was operating normally despite the much decreased ATP concentration, whilst urea synthesis was inhibited. It is possible that mitochondrial ATP content was severely depleted (the first step of urea synthesis requiring ATP is intra-mitochondrial) whilst cytosolic ATP was maintained at a concentration which allowed the Na-K ATPase to operate. These findings do not support the hypothesis of Folke (1974) that depletion of ATP deprives active membrane transport of energy supply (see introduction).

The values for glucose and lactate in table 7:7 differ from those found by Woods et al. (1970) because Woods used fed rats whereas cells in these experiments were prepared from 48 hour starved rats. Woods et al. (1970) found that after 80 minutes of liver perfusion

Table 7:7. The effect of fructose on adenine nucleotide, lactate, glucose and urea content of hepatocytes

Experimental details are described in Chapter 2 and the text. The values are means ± S.E.M. and the number of observations is in parenthesis. Fructose was added to an initial concentration of 10.0 mM. Values are expressed in µmol/g wet wt.

	Fructose	Incubation (min) 20	60
ATP	None	1.93 ± 0.07 (5)	1.94 ± 0.06 (3)
	10 mM	0.67 ± 0.05 (5)	1.04 ± 0.17 (3)
ADP	None	0.69 ± 0.12 (5)	0.55 ± 0.27 (3)
	10 mM	1.04 ± 0.17 (5)	1.49 ± 0.56 (3)
AMP	None	0.29 ± 0.03 (5)	0.25 ± 0.02 (3)
	10 mM	0.75 ± 0.06 (5)	0.63 ± 0.16 (3)
Total adenine nucleotides	None	2.91	2.74
	10 mM	2.46	3.16
Lactate	None	2.51 ± 0.79 (3)	2.48 ± 0.31 (3)
	10 mM	42.1 ± 0.45 (3)	87.9 ± 7.0 (3)
Alanine	None	0.52 ± 0.09 (3)	0.60 ± 0.06 (3)
	10 mM	2.71 ± 0.08 (3)	3.60 ± 0.22 (3)
Glucose	None	12.33 ± 0.92 (3)	14.13 ± 1.81 (3)
	10 mM	73.6 ± 4.35 (3)	201 ± 3.70 (3)
Urea	None	5.47 ± 0.77 (3)	-
	10 mM	4.10 ± 0.94 (3)	-

glucose was not elevated above control values, and lactate was elevated to a small extent. Table 7:7 shows that glucose and lactate were formed in considerable amounts in the starved rat liver. (The rates of lactate and glucose formation were 1.47 and 3.35 μmol/min/g wet wt. respectively.) Thus in the starved rat liver, fructose was a gluconeogenic substrate and in the fed rat it is chiefly utilized for oxidative metabolism or lipogenesis. (See also Sestoft, 1974.)

[Lactate] measured in the cells plus medium was 1.0 mM at 20 minutes and 2.1 mM at 60 minutes in the presence of fructose. Since $[lactate]_{inside}$ is approximately equal to $[lactate]_{outside}$, this means that at 60 minutes 2.0 mM lactate inside will be accompanied by an increase in 2.0 mM K^+. This cannot account for the 20 mM increase in $[K^+]_{inside}$ at 60 minutes with fructose (table 7:1).

v. Summary of Events which Occur on Treating Hepatocytes with Fructose

(The events in braces ({}) were not measured in this work, but are well established by other workers.)

0-10 minutes of incubation

a) {Fructose enters the cell and is rapidly phosphorylated by intracellular ATP with a subsequent accumulation of F-1-P.}

b) ATP content decreased (hydrolysis of ATP generates intracellular H^+); intracellular P_i utilized for ATP generation; ADP and AMP content did not change; intracellular P_i content decreased and extracellular P_i content unchanged;

c) intracellular $[Na^+]$ increased; intracellular $[K^+]$ unchanged.

d) Cell volume unchanged.

10-20 minutes of incubation

a) Fructose enters cell and is phosphorylated by ATP.

b) ATP content decreased further; continued hydrolysis of ATP generates H^+; ADP and AMP elevated; intracellular P_i content further decreased and extracellular P_i content also decreased.

c) Intracellular $[Na^+]$ not further changed; intracellular $[K^+]$ increased.

d) Lactate, glucose and alanine formation increased but urea synthesis decreased.

e) No change in cell volume.

20-60 minutes of incubation

a) Fructose in medium almost entirely utilized.

b) Intracellular ATP content increased; intracellular and extracellular ATP contents increased.

c) Elevated intracellular K^+ content maintained; intracellular $[Na^+]$ unchanged.

d) [Lactate], [glucose] and [alanine] further increased.

e) No change in cell volume.

Discussion

i. Comparison of Results With Those Found By Other Workers

Folke (1974) examined the effects of fructose (10 mM) in perfused rat liver (16-18 hour starved). He found an initial rise in plasma $[K^+]$ in the 0-10 minute period of perfusion, followed by a fall below control values for the 10-40 minute period. By 50 minutes, $[K^+]$ was restored to control values. He also found a decrease in plasma pH which reached a peak at 30 minutes and was not restored to control values by 50 minutes. Folke did not measure Na^+ or P_i. Folke's findings differ from those reported in this thesis in that I found no loss of intracellular $[K^+]$ at 10 minutes. Unlike Folke, I found no restoration of $[K^+]$ after 60 minutes of incubation. However, this may be due to the fact that a perfused rat liver of about 4-8 g, as used by Folke, would metabolize 10 mM fructose more rapidly than the 80 mg hepatocytes in my experiments. Results in this chapter do not substantiate Folke's hypothesis that fructose induced ATP depletion leads to inhibition of the Na-K ATPase, since at no time in my experiments was $[K^+]_{inside}$ decreased in the presence of fructose.

Lambotte (1977) measured plasma $[K^+]$, membrane potential and adenine nucleotides in dog liver perfused with 20 mM fructose. He

found an increase in plasma $[K^+]$ throughout a 60 minute period of incubation, and suggests that fructose produces an increase in P_K. This is contrary to my finding that $[K^+]$ in the medium is decreased (intracellular K^+ increased) and that P_K is decreased. However, direct comparison of results may be unjustified, as Lambotte used dog not rat liver. He fails to indicate whether he used fed or starved dogs. The state of the liver (fed or starved) may be important, for not only is the metabolic fate of fructose different between fed and starved animals (see section iv above, and Sestoft, 1974) but also the K^+ content of hepatocytes from fed liver differs from those of starved liver (see table 3:2). Lambotte measured a 15 mV rise in hepatocyte membrane potential in the presence of fructose (20 mM).

Dambach & Friedmann (1974b) measured membrane potential in 12-18 hour starved rat liver perfused with 20 mM fructose and found a hyperpolarization of the membrane. They related this effect to the metabolic process of glucose production from fructose, since they found a similar hyperpolarization with other gluconeogenic substrates such as lactate and alanine. They did not measure K^+, Na^+, P_i, adenine nucleotides or cell volume.

ii. The Effects of Fructose on the Distribution of Ions in Hepatocytes

From the results given in this chapter, it appears that there are at least three stages during metabolism of added fructose in hepatocytes. First, during the first ten minutes of incubation, fructose enters hepatocytes and is rapidly phosphorylated to F-1-P. P_i is mobilized from intracellular stores for the re-synthesis of depleted adenine nucleotides. Secondly, during the 10-25 minute period, P_i enters the cells from the medium. Thirdly, by 60 minutes, movement of P_i out of the cell occurs.

The mechanisms involved at each of these stages is discussed below.

Stage 1. Mobilization of Intracellular Stores of P_1

Intracellular P_i is chiefly stored in bound form, so release of bound P_i will lead to generation of anion (and cation, depending on the way it is bound) within the cell. (The fate of P_i is not relevant in this respect as long as the negative charge is not neutralized. Thus if P_i released from bound stores, or taken up from the medium, is incorporated into ATP, the negative charge will now be carried by the ATP molecule and a hydroxyl ion, which will continue to be additional to the total negative charge of the cell interior.) During this stage of fructose metabolism, rapid hydrolysis of ATP will lead to the generation of H^+ ions within the cell. The increased Na^+ content of hepatocytes reported here is consistent with an increase in intracellular H^+, since it has been demonstrated by Thomas (1976, 1977) that intracellular H^+ is exchanged with extracellular Na^+ to maintain a constant intracellular pH.

Stage 2. Movement of P_i from the Suspension Medium into Hepatocytes

Miller et al. (1952) reported transient lowering of serum P_i in human patients during intravenous fructose infusion. Similarly, the experiments reported in this chapter show that P_i is taken up by the hepatocytes from the suspension medium during the 10-20 minute period of incubation. This has two implications: first, influx of P_i involves influx of negative charge; secondly, influx of P_i may lead to release of H^+ in the medium and/or in the cells. I will discuss these in turn.

Influx of P_i Into Hepatocytes Involves Influx of Negative Charge. Influx of one molecule of P_i will involve influx of one, two or three negative charges, depending on whether it is taken up as $H_2PO_4^-$, HPO_4^{2-}, or PO_4^{3-}. $H_2PO_4^-$ and HPO_4^{2-} will be the predominant species in physiological conditions, and are the most likely candidates for transport. ($H_2PO_4^-$ was found to be the major species of P_i for inward transport by a carrier mechanism in yeast (Rothstein, 1963). Influx

of the negative charge carried by P_i can have two effects:

a) The electrogenic effect of entry of P_i into hepatocytes. If intracellular electroneutrality is not maintained, influx of P_i will lead to an increase in membrane potential. Membrane potential has not been recorded in this study, but the hyperpolarization of the membrane described by Lambotte (1977) corresponds in time with the period at which influx of P_i occurs. However, in my experiments intracellular K^+ content was greatly increased in the presence of fructose during this stage of incubation. This could have the effect of neutralizing, at least in part, the increased negative charge brought about by influx of P_i. This implies that even if an electrogenic machanism operates, it is not the only mechanism.

b) Movement of ions associated with movement of P_i into hepatocytes. Movement of cations inwards or anions outwards may be directly or indirectly associated with movement of P_i inwards. Results shown here indicate that at 20 minutes of incubation with fructose there was an increase of 28 µmol/g dry wt. K^+ within the cell. At the same time, there was an uptake of 26.2 µmol/g dry wt. P_i from the medium. Thus there was stoichiometry in inward transport of K^+ and P_i. Either K^+ transport could be directly linked with P_i transport in a specific carrier, or K^+ could move into the cell via a separate channel. By either mechanism, K^+ would neutralize additional P_i added to the cell.

It is not possible to differentiate between these two mechanisms at present. However, if K^+ movement was an indirect response brought about to balance additional P_i charge, then it might at first sight be thought that as one K^+ enters the cell together with one P_i molecule, the P_i must enter the cell as $H_2PO_4^-$ (HPO_4^{2-} would require 52.4 µmol/g dry wt. K^+ for maintenance of electroneutrality). This thought fails to take other factors into account:

1. Hyperpolarization of the membrane occurs at this period (Lambotte

1977; Dambach & Friedmann, 1974b). Charge carried inward by P_i is probably not fully neutralized by influx of K^+.

2. Movement of anions such as Cl^- and HCO_3^- may occur and would have to be taken into account. (These have not been measured.)
3. Charge may be generated inside the cell, for example H^+ may be generated by hydrolysis of ATP during F-1-P synthesis.

Hence at present it is not possible to deduce how much charge is carried in by P_i, because of the complexity of the events which occur simultaneously with P_i entry.

Changes in Hydrogen Ion Concentration in Cells and Medium During Influx of Phosphate. Apart from any changes in H^+ concentration within the cell related to metabolic events, movement of P_i across a membrane will lead to release of H^+ on one or other side of the membrane because of the re-establishment of the equilibrium:

$$H_3PO_4 \rightleftharpoons H_2PO_4^- + H^+ \rightleftharpoons HPO_4^{2-} + H^+ \rightleftharpoons PO_4^{3-} + H^+$$

If P_i moves into the cell as $H_2PO_4^-$ or H_3PO_4, then initially the pH of the extracellular compartment will rise while that of the intracellular compartment will fall. If P_i moves into the cell as as HPO_4^{2-} or PO_4^{3-}, then initially the pH of the extracellular solution will fall, and that inside the cell will rise. Decrease in plasma pH was recorded by Folke (1974) during perfusion of rat liver with fructose (10 mM) at a period at which P_i was likely to be entering the cell. Folke also reports a return of pH towards initial values ofter 30 minutes of incubation, which is the stage at which P_i starts reappearing in the medium. This is consistent with the idea that P_i enters the cell as HPO_4^{2-} or PO_4^{3-}. Influx of P_i as HPO_4^{2-} will lead to a rise in intracellular pH. This is consistent with the finding reported in this chapter that intracellular Na^+ content does not continue to rise after the first stage of fructose metabolism. This is because if the Na^+ increase is indeed related to decrease in cellular pH then, when P_i starts entering the cell and thus raises intracellular pH

(in the second stage of fructose metabolism), the Na^+ mechanism will be redundant.

Stage 3. Movement of P_i Out of the Hepatocyte

Values of lactate and glucose formation given in table 7:7 indicate that, by 60 minutes of incubation, 10 mM fructose (40 µmoles/flask) was almost entirely metabolized by 80 mgs of hepatocytes. By 60 minutes there was some recovery of intracellular ATP and P_i content. P_i was being transported out of the cells. The surprising finding reported in this chapter is that intracellular K^+ content remained elevated at this stage of fructose metabolism (intracellular K^+ was 41 µmol/g higher than that in control cells (table 7:1)). Metabolism of fructose to lactate (lactate is 95% ionised at physiological pH), which moves out of the cell down a concentration gradient, means that negative charge was transported out of the cell by lactate in addition to P_i. If indeed K^+ balances charge or is a linked co-ion in phosphate transport, efflux of K^+ in stage 3 would be expected. This issue is complicated by the fact that intracellular lactate itself leads to a concomitant increase in cellular K^+ (see Chapter 3 section 4).

Hence mechanisms in the three stages of change in ion distribution following acute fructose load are extremely complicated and have not been fully elucidated. Experiments in which intracellular and extracellular content of P_i, K^+, Na^+, Cl^-, H^+ and HCO_3^- are measured should shed light on this problem. Use of Cl^- and H^+ electrodes would be of great advantage in such a study.

iii. The Effect of Fructose on $^{42}K^+$ Influx into Hepatocytes

The findings indicate that a decrease in K^+ permeability in conditions of added fructose may occur. The mechanism involved is not yet understood.

iv. The Role of the Na-K ATPase in Transport of P_i into Hepatocytes

Ouabain had no significant effect on the influx of P_i into

hepatocytes during fructose loading. Influx of P_i across the hepatocyte membrane occurred independently of an equal influx of K^+ in these conditions. Since Na^+ content of cells was not increased, it is not clear how intracellular electroneutrality was maintained. This is a further instance of 'missing' cation in the presence of ouabain (compare Chapter 4 section v).

v. General Conclusions

Addition of fructose to rat hepatocytes gives rise to considerable perturbations in intracellular Na^+ and K^+ and P_i. It is likely that on addition of fructose Na^+ and K^+ content changes can be explained in terms of balance of intracellular charge during phosphate redistribution. However, one finding is puzzling in this respect: intracellular $[K^+]$ was sustained at a high value at 60 minutes despite the fact that intracellular P_i was moving out into the medium at this time. In these circumstances K^+ would be expected to move outwards concomitantly with P_i. This phenomenon may be related to increase in intracellular pH during outward P_i movement. This again raises the question of the species of phosphate which is transported through membranes, and, in conjunction with this, of the effect of inward and outward P_i transport on intracellular pH.

CHAPTER 8. DISCUSSION

One conclusion to be drawn from work presented in this thesis is that the isolated hepatocyte preparation is suitable for experiments involving the measurement of intracellular ion content of hepatocytes. Values for isolated hepatocyte Na^+ and K^+ content and concentration are similar to those found in whole liver. Isolated hepatocytes remain viable and maintain their Na^+ and K^+ content for 80 to 100 minutes of incubation. This gives sufficient time to investigate changes in ion content of cells which occur as a result of alterations in intracellular content of metabolic substrates or of inward transport of substrates. In addition, measurement of influx of ions with radio-active isotopes is possible under these conditions. However, one disadvantage of the preparation is that after 100 minutes the K^+ content declines and the Na^+ content of hepatocytes increases. Experiments requiring long incubations, such as those for measurement of efflux of radio-active ions, such as $^{42}K^+$, are difficult, since preloading of cells with $^{42}K^+$ in isolated hepatocytes would have to be done *in vivo* and $^{42}K^+$ would have to be added to all solutions used in preparation of cells. Since $^{42}K^+$ is a hazardous ion, substitution of potassium by rubidium, which is less hazardous but behaves in a similar manner to potassium, may be the answer to the problems involved in efflux experiments. Use of Rb^+ would require preliminary experiments to test whether it was a suitable substitute for K^+. Another approach to experiments measuring efflux would be to develop sustained incubation techniques such as those described by Dickson & Pogson (1977).

A general conclusion from the work in this thesis is that changes in Na^+ and K^+ content together with changes in rates of K^+ turnover can be related to changes in intracellular content of charge-carrying substrates such as lactic acid, ammonia, P_i and aspartic acid; with changes in cell volume; and with inward transport of metabolic substrates. In work presented here, the rate of a metabolic pathway

affects ion content chiefly in so far either as it leads to changes of intracellular content of substrate, or as it leads to further inward transport of substrates or to change in cell volume. Small intracellular changes in free Ca^{2+} or Mg^{2+} which may occur as a result of altered metabolic processes (see Bygrave, 1967) may well affect K^+ or Na^+ content or flux, but at present these changes are masked by the more gross changes outlined above.

In the future, ouabain may be a useful tool in investigating the relationships between metabolic processes and ion content and flux in hepatocytes. Addition of ouabain leads to a decrease in rates of urea synthesis and glucose formation from endogenous substrates and with added substrates. Ouabain is a specific inhibitor of the Na-K ATPase and therefore its primary action is to give rise to increase in cell Na^+ and decrease in cell K^+. Since ouabain is not known to have any direct effects on cell processes other than the Na-K ATPase, its effects on rates of glucose and urea formation are likely to be mediated through alterations in cell ion content. Ouabain has other advantages in that it neither directly affects cell volume nor leads to change in cell ATP content. The finding that intracellular ammonia content is significantly increased by ouabain indicates that cell contents of ions other than Na^+ and K^+ are indirectly affected by incubation with ouabain. This means that ions other than Na^+ and K^+ may be involved in changes in rates of glucose and urea formation seen with added ouabain. The finding that ouabain inhibits glucose production from lactate (added with small concentrations of pyruvate, NH_4Cl and oleate), but not from alanine, may shed light on the point of control at which ions are involved in control of rates of these processes. The pathway of glucose formation from alanine differs at some steps from that of the pathway from lactate (these differences are discussed by Krebs et al., 1976). Investigation into the effect of ouabain on rates of metabolic processes will require measurement of K^+, Na^+,

Cl^-, P_i and Ca^{2+} content in both cells, medium and mitochondria of isolated hepatocytes.

Festina lente (Suetonius)

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ION GRADIENTS AND FLUXES IN ISOLATED LIVER CELLS by G.M.Sainsbury, Lady Margaret Hall. Submitted for degree of Doctor of Philosophy in Hilary Term, 1978.

The overall aim of this thesis was to establish relationships between the ion content of isolated rat hepatocytes and metabolic processes such as glucose synthesis and fructose metabolism. In addition, a closely related subject, that of the mechanism of maintenance of the alleged ammonia gradient in liver, was also investigated.

Isolated rat hepatocytes were separated from the incubation medium for estimation of intracellular ion and metabolite values. Cell volume was estimated both from wet weight, dry weight and inulin space of cells, and from distribution of tritiated water between cells and medium. Cell volume increased in conditions where intracellular accumulation of amino acids occurred and when cells were treated anaerobically. The specific inhibitor of the Na-K ATPase, ouabain, did not affect cell volume.

Hepatocyte K^+ and Na^+ content, and K^+ turnover, were estimated in conditions of added substrates such as fructose, lactate, NH_4Cl, ethanol, asparagine, glutamine and alanine. Change in turnover of cell K^+, estimated with $^{42}K^+$, was not directly related to change in rate of metabolic processes such as glucose or urea synthesis. However, in all conditions in which increased cell volume occurred and in which amino acids were accumulated, K^+ turnover was increased. The effect of added alanine on cell ion and alanine content was examined in detail with special reference to the effects of ouabain and insulin.

Metabolism of added fructose led to redistribution of cell and medium inorganic phosphate. Movement of phosphate was concomitant with change in intracellular K^+ and Na^+ content.

Intracellular [ammonia] in isolated hepatocytes incubated without additions was 60 times greater than that of the medium. This constitutes an ammonia gradient only if intracellular ammonia was free in solution and not bound. Mechanisms which might be involved in maintaining high intracellular [ammonia] were investigated.

ABSTRACT

Two closely related areas of work are reported in this thesis: the relationships between metabolic processes, such as glucose synthesis and fructose metabolism, and Na^+ and K^+ content in hepatocytes; and the problems concerning the alleged ammonia gradient in rat liver.

Chapter 1.

The problems to be examined in this thesis are briefly introduced. Detailed introductions to specific aspects of relationships between metabolic processes and ion content of liver cells are made in the appropriate chapters.

Chapter 2.

The materials and experimental techniques employed are described. The work presented in this thesis is based chiefly on measurement of both intracellular and extracellular values for ions and metabolites; this requires separation of isolated hepatocytes from the incubation medium. The initial procedure used for separation of cells from medium was found to give rise to errors in estimation of both extracellular water space (inulin space) and of some intracellular metabolite and ion content values. Two distinct problems arising from the separation procedure were identified: the inulin space values were affected by position of the centrifuge tubes in the centrifuge; and the separation devices used for separation of cells from medium allowed loss of intracellular solutes during centrifugation. In order to obtain accurate values for both inulin space and intracellular solutes, a combination of two separation procedures was adopted within each experiment.

Two methods for determination of intracellular water content of isolated hepatocytes were compared, and were found to give similar results.

Chapter 3.

The endogenous Na^+ and K^+ concentrations and content of isolated

hepatocytes prepared from starved rats were measured and, after an initial 10 minutes of incubation, were similar to values found *in vivo* by other workers. Intracellular $[K^+]$ remained relatively constant for 100 minutes of incubation.

The effects of various concentrations of ouabain on decrease in cell $[K^+]$ and increase in cell $[Na^+]$ were compared over a 100 minute time course. A maximal effect was obtained with 2.0 mM ouabain. Ouabain was found to decrease hepatocyte oxygen consumption and also to decrease urea and glucose formation in most circumstances.

The effects of cyanide and anaerobiosis on the K^+ and Na^+ content of hepatocytes were examined. The rate of loss of K^+ in the initial stage of anaerobic incubation was found to be less than that found on addition of ouabain. This suggested that during the first 20 minutes of anaerobic incubation, the Na-K ATPase was not fully inhibited.

The effect of addition of various added substrates on K^+ turnover rate, K^+ content and cell volume were compared. The rate of K^+ turnover was increased in conditions in which amino acids were accumulated by hepatocytes against concentration gradients. K^+ turnover was also increased in conditions of increased cell volume. Cell content of Na^+ and K^+ was altered in the presence of added ionic substrates, for example, increased intracellular lactate (which is chiefly anionic at cell pH) gave rise to increased K^+ content whereas, increased intracellular NH_4^+ was accompanied by decreased K^+ content in hepatocytes. The changes in intracellular $[K^+]$ were stoichiometrically related to changes in intracellular [lactate] and $[NH_4^+]$. Addition of NH_4Cl in the presence of similar amounts of lactate did not affect the K^+ content of hepatocytes. These findings suggest that in these circumstances K^+ may act as a counter ion and moves inwards with lactate and outwards with NH_4^+ in order to maintain intracellular electroneutrality. It was not possible to establish a relationship between rates

of metabolic processes such as urea and glucose synthesis and rates of K^+ turnover in the experiments described in this section.

Chapter 4.

Intracellular water volume was estimated in conditions of added metabolic substrates and with metabolic inhibitors. A direct relationship existed between wet weight to dry weight ratio and intracellular volume on addition of ouabain and in all conditions in which added metabolic substrates led to increases in cell volume. This was because dry weight of cells, and extracellular water space were constant. With the metabolic inhibitor, cyanide, and in conditions of anaerobiosis, the relationship between wet weight to dry wieght ratio and cell volume was not direct because extracellular water volume and dry weight of cells were variable. Accurate measurement of intracellular water volume was not possible with cyanide or anaerobiosis after the first 20-40 minutes of incubation, chiefly because of differences in the viability of cells within each preparation, which led to cell lysis of a proportion of the preparation.

Increases in hepatocyte volume occurred in a number of metabolic conditions. In two of these, with alanine and in conditions of aspartic acid accumulation, relationships between cell volume changes and changes in K^+ and Na^+ content and concentrations were examined in some detail.

Chapter 5.

Hepatocytes accumulated added alanine against a concentration gradient. The ratio of alanine inside to outside was independent of extracellular alanine concentrations when [alanine] outside was in the range 2.5 - 10.0 mM. Accumulation of alanine was accompanied by increased K^+ content, however, when increased cell volume was taken into account, $[K^+]$ was lower than in cells incubated without alanine. Addition of alanine led to increased K^+ turnover in hepatocytes. On addition of ouabain, alanine was not accumulated by hepatocytes to any marked degree. Since ouabain is thought to have a specific effect on the Na-K ATPase, this suggests that the Na-K ATPase

is in some way involved in accumulation of alanine. Insulin, in relatively large concentrations, increased the alanine content of hepatocytes. K^+ turnover was also increased in the presence of alanine with insulin. The hypothesis that the effect of insulin in these conditions may be mediated by the Na-K ATPase is discussed.

Chapter 6.

The ammonia concentration of cells incubated without additions was found to be as much as 60 times as great as that of the incubation medium. The high ammonia ratio found in early stages of incubation decreased as incubation proceeded. This was because of increase in extracellular [ammonia] rather than decrease in intracellular [ammonia]. The high ammonia ratio was found only at physiological concentrations of ammonia. On addition of higher than physiological concentrations of NH_4Cl to the medium, the ammonia ratio decreased towards 1.0. A time course on addition of 10 mM NH_4Cl showed that ammonia entered hepatocytes rapidly down a concentration gradient such that the ammonia ratio was 1.0 within 2 minutes of incubation. The final steady state ammonia ratio of 2.0 in these conditions was reached in 40 minutes. $[K^+]$ in cells decreased concomitantly with [ammonia] increase on addition of added NH_4Cl. Experiments with $^{42}K^+$ indicated that NH_4^+ may compete with K^+ for entry into hepatocytes. The high endogenous ratio of ammonia was not abolished by ouabain, which indicated that although NH_4^+ may enter hepatocytes by the Na-K ATPase, the Na-K ATPase was not responsible for maintenance of the ammonia ratio. In conditions of anaerobiosis, the endogenous ammonia ratio was decreased. This was because not only was intracellular [ammonia] decreased, but also there was increase in extracellular [ammonia]. This suggests that metabolic energy is required for maintenance of the high endogenous ammonia ratio. Separation of mitochondria from cytosol and subsequent measurement of cytosolic and mitochondrial ammonia content showed that high ratios of ammonia existed between cytosol and medium , and between mitochondria and

cytosol. In other words, ammonia was not equally distributed throughout the cell interior. Since mitochondria have been shown to be readily penetrated by ammonia, the mechanism for maintenance of an ammonia gradient between mitochondria and cytosol is obscure. In light of the problems raised by the experiments above, the hypothesis that part of intracellular ammonia is bound and not freely in solution is discussed.

Chapter 7.

Addition of fructose to liver cells is known to lead to a rapid depletion of cellular ATP and inorganic phosphate as a result of rapid phosphorylation of fructose to fructose-1-phosphate. Intracellular fructose-1-phosphate is accumulated within liver cells and intracellular phosphate is replenished by inward movement of extracellular phosphate. Since both phosphate and fructose-1-phosphate are negatively charged at cell pH, the effect of changes in their concentrations on cell Na^+ and K^+ was investigated. Initially intracellular Na^+ increased, but by 60 minutes Na^+ was restored to control values and intracellular $[K^+]$ was significantly increased. The permeability of the cell to K^+ was slightly decreased in the presence of added fructose. It was concluded that addition of fructose to liver cells indirectly gives rise to significant changes in the distribution of Na^+, K^+ and inorganic phosphate between cells and medium.

Chapter 8.

Work in this thesis has established that the isolated hepatocyte preparation is suitable for the study of ion content and flux in rat liver. It is concluded that changes in concentrations of intracellular metabolites such as lactate, NH_4Cl, alanine, fructose-1-phosphate, and aspartic acid are accompanied by changes in $[K^+]$ and $[Na^+]$ in order to maintain intracellular ion balance.

Proposals are made for the next stage of experimental work in this investigation. The next step in this work is to ascertain the distribution

of Ca^{2+}, Mg^{2+}, P_i, and Cl^- in circumstances in which K^+ and Na^+ perturbations occur. Ultimately it is hoped to ascertain the role of changes in ion content in control of metabolic processes such as glucose and urea synthesis.