Metabolic Aspects of Ion Transport

in the Foetal Gastric Mucosa.

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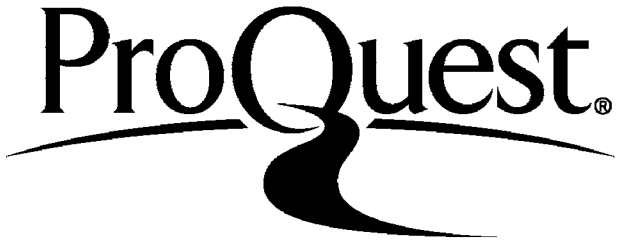

ABSTRACT

The rabbit foetal gastric mucosa actively absorbs Na^+ and secretes a small amount of acid. The mechanisms by which metabolic energy is utilized for active ion transport in this tissue were investigated.

The history and main theories of gastric secretion and oxidative phosphorylation were reviewed briefly. Na^+ transport in amphibian and mammalian gastric mucosae was described and discussed.

Oxygen consumption, adenosine triphosphate, adenosine diphosphate, and creatine phosphate concentration changes and active ion transport in the rabbit foetal gastric mucosa were investigated in the presence and absence of exogenous glucose, during anoxia and reoxygenation and in the presence of ouabain, oligomycin, and dinitrophenol.

Investigation of the effect of the presence and absence of exogenous glucose suggested that the tissue contained an energy source other than creatine phosphate. It was also shown that creatine phosphate could however be used to some extent in adverse conditions, such as during anoxia.

Experiments on the effect of anoxia and subsequent reoxygenation showed that isolated foetal gastric mucosal cells were capable of resynthesizing adenosine triphosphate after large tissue concentration decreases of both adenosine triphosphate and adenosine diphosphate. However, ion transport did not seem to be solely dependent on the absolute tissue concentration of adenosine triphosphate. The adenosine triphosphate/adenosine diphosphate ratio also did not seem to be a direct controlling factor.

The effects observed with ouabain strongly suggested direct involvement of adenosine triphosphate in active Na^+ transport,

possibly through a $(Na^+ + K^+)$-stimulated adenosine triphosphatase. However, the effects of ouabain on acid secretion were thought to be indirect. The experiments with oligomycin and dinitrophenol showed that respiratory energy could not be utilized directly for active ion transport. A high-energy phosphorylated intermediate or adenosine triphosphate seemed necessary. Nevertheless, acid secretion seemed also to be dependent on oxidative processes.

To My Parents,

Krysia

and Cam.

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ABBREVIATIONS

ADP	Adenosine 5'-diphosphate
AMP	Adenosine 5'-phosphate
ATP	Adenosine 5'-triphosphate
ATPase	Adenosine triphosphatase
CP	Creatine phosphate
cyt	Cytochrome
d.c.	Direct current
DNP	2,4-dinitrophenol
EDTA	Ethylenediaminetetra-acetate
FP	Flavoprotein
NAD^+	Nicotinamide-adenine dinucleotide, oxidised
NADH	Nicotinamide-adenine dinucleotide, reduced
$NADP^+$	Nicotinamide-adenine dinucleotide phosphate, oxidised
NADPH	Nicotinamide-adenine dinucleotide phosphate, reduced
ox.	Oxidised
p.d.	Potential difference
Po_2	Partial pressure of oxygen
red.	Reduced
s.c.c.	Short-circuit current
Tris	2-amino-2-hydroxymethylpropane-1,3 diol
UQ	Ubiquinone

CHAPTER 1

INTRODUCTION

A. GASTRIC SECRETION

Although scientific interest in the processes involved in digestion began during the 17th century, little progress was made in the field of gastric physiology until the 19th century, following the great advances in physics and chemistry.

Prout (1824) identified the acid of gastric juice as hydrochloric acid. During the latter half of the 19th century, increasing use of the microscope led to the identification of a heterogeneous cell population in the gastric mucosa. The combined use of dyes, pH indicators and microscopy led to the view that parietal cells (later renamed oxyntic cells) seemed to be linked with acid secretion. This was the result of the work of Swiecicki, Heidenhain, Langley, Claude Bernard, Frankel and others (see Conway, 1953).

During this time progress in physical chemistry, particularly the development of thermodynamics and electrochemistry was of considerable importance. The idea that electrolytes in solution are permanently dissociated into pairs of oppositely charged ions became established. Fick (1855) evolved his law of diffusion and Nernst (1889) combined the laws of thermodynamics and electrochemistry to produce an equation relating the electromotive force (E.M.F.) of a system to electrolyte concentration gradients. This relationship was of particular importance to physiologists who were attempting to explain the ionic and potential differences between living cells and their environment.

Galvani (1792) pioneered the field of electrophysiology with the first demonstration of electrical potentials in living systems using the frog muscle. In 1834 Donné observed an electrical p.d. across the gastric wall and associated it with acid secretion. A few years later, Du Bois Reymond (1848) showed that the frog skin was a site of

E.M.F. and could produce a current flow. These findings were confirmed and extended by Galeotti (1904). He demonstrated that the p.d. across the frog skin depended on the presence of sodium salts in the bathing solution and that the p.d. persisted when identical solutions bathed the frog skin on both sides. Therefore diffusion could not explain these results, nor could they be explained in other simple physical or chemical terms.

At the end of the 19th century, the membrane theory of cell permeability arose, originating from the work of Pfeffer, de Vries and Overton on plant and animal cells. The presence of a semi-permeable cell membrane which was easily permeable to water, but only with difficulty or not at all to the molecules of water-soluble substances was postulated. These investigators, especially Pfeffer, pointed out the great importance of the part that the semi-permeable membrane must play in metabolism, regulating the entrance of substances into the cell and their excretion from the cell, thereby influencing the course of cellular metabolism (see Troshin, 1966).

Investigations into all aspects of membrane phenomena gathered momentum in the 20th century, coupled with continuing advances in chemistry, physics and biochemistry. However, the emphasis in gastric physiology continued to be on searching for the actual site of acid secretion and on obtaining knowledge of the composition and control of gastric secretion, using Pavlov pouches. To explain the observation that the acidity of gastric juice increased as the rate of secretion increased, Pavlov (1910) developed the hypothesis that parietal cell secretion was high and constant throughout and that the acidity varied because of the varying degree of neutralization and dilution from non-parietal cells. Several investigators confirmed and extended Pavlov's

findings by estimating, using indirect methods, the inorganic composition of parietal and non-parietal secretions (Hollander, 1931; Gray, 1943). The composition of parietal cell secretion estimated by Gray (1943) was 166 m-equiv/l. Cl^-; 159 m-equiv/l. H^+ and 7 m-equiv/l. K^+ which remained constant even during large variations in acidity and secretory rate. With slight variations in these figures, this work was confirmed by other investigators. The striking feature was that the chloride exceeded the amount of hydrogen ions. Non-parietal cell secretion was thought to consist of mainly chloride ions, some bicarbonate ions and an equivalent amount of cations. From these investigations the component theory of gastric acid secretion was put forward. It regarded gastric juice as a primary secretion of acid by parietal cells, modified by non-parietal secretion.

However, Teorell (1939, 1940) suggested the concept of the gastric mucosa as an ion permeable membrane and used this as the basis of a diffusion theory to account for the variation of acidity with secretory rate. He observed the passage of alkali and chloride from the blood to the stomach and explained the excess of chloride over hydrogen ions as being the result of the diffusion of secreted hydrogen ions down their electrochemical potential gradient back into the blood. At the same time, sodium ions diffused down their electrochemical potential gradient from blood to the gastric lumen. In other words, he suggested a passive H^+/Na^+ exchange. Evidence in support of Teorell's findings has since been reported (Bornstein, Dennis & Rehm, 1959; Berkowitz & Janowitz, 1966; Bugajski, Code & Schlegel, 1972). However, the slight difference between the osmolarity of plasma and gastric juice can also be explained on the basis of the component theory: the hydrochloric acid being neutralized by the sodium bicarbonate secreted by non-parietal

cells with formation of sodium chloride, water and a loss of carbon dioxide.

Gray, Adkison & Zelle (1940) showed that the formation of acid was an actual secretory process, by mounting an isolated frog gastric mucosa between two chambers with known solutions on each side and recording pH changes that occurred. With sodium chloride solution bathing the mucosal side and Ringer solution bathing the serosal side, the mucosal solution became acidic. When the tissue was reversed so that the sodium chloride solution was bathing the serosal side and Ringer solution the mucosal side, the pH of the serosal solution increased, suggesting a release of alkali. The isolated mucosae of other tissues did not produce the same result, indicating that acid secretion was a particular function of the gastric mucosa. Gray & Adkison (1941) showed that unlike the sodium ion, the potassium ion seemed essential for acid production. However, Patterson & Stetten (1949) obtained acid secretion from an in vitro preparation of the rat stomach for about one hour even when the bathing solutions contained no salts, but were simply 5% (w/v) glucose solutions.

Ussing and his collaborators in their studies of ion movements across the frog skin developed concepts and techniques which were of great importance to similar studies in other tissues, including the gastric mucosa. Ussing (1949a) clearly defined active transport as the transport of an ion against its electrochemical potential gradient. Energy other than the kinetic energy of the ion or that due to the electrical or chemical gradients must be used for this active transport. Once it has been established that a given ion is actively transported, it is usually assumed that a metabolic process must be present that performs osmotic work at the expense of cellular chemical energy.

Ussing (1949b), using isotopic tracers, found that the isolated frog skin could transport sodium from the outer face to the inner face against a steep concentration gradient. Ussing and his colleagues then put forward the idea that the p.d. across the frog skin was very likely to be the consequence of the inward transport of positively charged sodium ions. Proof of the relationship between the electrical asymmetry of the frog skin and the active transport of sodium was obtained by the short-circuit current technique (Ussing & Zerahn, 1951). The reasoning behind this technique is as follows.

If the skin is bathed with identical solutions on both sides and active ion transport is taking place at the same time, the resulting movement of the charge will set up a p.d. which will then tend to be partially short-circuited by movement of the passive ions in the system. If the two sides of the skin are then connected by reversible electrodes and a circuit of very low resistance, a current will pass which will be exactly equivalent to the rate of active transport of charge. However, the available reversible electrodes have too high a resistance to effect a total short-circuit of the skin. This difficulty was overcome by applying an E.M.F. of appropriate sign and magnitude across the skin to reduce the spontaneous p.d. to zero. The current then passing in the external circuit is equal to the short-circuit current. Ussing & Zerahn (1951) found that the rate of net sodium transport across the skin, measured using radiosodium, was exactly equal to the short-circuit current, thus proving that the sodium ion is the only ion subjected to active transport in this system.

From an analysis of the electrical p.d. across the mammalian stomach and the concentrations in the blood and gastric juice respectively, Rehm (1950) concluded that both chloride and hydrogen

ions were actively transported across the gastric mucosa. Hogben (1951, 1952, 1955) was the first to apply the in vitro Ussing technique to the gastric mucosa and he showed in the frog that the s.c.c. accounted for the net Cl^- transport minus the H^+ transport, thus quantitatively supporting Rehm's earlier proposal. Within the limits of experimental error, no evidence was found for active Na^+ or K^+ transport. The movement of these ions was largely passive, down their electrochemical potential gradients. Both the H^+ and Cl^- pumps are active and hence they are both dependent on energy made available by cellular metabolism. The study of the relationship between the secretory processes and metabolism would provide further understanding of the mechanisms by which osmotic work is performed at the expense of cellular chemical energy.

B. OXIDATIVE PHOSPHORYLATION

The main cellular mechanism for production of energy is mitochondrial oxidative phosphorylation. Although mitochondria had been observed in cells as early as the second half of the 19th century (1850-1890) by cytologists, it was not until 1949 that Kennedy & Lehninger first established the role of mitochondria as the seat of oxidative phosphorylation.

In 1898 Benda introduced crystal violet as a stain for mitochondria (see Fruton, 1972) and Michaelis (1900) introduced the principle of supravital staining of mitochondria with Janus green. The work of Michaelis was particularly significant since it showed that mitochondria in living cells could bring about oxidation-reduction changes in a dye. Although during the early part of the 20th century the study of mitochondria was expanding, most early cytologists' speculations centred on a genetic role for mitochondria. It was not until 1912 that attention was called to the possibility that mitochondria were

the sites of cellular oxidations. However, although Warburg (1913) found respiration to be associated with granular, insoluble elements of cell structure which he recovered by filtration of tissue dispersions, the significance of his observation was not developed further by cytologists at that time.

The study of animal respiration dates from the 18th century. However, the modern era of research on respiration began at the turn of the 20th century with the discovery of dye-reducing dehydrogenases in cell-free extracts of tissues by Schardinger in 1902 (see Fruton, 1972), by Batelli & Stern (1911), and by Thunberg (1920). The unifying chemical basis for biological dehydrogenation reactions was put forward by Wieland (1913). Warburg (1924) developed the concept that most, if not all utilization of oxygen by cells takes place through the intervention of an iron-containing catalyst that could be inhibited by cyanide and by carbon monoxide. This view was supported by the work of Keilin (1925), who rediscovered the cytochromes and showed them to undergo characteristic changes in oxidation state in intact insect muscles.

The two lines of investigation on enzymic activation of substrates by dehydrogenases and activation of oxygen by an iron-containing catalyst came together in the 1930's. The isolation and determination of structure of the pyridine nucleotides, as well as the principles of their action with dehydrogenases were initiated by Euler and his co-workers (Euler, Myrback & Nilsson, 1928) and carried to a new stage of understanding by Warburg & Christian (1936, 1939). At this time also came the discovery of the flavoproteins and flavin nucleotides by Warburg & Christian (1932, 1933) and Theorell (1934, 1935), which led finally to the concept that the flavoproteins are mediators between

the pyridine nucleotides and the cytochrome system. Also during this period,Keilin (1930) isolated cytochrome 'c' and performed his first reconstructions of electron transport with heart particles. Emerging slowly from all this work, the basic form of the respiratory chain as the sequence: dehydrogenases ⟶ flavoproteins ⟶ cytochromes ⟶ O_2 began to take shape, a concept that was also supported by work of Szent-Györgyi (1937).

On the basis of simple but penetrating experiments on the respiration of muscle suspensions, Krebs (1937) postulated the citric acid cycle as the primary cellular mechanism for the oxidation of carbohydrate. This concept of a cyclic multienzyme system for stepwise oxidative degradation of the carbon skeleton of pyruvate had a profound impact on both experimentation and thought in biological oxidations. Some years, however, were to elapse before isotopic tracer experiments provided the final proof for the citric acid cycle mechanism.

During the time of these developments and advances in biological oxidations, relatively little attention was focused onto the mechanism of energy recovery from respiration, although it had long been appreciated from physiological and calorimetric studies that combustion of carbohydrate produced ultimately large amounts of heat. Lohmann (1929) discovered ATP, but it was at first considered to be peculiar to muscle. Its real significance in cellular energy transformation was not appreciated until the important demonstration by Warburg & Christian (1939) of the formation of ATP coupled to the enzymic oxidation of glyceraldehyde phosphate and the work of Meyerhof & Kiessling (1935) on the formation of ATP from phosphoenolpyruvate. ATP is thought to be the main,direct source of energy required by a living system. Hydrolysis of the two terminal phosphate bonds may release energy to

the value of 12,000 cal/mole for each bond under intracellular conditions (see Lehninger, 1965). These bonds were named high-energy phosphate bonds by Lipmann (1941) and are usually denoted by the symbol ~. Kalckar (1937) first described aerobic or oxidative phosphorylation coupled specifically to respiration, which Belitser & Tsybakova (1939) soon postulated on theoretical grounds to be associated with electron transport from substrate to oxygen via flavoprotein and cytochromes. For further detail of the historical background of oxidative phosphorylation see Keilin & Slater (1953-1954); Lehninger (1964); Keilin (1966); Kalckar (1969) and Fruton (1972).

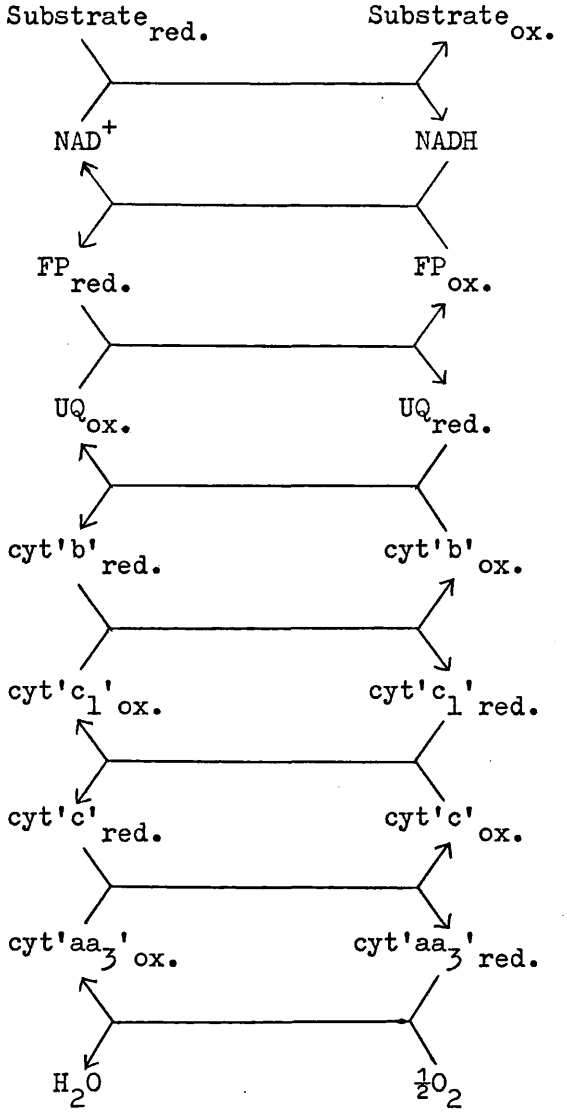
In 1948, problems connected with the isolation of intact mitochondria were finally overcome when Hogeboom, Schneider & Palade (1948) reported that when a medium of 0.88 M sucrose is used for dispersion of rat liver cells, the nuclei, mitochondria and microsomes could be easily separated by differential centrifugation. However, most important was that the liver mitochondria could be recovered in the elongated form characteristically seen in intact liver cells. On the other hand, preparations obtained from saline media or from media containing lower sucrose concentrations were spherical and swollen. Using this new method of isolating intact mitochondria from 0.88 M sucrose medium, Kennedy & Lehninger (1949) showed that isolated rat liver mitochondria carried out the oxidation of all the Krebs citric acid cycle intermediates at a rate compatible with that of the intact liver and most importantly, that oxidative phosphorylation accompanied these organized oxidations.

In oxidative phosphorylation, the hydrogen atoms derived from metabolic substrates enter the electron transport chain through NAD^+; eventually being used to reduce oxygen to water. The electron transport

chain is a series of coupled oxidation/reduction reactions which occur in the following sequence:

Substrate $\rightarrow NAD^+ \rightarrow FP \rightarrow UQ \rightarrow$ cyt'b' $\rightarrow$ cyt'c_1' $\rightarrow$ cyt'c' $\rightarrow$ cyt'aa_3' $\rightarrow O_2$.

Oxidation of the substrate causes reduction of NAD^+, the reoxidation of which, is coupled with the reduction of the next component of the chain, FP and so on through the whole electron transport chain.



However, in the case of succinate, the electrons are passed directly to FP. When electron transport occurs, there is simultaneous phosphorylation of ADP to ATP. The sites of formation of ATP occur when there is a large change in energy between the oxidised and reduced products.

Various methods have been used to locate the phosphorylation steps in the respiratory chain. However, there continues to be controversy about the precise location of these sites. The general scheme is that site I is between NAD^+ and FP (Copenhaver & Lardy, 1952); site II between FP and cytochrome 'c' (Slater, 1955) and site III between cytochrome 'c_1' and oxygen (Lehninger, 1954). Evidence for the more precise localization of the sites of phosphorylation is given by Slater (1966). This system is usually called respiratory chain phosphorylation.

The net result of much work in many laboratories in the 1960's can be summarized as follows. Electron or hydrogen transfer in mitochondria builds up an 'energy pressure' that may be used to synthesize ATP and for energy-requiring reactions in the mitochondria: movement of cations into and out of mitochondria against a concentration gradient (Brierly, Murer & Green, 1963; Carafoli, 1965) and the reduction of $NADP^+$ by NADH beyond the equilibrium point, usually called the energy-linked transhydrogenation reaction (Lee, Azzone & Ernster, 1964; Lee & Ernster, 1964). All reactions except the transhydrogenation are readily reversible. The first proof of the existence of this 'energy pressure' came with the discovery of the reversal of electron flow in the respiratory chain (Chance and Hollunger, 1957, 1960). Snoswell (1961, 1962) and Slater, Kemp & Tager (1964) showed that oligomycin had no effect on the utilization of the energy of respiration for reversal of the respiratory chain. Thus the energy need not be provided by ATP formed by oxidative phosphorylation, but can be provided by the 'energy pressure'. The energy may also be dissipated by an uncoupler such as DNP, which works catalytically. Although something is known about the peripheral reactions, the nature of the

primary 'energy pressure' is still unknown.

There are two main hypotheses concerning the nature of the primary energy-conserving process. (1) According to the so-called CHEMICAL HYPOTHESIS (Slater, 1953), modelled on the mechanism of substrate-linked phosphorylation, the energy is conserved in an energy-rich compound (a high-energy intermediate) between one of the products of the redox reaction and a ligand variously indicated as C (Slater, 1953) or I (Chance & Williams, 1956).

$$AH_2 + B + C(I) \rightleftharpoons A\sim C(I) + BH_2$$

$$A \sim C(I) + ADP + P_i \rightleftharpoons A + C + ATP$$

SUM: $AH_2 + B + ADP + P_i \rightleftharpoons A + BH_2 + ATP$

where AH_2 and B are both components of the electron transport chain. This hypothesis has been extended to include two high-energy compounds and possibly a phosphorylated high-energy intermediate (Chance & Williams, 1956; Ernster, 1963; Slater, Lee, Berden & Wegdam, 1970a, b) as follows:

$$AH_2 + B + I \rightleftharpoons A \sim I + BH_2$$

$$A \sim I + X \rightleftharpoons X \sim I + A$$

$$X \sim I + P_i \rightleftharpoons X \sim P + I$$

$$X \sim P + ADP \rightleftharpoons X + ATP$$

A conformational hypothesis, which is formally rather similar to the chemical one was put forward by King, Kuboyama & Takemori (1965) and Boyer (1965). They proposed that the primary energy-conservation process is a conformational change in a respiratory protein (a flavoprotein, an iron-sulphur protein or a cytochrome) and that this energy may be utilized to make ATP.

$$AH_2 + B \rightleftharpoons A^* + BH_2$$

$$A^* + ADP + P_i \rightleftharpoons A + ATP$$

SUM: $AH_2 + B + ADP + P_i \rightleftharpoons A + BH_2 + ATP$

Here the energy is locked away in the folding of the chains of the protein instead of in a covalent bond between a component of the respiratory chain and a ligand.

(2) According to the CHEMIOSMOTIC HYPOTHESIS of Mitchell (1961a, 1966, 1968), the primary energy-conserving act is the translocation of protons across the mitochondrial inner membrane, uncompensated by the movement of cations in the opposite direction or of anions in the same direction. The resultant membrane potential is utilized to drive ATP synthesis. Mitchell's theory dispenses with the high-energy intermediates of the chemical theory. A third hypothesis which has been put forward recently by Skulachev (1970, 1971) is basically a combination of the chemical and chemiosmotic hypotheses, in which energy is conserved through the formation of a high-energy compound leading to the generation of a membrane potential.

The unproven assumption of the chemical hypothesis is the existence of the high-energy intermediate (or conformation). The unproven assumption of the chemiosmotic hypothesis is that the transfer of reducing equivalents along the transport chain is coupled with the translocation of protons, leading to a membrane potential. Direct experimental proof for one or other hypothesis is very difficult to obtain. There has been no success in isolating any high-energy intermediate, possibly because it cannot survive outside the membrane. Recently, however, spectroscopic and potentiometric evidence has been put forward in support of the existence of high-energy forms of cytochrome 'b' and cytochrome 'a_3' (Wilson & Dutton, 1970a, b; Chance,

Wilson, Dutton & Erecińska, 1970; Slater et al., 1970a, b). The amount of H^+ transport required to build up an appreciable membrane potential is undetectably small and the direct electrical measurement of a membrane potential on the two sides of the membrane is technically very difficult. There is evidence in favour and against both of these hypotheses, which is summarized and discussed by Slater (1971) who concludes that there seems to be more evidence in favour of the chemical hypothesis than for the chemiosmotic hypothesis. However, further work is necessary to elucidate the precise nature and mechanisms involved in the primary energy-conserving process in mitochondria.

C. ENERGETICS OF GASTRIC SECRETION

The gastric secretory product may achieve acidity as high as 160 mM (pH approximately 0.8) as compared to the blood of pH 7.4. The minimum free energy (ΔG) required for the transport of an ion may be estimated from the concentration and electrical gradients and is described by the following equation: $\Delta G = nRT \ln a_1/a_2 + nzFE$, where n is the number of gram ions secreted, R is the gas constant, T is the absolute temperature, a_1 and a_2 are the activities of the ion on either side of the limiting membrane, z is the net charge of the ion, F is the Faraday constant and E is the electrical potential difference across the limiting membrane. Using the concentration and electrical gradients observed in various gastric preparations, it can be calculated that about 10,000 cal/g mole are required for the secretion of 160 mM H^+ in the mammal and about 8,800 cal/g mole are required for the secretion of 120 mM H^+ by the amphibian gastric mucosa. These figures represent the minimum free energy requirement if the system was 100% efficient. From simultaneous measurements of the rate of acid secretion and oxygen consumption, it has been found that for the frog gastric mucosa

approximately 2 moles of HCl are produced per mole of O_2 consumed by the entire tissue (Teorell, 1949a; Davenport, 1952; Forte & Davies, 1963). Since the complete oxidation of glucose to CO_2 and H_2O yields about 114,000 cal/mole O_2 utilised, there is ample energy available within the system to account for the acid secretion.

Acid secretion is dependent on oxidative metabolism (Delrue, 1930; Rehm, 1946; Crane, Davies & Longmuir, 1946; Davenport, 1947; Patterson & Stetten, 1949). Based on this observation and the operational simplicity of the oxidation-reduction mechanism developed by Lund (1928) and Lundegårdh (1939), three groups of workers independently proposed an analagous system to account for the formation of gastric acid - the redox pump hypothesis (Robertson & Wilkins, 1948; Conway & Brady, 1948; Crane & Davies, 1948; Conway, 1951). The basic feature of this scheme is that hydrogen ions are produced from substrate hydrogen with molecular oxygen acting as the final electron acceptor via the cytochrome system. If transport of any monovalent ion were dependent solely on this process, a quantitative upper limit of 4.0 (the electrochemical equivalent of oxygen) would exist between the number of ions transported and oxygen consumed.

Theestimates of approximately two moles of HCl produced per mole O_2 consumed by gastric tissue are well within the stoichiometric limit for a redox pump. However, these respiration measurements involved the entire tissue and hence included processes and cell types other than those connected with acid secretion. Various workers have attempted to measure the portion of tissue oxygen consumption which is directly coupled to acid secretion. Crane & Davies (1951) using gastric mucosae from a species of frog which did not secrete acid spontaneously, measured the increase in O_2 uptake (ΔQ_{O_2}) and the net acid secretion

(ΔQ_{HCl}) upon stimulation of the preparations by the addition of histamine. They found that in the majority of cases, the ratios $\Delta Q_{HCl}/\Delta Q_{O_2}$ were above 4.0. The basic assumption made in calculating these ratios was that the whole of the increase in oxygen consumption of the mucosa was utilized for acid secretion.

Davenport (1952) and Davenport & Chavré (1953) attempted to evaluate the Q_{HCl}/Q_{O_2} from the slope of the relationship between these two parameters for large populations of frog and mouse gastric mucosae. They concluded that mean ratios of Q_{HCl}/Q_{O_2} were below 3.0 for histamine-stimulated as well as unstimulated mucosae. However, many of the individual points fall above the 4.0 mark. Forte & Davies (1963, 1964) using both total oxygen consumption and change in oxygen consumption associated with reversible inhibition of acid secretion by thiocyanate and by an opposing current, obtained values for $\Delta Q_{HCl}/\Delta Q_{O_2}$ of above 4.0 (range 5.0 - 15.0). In this work the underlying assumption was that the residual respiration after inhibition of acid secretion was not associated with the secretory process, nor could it be channelled into secretory work.

Bannister (1965a, b, 1966) used single, everted sacs of frog gastric mucosae and in the majority of his experiments obtained a linear relationship between oxygen uptake and acid secretion, and a mean ratio for Q_{HCl}/Q_{O_2} of 2.4 ± 1.0 (mean $\pm$ S.D.). He also found that oligomycin and arsenate did not affect significantly the value of the ratio between acid secretion and the associated oxygen uptake. These results coupled with the fact that anoxia inhibits acid secretion in the frog gastric mucosa, support a redox mechanism for acid secretion. The argument used by supporters of the redox theory to explain results of ratios above 4.0, is that the oxygen consumption in

the resting state is not the same as the non-acid producing oxygen consumption. Bannister (1965a) found in some experiments that the measured resting rate of oxygen consumption in acid secreting mucosae was higher than the calculated non-acid producing oxygen consumption. Therefore the resting rate of oxygen consumption would appear to contain a component of oxidative metabolism which may become available for acid secretion.

The other main theory advanced is that the energy available from the hydrolysis of ATP is transferred to a proton carrier, which as a result moves protons against an electrochemical gradient. In most tissues which transport Na^+ and K^+ actively, a (Na^+ + K^+)-stimulated ATPase located in the membrane has been isolated, demonstrating the direct dependence of the active transport process on ATP (e.g. in nerve, Skou, (1957); in red blood cells, Glynn (1962); in toad bladder, Solinger,Gonzalez, Shamoo, Wyssbrod & Brodsky (1968) among others). Strong evidence in favour of this view is that both the active Na^+ transport and the (Na^+ + K^+)-stimulated ATPase are inhibited by cardiac glycosides, particularly ouabain. A good review on this subject is one by Glynn (1964). However, in the case of active transport of other ions, in particular the hydrogen ion, indirect evidence has been found in support of the involvement of ATP. One of the earliest arguments for such a proposal was that uncoupling agents such as DNP, are well known to inhibit acid secretion by the gastric mucosa, whereas respiration is stimulated or unchanged (Davies, 1951; Davenport & Chavré, 1953; Heinz & Durbin, 1957; Sachs, Collier, Shoemaker & Hirschowitz, 1968). Although it could be argued that in gastric tissue uncoupling agents have inhibitory sites other than those typically associated with oxidative phosphorylation, Forte, Forte, Gee

& Saltman (1967) showed that mitochondria isolated from the rabbit gastric mucosa undergo typical phosphorylating reactions which are uncoupled by DNP. Therefore there does not appear to be a specialized system peculiar to gastric mitochondria.

However, uncouplers act as inducers of proton translocation in artificial lipid systems (Chappell & Haarhoff, 1967) and in mitochondria (Mitchell, 1961b; Cunarro & Weiner, 1973) and some investigators feel that uncoupling is dependent on this capacity to increase proton conductance in membranes (Mitchell & Moyle, 1967). Therefore the action of uncouplers could be explained by mechanisms not requiring inhibition of ATP synthesis. However, Durbin & Kasbekar (1965) found that at concentrations which block electrical and secretory activities, DNP reduced the ATP concentration in the frog gastric mucosa. Amytal, which blocks mitochondrial electron transport, inhibits acid secretion in the frog gastric mucosa and causes a decrease in the tissue ATP concentration (Sachs, Shoemaker & Hirschowitz, 1967; Sachs et al., 1968). Phosphorylative inhibitors, such as galegine sulphate and aurovertin, inhibit acid secretion and oxygen consumption and so does atractylate, which inhibits ATP-ADP translocation across the mitochondrial membrane (Sachs et al., 1968). From these results it would appear that a simple redox theory for gastric acid secretion would require many secondary assumptions to explain the inhibitory action of compounds such as aurovertin or atractylate, while the ATP theory can easily explain these actions.

Forte, Adams & Davies (1965) studied the relationship between phosphate metabolism and acid secretion. Bullfrog gastric mucosae were divided in half and one part was used as a control,while the other half was subjected to various conditions during which the rate of acid

secretion was measured. ATP, ADP and AMP concentrations were measured in both control and experimental tissue. A linear correlation was found to exist between the rate of acid secretion and the concentration of ATP within the mucosal tissue. Anoxia decreased the rate of acid secretion, but a significant rate was still measureable after 1 hour in oxygen-free solution, and anaerobically produced ATP seemed able to maintain some acid secretion until levels fell below 0.4 - 0.5 μ-mole/g tissue. Inhibition of anaerobic glycolysis by iodoacetate caused acid secretion to fall to zero and tissue ATP to fall to very low levels. On reoxygenation of anaerobic mucosae, acid secretion only returned when the ATP concentration was restored. Thiocyanate (SCN^-) caused 90% inhibition of acid secretion, although the ATP concentration in the tissue was not significantly altered. Thus they postulated that SCN^- affected the acid secreting mechanism at some point distal to the production of energy metabolites. Forte, Adams & Davies (1965) estimated that at least 1.5 equivalents of hydrogen ions could be produced per mole of ATP utilized.

Durbin (1968), investigating the same problem measured mucosal concentrations of CP and ATP. In general his results are in agreement with those of Forte, Adams & Davies (1965). Kasbekar & Durbin (1965), in support of the direct involvement of ATP in acid secretion, isolated an ATPase in the microsomal fraction from homogenates of frog gastric mucosae. The enzyme was not stimulated by sodium and potassium or inhibited by ouabain. However, the gastric microsomal ATPase activity was depressed by SCN^- and stimulated by HCO_3^-. These results were confirmed by Forte, Forte & Bils (1965) and Sachs, Mitch & Hirschowitz (1965). Thiocyanate also rapidly and reversibly inhibits gastric HCl

secretion (Crane, Davies & Longmuir, 1946; Davenport, 1940; Rehm & Enelow, 1945; Forte, Adams & Davies, 1965). However, it was found that ATPases from other tissues are inhibited by SCN^- and stimulated by HCO_3^- (Sachs, Mitch & Hirschowitz, 1965), which indicates that the ATPase is not unique for H^+ secretory tissues. Wiebelhaus, Sung, Helander, Shah, Blum & Sachs (1971) succeeded in separating oxyntic cells from the adult Necturus gastric mucosa and found that the SCN^--inhibited, HCO_3^--stimulated ATPase is localized exclusively in the oxyntic cells. They also found an ATPase with similar characteristics in liver, pancreas and brain. There is, however, evidence that mitochondrial ATPase is also stimulated by HCO_3^- (Racker, 1962) and inhibited by SCN^- (Sachs, Wiebelhaus, Blum & Hirschowitz, 1972). Sachs, Wiebelhaus, Blum & Hirschowitz (1972) further purified the gastric enzyme, solubilized it and found sufficient evidence that it is derived from vesicles found at the luminal surface of the acid secreting cell, although there may be at least a mechanistic similarity between mitochondrial ATPase and the gastric vesicular ATPase. The similarity between these two enzymes suggests that careful study of a tissue is required to establish that a non-mitochondrial HCO_3^--stimulated ATPase is functioning in that tissue and may explain the widespread distribution of this enzyme.

Forte, Forte & Saltman (1967) found a K^+-dependent p-nitrophenyl phosphatase in the gastric microsomal fraction and Sachs, Rose, Shoemaker & Hirschowitz (1966) found a K^+-dependent acetyl phosphatase in homogenates of the frog gastric mucosa. Limlomwongse & Forte (1970) found that the K^+-stimulated phosphatase and the SCN^-- inhibited, HCO_3^--stimulated ATPase described by Kasbekar & Durbin (1965) first appeared in the morphological development of the frog at the stage where

H^+ secretion was first observed. Although these findings are of substantial interest, they obviously do not establish the necessity for ATP in gastric secretion.

In search for even more direct support for the theory that ATP hydrolysis provides the energy for hydrogen ion secretion in the gastric mucosa, Kidder (1971) investigated the effect of exogenous ATP on acid secretion in the bullfrog gastric mucosa. Clearly, if one could demonstrate that exogenous ATP could support acid secretion in a mucosa which was otherwise incapable of secretion, this would constitute strong support for this theory. On the other hand, a failure of this experiment could be explained by assuming that the added ATP did not penetrate the tissue and thus was not getting to the active site. However, if it could be proven that sufficient ATP was entering the cells, but failed to stimulate acid secretion, the ATPase hypothesis would be in serious difficulty. It is of great interest that Armstrong & Gerencser (1971) and Gerencser & Armstrong (1972) have demonstrated that ATP added to the mucosal bathing solution can cause sustained sodium transport in energy-depleted frog intestinal preparations.

Kidder (1971) found that exogenous ATP failed to maintain acid secretion in anaerobic conditions and it did not prolong the time course for the decay of secretion upon changing to anaerobic conditions. More recently (Kidder, 1973) provided indirect evidence that exogenous ATP probably could enter the cells and would enter at a rate sufficient to produce measureable acid secretion under anoxic conditions, were acid secretion driven by ATP alone. Therefore, these results indicate that although ATP enters the cells at a sufficient rate, it cannot by itself sustain acid secretion in the absence of oxygen. The experiments

of Kidder, Curran & Rehm (1966) and Kidder (1970a) indicated that acid secretion was closely associated with a process which caused cytochrome 'c' to shift to a more reduced state, but as ATP seems also to be involved as indicated by other evidence, suggested that both an intact electron transport system and oxidative phosphorylation were required for H^+ transport. In aerobic conditions, ATP caused a transient increase in secretory rate, followed by return to a steady state somewhat higher than the control state and a similar correlation between secretion and cytochrome 'c' steady-state shifts was found (Kidder, 1971).

However, the experiments of Hersey & Jöbsis (1969) and Hersey (1971) show changes in the redox steady state of all the cytochromes with secretory rate changes in intact mucosa, which is almost at complete variance with the work of Kidder et al. (1966). Kidder (1970b) has argued that Hersey & Jöbsis had very thick unstirred layers with their technique and were dealing with partially anoxic preparations. However, these studies have been hindered by a number of technical difficulties.

D. SODIUM TRANSPORT IN THE STOMACH

In the isolated frog gastric mucosa, Hogben (1951, 1955) found no evidence for active Na^+ transport. There was no net Na^+ transfer in the absence of an electrochemical potential gradient and the movement of Na^+ in the presence of an electrochemical potential gradient suggested that its movement was largely passive. Variations in the mucosal Na^+ concentration (Rehm, 1962) and serosal and mucosal Na^+ concentrations of about 10 mM only (Harris & Edelman, 1964) had no effect on the frog gastric p.d. or H^+ secretion. However, when Na^+ in the bathing solutions was replaced by choline, the p.d. and H^+

secretion were severely depressed (Sachs, Shoemaker & Hirschowitz, 1966). This suggests that Na^+ does have an essential role in amphibian gastric secretory function, but the effects of Na^+ removal appear to be modified by the nature of the buffers and replacement ions used (Davenport, 1963). However, the Na^+ distribution does not appear to be in electrochemical equilibrium between the serosal solution and the epithelial cell interior (Davenport & Alzamora, 1962). In addition, ouabain (10^{-3} to 10^{-6}M) inhibited frog gastric acid secretion and caused a fall in the tissue K^+ concentration and an increase in the tissue Na^+ concentration (Davenport, 1962).

For the early tadpole stomach, similar results were obtained (Forte, Limlomwongse & Kasbekar, 1969). The transmucosal p.d. was dependent upon the presence of Na^+ in the serosal bathing solution, although a significant change in the p.d. was observed only when the serosal Na^+ concentration was below 15 - 20 mM. The p.d. was sensitive to ouabain (10^{-3}M) only when added to the serosal side and to the complete omission of K^+ from the serosal solution. However, isotopic flux analysis showed that the transepithelial movement of Na^+ was consistent with passive diffusion. These results both in the adult frog gastric mucosa and tadpole stomach can be explained by a hypothesis for an active Na^+ transport system (a Na^+ pump), orientated such that its operation would be in the direction of cell interior to the serosal side. Since the mucosal membrane of these cells is relatively impermeable to Na^+ (and K^+ as well) as shown by the asymmetry of the effects described, the pump would not provide significant transepithelial transport of Na^+, but might simply be regarded as a pump-leak system at the serosal interface analagous to transport systems.in many cell membranes such as the red blood cell, muscle and

nerve. In systems in which net transepithelial Na^+ flux is functionally important, a relatively low resistance to Na^+ movement across the outer facing membrane (or control thereof) is an essential feature (Koefoed-Johnsen & Ussing, 1958; Curran & Gill, 1962; Frazier, Dempsey & Leaf, 1962; Schultz, Curran, Chez & Fuisz, 1967).

However, more recently, Flemström & Öbrink (1970) and Flemström (1971) found that in hypoxic conditions (O_2 tension of 300 mm Hg in the bathing solutions) Na^+ was actively transported from the mucosal to the serosal side of the isolated frog gastric mucosa as measured by the flux ratio (Teorell, 1949b; Ussing, 1949c). The s.c.c. remained unchanged although the H^+ secretion decreased when the O_2 tension was reduced from 700 to 300 mm Hg which might be interpreted as a contribution of the active transport of Na^+ to the s.c.c. At a high (700 mm Hg) and two lower (150 and 40 mm Hg) O_2 tensions, no active transport of Na^+ was observed. Ouabain (10^{-7}M) inhibited the s.c.c. in hypoxic mucosae (Po_2 = 300 mm Hg) only when added to the serosal side. In fully oxygenated mucosae (Po_2 = 700 mm Hg), no such effect was obtained (Flemström & Öbrink, 1972). These results were explained by an active Na^+ pump asymmetrically distributed in the gastric acid secreting cells (i.e. present only at the serosal side), which was postulated to be stimulated at an O_2 tension of 300 mm Hg for the following reasons. Under conditions of full oxygenation, the Na^+ pump is less effective due to an intracellular pH which is high possibly because of an intracellular liberation of base during H^+ secretion and therefore well above the pH optimum of the ($Na^+ + K^+$)-stimulated ATPase, which is about 7.4 (Skou, 1965). When the O_2 tension is moderately reduced, there would be a small reduction of intracellular pH thus favouring the ($Na^+ + K^+$)-stimulated ATPase and stimulating the

Na^+ pump mechanism. On account of an asymmetric distribution of the Na^+ pump, this would result in active transport of Na^+ from the mucosal to the serosal side. Further reduction of the O_2 tension would inhibit all active transport mechanisms together with all other aerobic cell activities. It would be of great interest to investigate the effect on the p.d. and s.c.c. of Na^+ removal from the bathing solutions at an O_2 tension of 300 mm Hg and to compare the results with those described previously in the fully oxygenated state. A possible function of the active Na^+ transport mechanism in the frog gastric mucosa would be to provide specific ion gradients, especially the maintenance of an optimal intracellular K^+ concentration, which appears to be required for normal biochemical (Forte, Forte & Saltman, 1967) and secretory function (Davenport, 1963, Davis, Rutledge, Keesee, Bajandes & Rehm, 1965; Sedar & Wiebelhaus, 1972).

For mammalian gastric mucosae, there is direct evidence (in some cases) for transepithelial active Na^+ transport. Wright (1962, 1964) found that the p.d. and s.c.c. of the foetal rabbit stomach depended totally on the presence of mucosal Na^+ up to the 22nd day of gestation. This also indicates that the mucosal side in this tissue must be permeable to Na^+. After this time (23rd day onwards) a small amount of hydrochloric acid secretion was shown, and approximately 70% of the s.c.c. continued to be dependent on the presence of mucosal Na^+. The movement of K^+ was passive, down the electrochemical potential gradient and therefore does not contribute to the s.c.c. (Wright, 1964). Kendall & Wright (1967) confirmed by the use of radiosodium (^{22}Na and ^{24}Na) that the Na^+ dependent s.c.c. in the 28 day old rabbit foetal stomach is an exact measure of the active transport of Na^+ from mucosa to serosa. The Na^+ independent fraction of the s.c.c. was not precisely

characterized because of the difficulty in measuring directly the rate of acid secretion of a preparation mounted in the Ussing-type of chambers (Ussing & Zerahn, 1951). However, from the work of Wright (1962, 1964) and Kendall & Wright (1967), the Na^+ independent s.c.c. can be associated with the secretion of hydrogen and chloride ions from serosa to mucosa. Active transepithelial Na^+ transport from mucosa to serosa, as well as modest or no acid secretion from serosa to mucosa has been reported to be present in several other in vitro mammalian gastric preparations (Cummins & Vaughan, 1963, 1965a, b; Kitahara, 1967; Kitahara, Fox & Hogben, 1969; Chez, 1970; Forte, Forte & Machen, 1972).

However, investigations using mammalian stomachs have been limited by the relatively short in vitro survival of the tissue, compared to the amphibian gastric mucosa. Before the work of Wright (1962) on the foetal rabbit stomach, the only mammalian stomachs that seemed able to survive in vitro conditions were the rat stomach (Patterson & Stetten, 1949) and the mouse stomach (Davenport & Chavré, 1951, 1953). In both cases the p.d. and s.c.c. were not measured, but Patterson & Stetten found that acid secretion increased over a period of 3 to 6 hours, indicating that the preparation was viable. Davenport and Chavré found that they had to raise the O_2 tension in the bathing solutions to about 3,200 mm Hg to minimize lactic acid production and obtain maximal acid secretion. This, they concluded was necessary for complete oxygenation of the cells. The dog stomach was found to survive only if its blood supply was left intact (Rehm, 1945, 1953; Bornstein, Dennis & Rehm, 1959). The rabbit foetal stomach in vitro maintains a steady p.d. and s.c.c. for 4 to 6 hours (Wright, 1962).

The crucial condition for in vitro survival is adequate oxygenation of the tissue, which seems to be dependent on the thickness of the muscle coat if not removed, or on the thickness of the separated mucosa. In the stomach of the 28 day old rabbit foetus, the muscle coat is very thin and therefore adequate oxygenation can be achieved whether the preparation is used as a sac or as a membrane clamped in the Ussing-type of chambers (Wright, 1962, 1964). Stomachs of rabbits more than one day old were unable to maintain a p.d. for more than a few minutes in vitro (Wright, 1964). The transmural p.d. of the isolated rat stomach (Cummins & Vaughan, 1963, 1965a, b) and of the cat, dog, monkey and human stomach (Kitahara, Fox & Hogben, 1969) decreased up to 50% in 3 hours. In the cat, monkey and human preparations, this decrease was predominantly associated with a reduced s.c.c. indicating diminishing active ion transport. However, Kitahara (1967) and Sernka & Hogben (1969) found somewhat greater stability of the p.d. and s.c.c. when the gastric mucosae of the cat, rat and guinea pig were used after gentle removal of the muscle coat.

Recently, Forte, Forte & Machen (1972) found that the isolated piglet gastric mucosa maintained a steady transepithelial p.d. for up to 8 hours. The muscle coat had been removed by a 'bubble' technique. This consisted of injecting about 5 ml. of Krebs solution through a hypodermic needle inserted just beneath the serosal muscle layer in the fundic region of the stomach so as to form a 'bubble' between the muscle and the gastric mucosa. The serosal muscle could then be dissected away without difficulty, leaving only the gastric mucosa and the muscularis mucosa. The s.c.c. of the piglet gastric mucosa could be accounted for by a net flux of Cl^- from serosa to mucosa, a net

flux of Na^+ in the opposite direction and a small amount of H^+ secretion. When histamine was added to the serosal bathing solution, acid secretion and net Cl^- flux from serosa to mucosa were stimulated and the net Na^+ flux from mucosa to serosa was decreased. The sum of the active Cl^-, Na^+ and H^+ movements again agreed fairly well with the s.c.c.

In the amphibian gastric mucosa which survives well in vitro, active Na^+ transport may be difficult to detect because of the predominance of acid secretion compared to the adult or foetal mammalian gastric preparations. This line of reasoning seeks a common basis with which to explain the effects and role of Na^+ in the secretory activity of various gastric preparations. Other explanations stressing inherent species differences or variable progressive deterioration of the mucosae must also be taken into account.

Active Na^+ transport and acid secretion in the foetal rabbit stomach show a definite dependence on oxidative metabolism as shown by Kendall (1968) through the use of metabolic inhibitors. Similar results have also been found in the rat stomach (Cummins & Vaughan, 1965a) and in the mouse stomach (Davenport, 1947). The experiments of Kendall (1968) fall into two main groups: those involving inhibitors which directly affect the respiratory chain (e.g. anoxia, amytal, rotenone, azide and antimycin A) and those where the inhibitors act on the energy producing (oligomycin, iodoacetate, fluoride and DNP) or utilizing (ouabain) mechanisms. Considering both active transport mechanisms, inhibition by the former group is rapid and immediate and the maximum inhibition is unaffected by anoxia. Inhibition by the latter group commenced often with a time lag (except for ouabain and oligomycin) and it is possible that the inhibition

started only because there was a general lack of ATP in the system. Thus the metabolic breakdown of glucose could not proceed because there would be no ATP to enable the formation of glucose-6-phosphate and hence lack of metabolite for the Krebs' cycle and reduced co-enzymes for the cytochrome chain. Inhibition by ouabain of active Na^+ transport strongly suggested the involvement of ATP in this process, as found in other Na^+ transporting tissues, but Kendall (1968) was unable to demonstrate the presence of a ($Na^+ + K^+$)-stimulated ATPase in foetal rabbit gastric microsomes. However, after treatment with 1 M urea, 25% inhibition of the total Mg^{++}-activated ATPase was obtained with ouabain, which probably indicated the presence of($Na^+ + K^+$)-stimulated ATPase. Kendall (1968) also found no ($Na^+ + K^+$)-stimulated ATPase present in the adult rabbit gastric mucosa. However, Nakao, Tashima, Nagano & Nakao (1965) reported the presence of ($Na^+ + K^+$)-stimulated ATPase in the adult rabbit gastric mucosa after treatment of the tissue homogenate with sodium iodide. Therefore Kendall's failure to show the presence of ($Na^+ + K^+$)-stimulated ATPase in the rabbit foetal gastric mucosa may have been due to inadequate experimental procedures used. Cummins & Vaughan (1965a) have shown the presence of a ($Na^+ + K^+$)-stimulated ATPase in the rat gastric mucosa which transports Na^+ actively.

Although acid secretion in the foetal rabbit stomach seemed to have a more direct link with metabolic oxidation than did the active Na^+ transport, a direct role for ATP could not be ruled out, especially since an SCN^--inhibited, HCO_3^--stimulated ATPase was found (Kendall, 1968).

E. THE PRESENT PROBLEM

The rabbit foetal stomach is particularly suitable for the study

of mammalian gastric ion transport mechanisms because of its ability to survive well *in vitro*. As described in the previous section, the rabbit foetal stomach mainly performs transepithelial active Na^+ transport accompanied by a small amount of acid secretion. A definite dependence of both the active Na^+ transport and acid secretion on metabolism has been shown, and in both cases the direct involvement of ATP has been suggested. However, the mechanisms involved are not fully understood.

The present work was carried out as an extension of the previous work of Kendall (1968) in order to try and elucidate further the mechanisms by which metabolic energy is utilized for the active transport of ions in the foetal rabbit gastric mucosa. Oxygen consumption, ATP, ADP and CP concentration changes and active ion transport in the rabbit foetal gastric mucosa were studied in similar metabolic conditions.

CHAPTER 2

MATERIALS AND METHODS

A. MATERIALS

All laboratory chemicals used were of Analar grade and obtained from the British Drug House Ltd. Other materials were obtained as follows:

Adenosine 5'-diphosphate (trisodium salt) .... Boehringer Mannheim

Adenosine 5'-triphosphate (crystalline disodium salt) ..... " "

Creatine kinase (rabbit muscle); EC 2.7.3.2 .. " "

Creatine phosphate (crystalline disodium salt) " "

2,4-dinitrophenol ..... The British Drug House

Glucose-6-phosphate dehydrogenase (yeast); EC 1.1.1.49 ..... Boehringer Mannheim

Hexokinase (yeast); EC 2.7.1.1 ..... " "

Lactate dehydrogenase (beef heart); EC 1.1.1.27 ..... " "

Liquid nitrogen ..... British Oxygen Company Ltd.

Nembutal (pentobarbitone sodium, B.Vet.C) .... Abbott Laboratories Ltd.

Neutralit indicator paper, pH 5-10 ..... E. Merck, Darmstadt

Nicotinamide-adenine dinucleotide, reduced (disodium salt, grade II) ..... Boehringer Mannheim

Nicotinamide-adenine dinucleotide phosphate, oxidised (disodium salt) ..... " "

Oligomycin ..... Sigma Chemical Co.

Ouabain (strophanthin G) ..... The British Drug House Ltd.

Penicillin V potassium ..... Eli Lilly & Co.

Phosphoenolpyruvate (crystalline tricyclohexylammonium salt) ..... Boehringer Mannheim

Pyruvate kinase (rabbit muscle); EC 2.7.1.40 . " "

Rabbits (female, albino) ..... Cheshire Rabbit Farm

Silicone antifoam emulsion ..... Hopkins & Williams Ltd.

Streptomycin sulphate ..... Glaxo Company Ltd.

B. METHODS

(i) Operative procedure.

Adult, female albino rabbits were mated, so that the time of conception was known to within 12 hours. On the 28th day of pregnancy, the rabbit was anaesthetized with Nembutal. The foetuses were taken out by Caesarean section and killed by a blow on the head. The whole foetal stomach was removed rapidly, washed with ice-cold Krebs bicarbonate Ringer solution and stored at $4^{\circ}C$ in this solution. Under these conditions, the stomachs remained viable for up to five days as judged by their ability to develop the same order of magnitude of potential difference and short-circuit current. Maximum p.d. of fresh and 24 hour-stored stomachs was obtained within one hour, while stomachs stored for five days could take up to six hours. Therefore, only fresh and 24 hour-stored stomachs were used throughout the work.

(ii) Solutions.

The Krebs bicarbonate Ringer solution had the following composition (mM): Na^+, 129.14; K^+, 19.41; Ca^{++}, 5.17; Cl^-, 139.48; HCO_3^-, 19.41; glucose, 24. It was oxygenated with 95% O_2 + 5% CO_2 before use. This solution will be referred to hereafter as the medium.

Experimental media were modified Krebs bicarbonate Ringer solution, the modifications being specified with each experiment. Na^+-free medium had the sodium replaced by an equivalent amount of choline. For experiments lasting longer than 5 hours, and in all experiments where the oxygen consumption of cells was measured, the media contained

the following antibiotics: streptomycin sulphate, 50 μg/ml. and penicillin, 50 μg/ml. This was done to minimize bacterial contamination. Throughout the work, 95% O_2 + 5% CO_2 was used for oxygenation.

(iii)Measurement of the short-circuit current.

Kendall & Wright (1967) showed that the total s.c.c. of the gastric mucosa of a 28-day old rabbit foetus consisted of two components: a Na^+ dependent component (approximately 70%) and a Na^+ independent component (approximately 30%). From measurements of the net flux of Na^+ using radiosodium, and the s.c.c., it was found that the Na^+ dependent component of the s.c.c. (I_{Na}) could be used as an exact measure of the net active transport of Na^+ from mucosa to serosa. The Na^+ independent s.c.c. could be associated with the secretion of hydrogen and chloride ions from the serosa to the mucosa (Wright, 1962,1964; Kendall, 1968). Therefore the total s.c.c. and the Na^+ independent s.c.c. were measured using the method described by Kendall & Wright (1967).

The foetal stomach was cleared of adherent tissue and cut open through the cephalic surface. The stomach contents were washed out with cold experimental medium. The stomach was opened out to form a flat membrane (consisting of muscle and mucosa) which was then sandwiched between two Perspex chambers (Fig.1A), based on the type used by Ussing & Zerahn (1951). A membrane with an area of 0.6 cm^2 was obtained. The volume of each chamber was 5 ml. The chambers were provided with an oxygen lift which oxygenated and stirred the solutions, which contained one or two drops of silicone antifoam emulsion to prevent frothing. Two holes in the chambers enabled salt bridges (thin polythene tubes filled with 3M-KCl in 2% (w/v) agar)

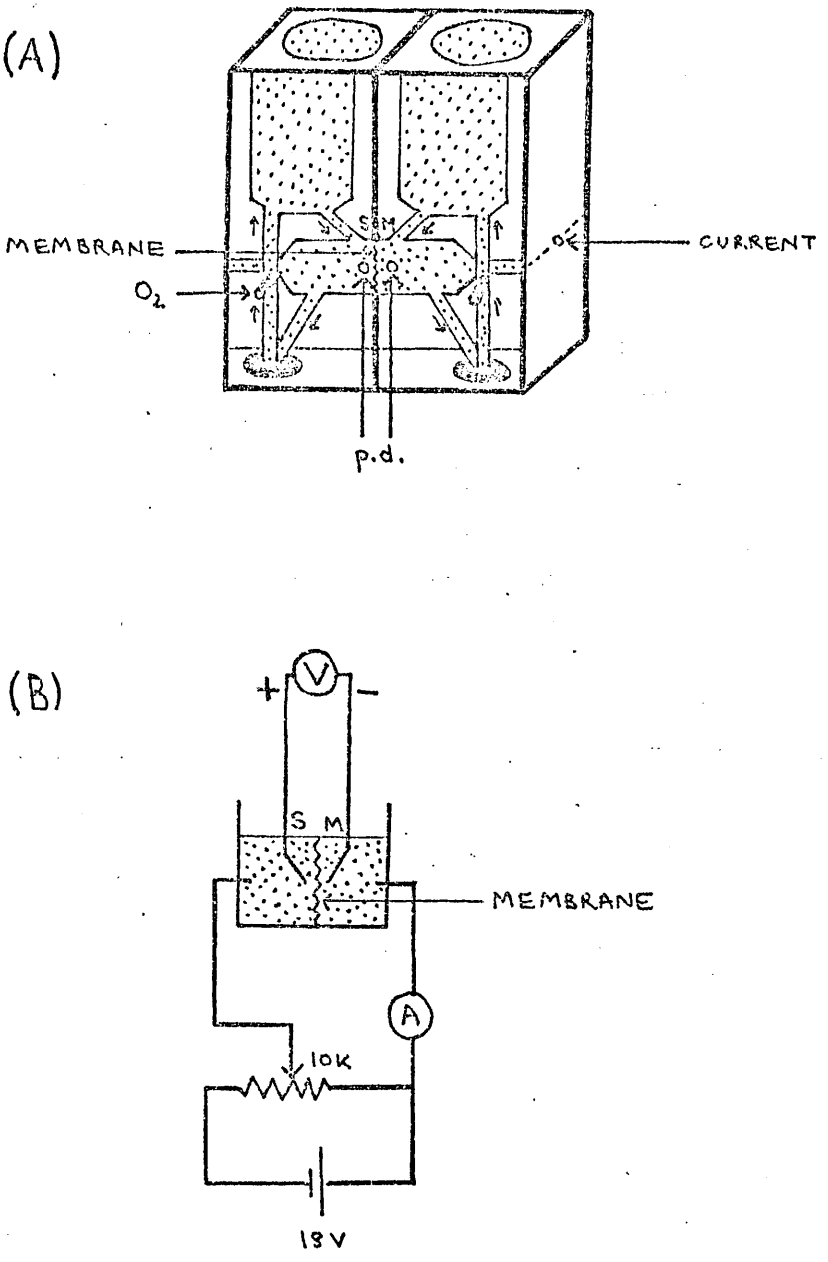


Fig.1 (A) Perspex chambers between which the foetal stomach membrance was clamped.

(B) Circuit used for passing current and recording the p.d. across the foetal stomach membrane.

M, mucosal side; S, serosal side.

to be inserted with their tips close to the membrane. The other ends of these bridges were connected to calomel electrodes, connected to a Vibron 33B electrometer, which was used to measure the p.d. across the membrane. The chambers had a second pair of holes, so that two other salt bridges could be inserted with their tips lying along the normal to the plane of the membrane at its centre. These bridges were connected with a pair of silver/silver chloride electrodes to a circuit for passing current through the system (Fig.1B). By means of these two pairs of electrodes, it was possible to measure the s.c.c. and d.c. resistance of the preparation, as well as the open-circuit p.d. The chambers were placed in a water-bath, the temperature of which was adjusted so that the solutions in the chambers were maintained at a constant temperature of 35°C.

The total s.c.c. was measured with experimental medium containing Na^+ on both sides of the membrane. The Na^+ independent s.c.c. was measured by substituting 154 mM-choline chloride solution on the mucosal side, after two or three washes. The foetal stomach membrane area of 0.6 cm^2 yielded 0.105 $\pm$ 0.008 g wet weight of mucosa (mean $\pm$ S.E. of mean, number of experiments = 8).

(iv) Measurement of oxygen consumption.

The oxygen consumption of foetal gastric mucosal cell suspensions was measured using a Radiometer Po_2 electrode, type E 5046, a Clark-type oxygen electrode. It consists of a combined platinum cathode (20 μthick) and silver/silver chloride anode, mounted in an electrode jacket and covered with a plastic membrane. A thin layer of electrolyte solution (supplied by Radiometer) separates the membrane from the electrode. The electrolyte solution is a phosphate buffer to which some potassium chloride has been added to stabilize the

potential of the anode (reference electrode). A polarizing voltage of -0.64 V is applied to the platinum electrode. The electrode is placed close to the membrane and because of the polarizing voltage, oxygen diffusing through the membrane is reduced at the platinum electrode: $O_2 + 2H^+ + 2e^- \longrightarrow H_2O_2$. This reduction process produces a current through the Po_2 electrode. The current is proportional to the partial pressure of oxygen outside the membrane, since this pressure is the motive power in the oxygen diffusion.

A water-jacketed glass chamber (Fig.2) was used for the measurement of the oxygen consumption of foetal gastric mucosal cell suspensions. The electrode was inserted at one side of the chamber, with the membrane in contact with the solution in the chamber. The membrane used was a Teflon membrane, 0.0005 inches thick. The solution was stirred at a constant rate using a magnetic stirrer. A Churchill water-bath was used to circulate water through the glass chamber. The temperature of the water-bath was adjusted so that the solution in the chamber was maintained at a constant temperature of 35°C. The electrode current was passed through a variable resistance (very small compared to the resistance of the electrode) and the voltage drop fed to a Bryants 27,000 potentiometric chart recorder. The recorder was adjusted to read zero Po_2 when the electrode was placed in an oxygen-free solution. This zero adjustment was necessary to be able to read the Po_2 of a solution directly from the recorder. The electrode was then calibrated using the solution of zero Po_2 and a solution of known Po_2. The characteristic of the electrode is strictly linear and before every experiment, this was checked using air-equilibrated tap water (Po_2 = 150 mm Hg) and tap water equilibrated with 95% O_2 + 5% CO_2 (Po_2 = 722 mm Hg). From these two readings, the zero current was also

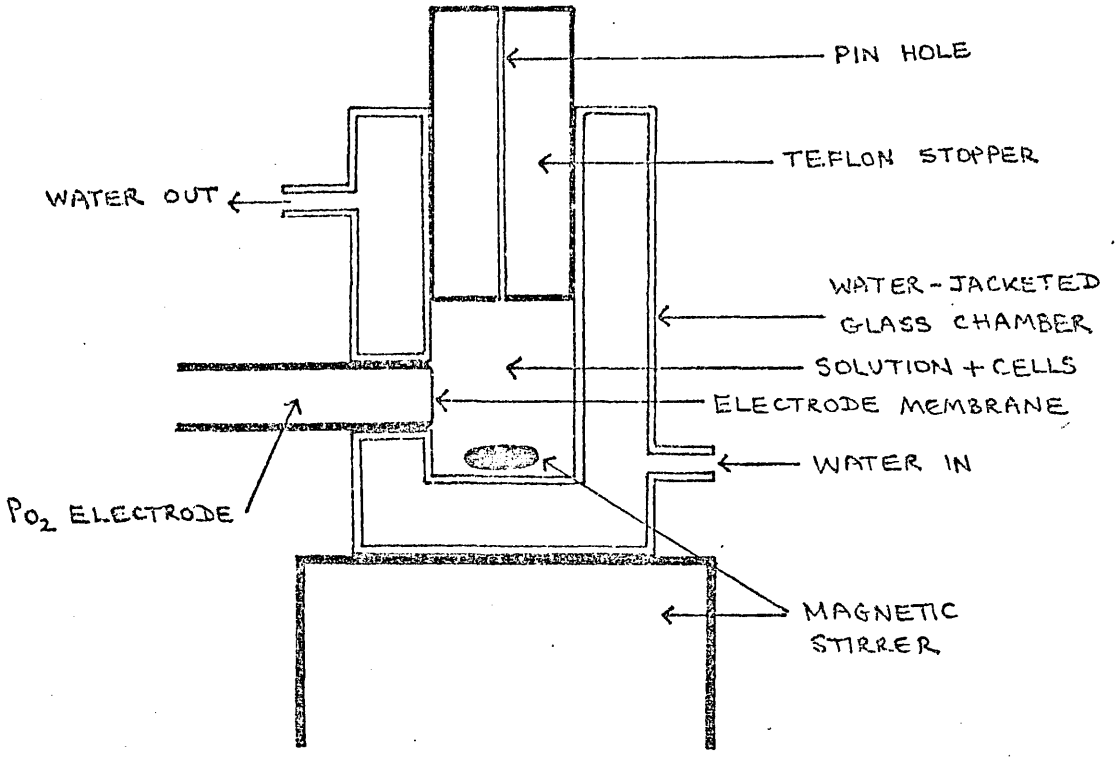


Fig.2 Water-jacketed glass chamber in which the oxygen consumption of foetal gastric mucosal cells was measured.

calculated. It was always very small, but if it corresponded to more than 5 mm Hg, the zero adjustment was repeated. This procedure also served as a check of the response time of the electrode. When the 0.0005 inch thick Teflon membrane was used, it took less than 60 seconds to give a value, 99% of the final level. A quick response time is necessary to be able to follow small, rapid changes in the Po_2 of a solution accurately.

The oxygen consumption of foetal gastric mucosal cell suspensions was always measured over the Po_2 range of 700 to 400 mm Hg. In this range, the oxygen consumption remained constant (a linear drop of the Po_2 with time was always obtained), indicating that the cells were not being subjected to anoxic conditions. The short-circuit current of a foetal stomach also remained constant in this range (Wright, 1970). Before an experiment was started, the oxygen consumption of blank experimental media was measured and was found to be greater than 10% of the oxygen consumption of cell suspensions subsequently measured. Controls, using sterilized media indicated that this was not due to bacterial contamination. The adsorption of oxygen on the Teflon stopper was probably the cause. For this reason, blank oxygen consumption measurements were always subtracted from subsequent measurements of the oxygen consumption of cell suspensions. At least two blank measurements were made to check that they were constant, before cells were added to the medium.

A known volume of blank experimental medium, containing a few drops of silicone antifoam emulsion was added to the chamber and oxygenated thoroughly with 95% O_2 + 5% CO_2. The Teflon stopper was inserted rapidly into the chamber and all the air was expelled through the pin-hole. The fall in Po_2 over a measured time interval, at

constant temperature and in a known volume was followed on the recorder. Using Henry's Law, the amount of oxygen consumed per hour was calculated. The stopper was removed and the medium was reoxygenated.

The foetal stomachs were cleared of adherent tissue, incised through the cephalic surface and their contents washed out with cold experimental medium. After opening out the stomachs, the mucosae from three foetal stomachs were scraped off the underlying tissue using a blunt spatula, and transferred to the chamber. The sensitivity of the Teflon membrane was affected by the presence of clumps of cells being stirred. Therefore, after time was allowed for thorough oxygenation of the cells, and for warming up of the cells to 35°C, a readjustment was made to give the initial reading of 722 mm Hg on full oxygenation with 95% O_2 + 5% CO_2. The Teflon stopper was inserted rapidly as described for blank measurements and the fall in Po_2 of the solution was followed over a timed interval, never allowing the Po_2 to fall below 400 mm Hg. At the end of each experiment, the volume of experimental medium containing the cells was measured. The cell suspension was centrifuged at 1700 x g for 10 minutes and the supernatant was discarded. The cell pellet was weighed and then dried to constant weight in an oven. The results were expressed as μ-moles O_2 consumed/mg dry weight/hour. The dry weight of mucosal cells was found to be 8.54 ± 0.36% of the wet weight (mean ± S.E. of mean, number of experiments = 47). The oxygen consumption experiments were divided into two distinct groups: (a) those in which cells were incubated in experimental medium containing Na^+, and (b) those in which cells were incubated in Na^+-free experimental medium. Specific metabolic conditions were then imposed upon these two groups.

(v) Measurement of ATP, ADP and CP concentrations.

(a) Experimental procedure.

Since the amount of tissue available from one foetal stomach was insufficient for ATP, ADP and CP determination by the methods used, pooled mucosae from three foetal stomachs (approximately 0.3 g of tissue) were used.

The stomachs were cleared of adherent tissue, incised through the cephalic surface and their contents washed out with cold experimental medium. After opening out the stomachs, the mucosae from three foetal stomachs were scraped off the underlying tissue using a blunt spatula and transferred to 20 ml. of cold experimental medium in a 50 ml. volumetric flask, kept on ice until the experiment was started. A few drops of silicone antifoam emulsion were added to each flask to prevent excessive frothing on gassing. The cell suspensions were incubated with shaking and gassing at 35°C for the required length of time. All experiments were divided into two distinct groups: (a) those in which cells were incubated in experimental medium containing Na^+, and (b) those in which cells were incubated in Na^+-free experimental medium. Specific metabolic conditions were then imposed upon these two groups.

At the end of the experiment, the cell suspensions were centrifuged at 1700 x g for 10 minutes. The pellet of cells was removed immediately, weighed and frozen in liquid nitrogen. ATP, ADP and CP were extracted and assayed by a modification of the method of Lowry, Passonneau, Hasselberger and Schulz (1964).

(b) Extraction procedure.

The frozen cell pellet was placed on 3M-perchloric acid (0.3 ml./125 mg tissue) previously frozen in a test-tube, which

subsequently was kept on ice. A glass rod was used for mixing to ensure that on thawing the perchloric acid had completely penetrated the pellet (3-5 minutes). Cold 2M-$KHCO_3$ was added to neutralize the perchloric acid extract. The precipitate was spun down and discarded. The volume of the supernatant was measured and Neutralit indicator paper, pH 5-10, was used to check that the pH of the sample was between 7.5 and 8.0. The pH was further adjusted, if necessary. The samples were frozen and stored at -10°C, except for brief intervals when they were thawed to remove aliquots for analysis.

The method of Lowry et al. (1964) was modified as follows. (1) The extraction of the tissue was performed on ice (0°C) and not at -10°C. This had no effect on the concentration of ATP, ADP or CP subsequently measured. (2) Omission of EDTA from the extraction procedure did not cause any loss of ATP, ADP or CP. (3) Neutralization of the perchloric acid extract, carried out in the presence of the perchloric acid-insoluble material, had no effect on ATP, ADP or CP tissue concentrations. This confirmed that there was no interference by certain enzymes (for example, adenylate kinase, EC 2.7.4.3 and aldolase, EC 4.1.2.7) which are not destroyed by acid treatment and can possibly be reactivated by neutralization. (4) Since facilities were not available for storage of the samples at -80°C, experiments were carried out which showed that no appreciable loss of ATP, ADP or CP occurred in the neutralized tissue extracts, when stored at -10°C for one week. Samples were always analysed within one week of extraction.

The loss of ATP, ADP and CP due to possible incomplete extraction was investigated. A second extraction with 3M-perchloric acid yielded less than 5% of the values obtained in the first

extraction. Therefore, one extraction was considered sufficient. Recovery of ATP, ADP and CP standard solutions processed through the extraction procedure, was always greater than 85%. No corrections were made of the concentrations measured in foetal gastric mucosal cells, since absolute concentrations of ATP, ADP and CP were not crucial to the present work.

(c) General assay procedure.

The ATP, ADP and CP assays are dependent on the fluorimetric measurement of the appearance of NADPH, or the disappearance of NADH, upon addition of the appropriate enzymes. NADH and NADPH absorb light at 340 nm and emit a fluorescent band at a longer wave-length, which has a peak at 460 nm. All assays were carried out using a Locarte single sided fluorimeter MK4 (Locarte LF/2 primary filter, 340-380 nm; Locarte LF/5 secondary filter, 440 nm photomultiplier cut-off) or an Aminco fluoro-microphotometer (4-7113 primary filter, 360 nm peak wave-length; 4-7116 secondary filter, sharp cut-off, 415 nm and above). A blank and freshly made up standard solutions of appropriate concentration were assayed with every batch of samples analysed, to ensure that the reaction was complete at the time selected. The blank and standard solutions were similar to the tissue samples in volume and in perchloric acid and $KHCO_3$ composition. All assays were carried out in duplicate.

The concentrations of three ATP and three ADP standards were calculated from measurements of their absorption at 260 nm, using a Beckman DB spectrophotometer. ATP and ADP standard lines were plotted. One CP standard of unknown concentration was assayed to check that the reaction was working. Allowing for volume changes, the ATP standard line was used to calculate CP concentrations, on the

assumption that 1 mole of ATP is formed from 1 mole of CP. Internal ATP, ADP and CP standards were used to check whether the tissue extracts contained any factors which could affect the reaction. Calculations of ATP, ADP and CP concentrations were carried out using the Control Data Corporation 6600 computer (Fortran, computer language) of the University of London.

Three modifications were introduced to the assay procedures of Lowry et al. (1964). (1) Bovine serum albumin was omitted from all solutions; (2) the enzymes were not diluted; (3) the enzymes were not added as a mixture. Modifications (1) and (2) did not affect the reactions. Modification (3) was introduced for greater accuracy. The enzyme which started the reaction was always added last. The other enzyme was added before the initial reading was taken, to remove any intermediate which could have been present in the tissue extract.

(d) ATP assay.

The enzyme, hexokinase catalyses the phosphorylation of glucose by ATP in the presence of Mg^{++} according to the equation:

$$\text{glucose} + \text{ATP} \xrightarrow{Mg^{++}} \text{glucose-6-phosphate} + \text{ADP}.$$

The enzyme, glucose-6-phosphate dehydrogenase catalyses the oxidation of glucose-6-phosphate by $NADP^+$ according to the equation:

$$\text{glucose-6-phosphate} + NADP^+ \longrightarrow \text{6-phosphogluconate} + NADPH + H^+.$$

The equilibrium constant for this reaction is greatly in favour of NADPH formation, permitting quantitative measurement of ATP according to the overall reaction, described by the following equation:

$$\text{glucose} + \text{ATP} + NADP^+ \longrightarrow \text{ADP} + NADPH + H^+ + \text{6-phosphogluconate}.$$

The increase in fluorescence accompanying the conversion gives a quantitative measure of ATP if glucose is present in excess.

The ATP assay mixture contained glucose, 1 mM; $NADP^+$, 0.03 mM;

$MgCl_2$, 5 mM; Tris buffer pH 7.5, 100 mM; glucose-6-phosphate dehydrogenase, 2 μg/ml. (140 U/mg) and the sample (0.1 ml.) in a final volume of 1 ml. The initial reading was taken. The reaction was started by the addition of hexokinase, 5 μg/ml. (140 U/mg). The mixture was incubated at 27°C for 10 minutes and the final reading was taken.

The Tris buffer was prepared by adjusting the pH of a solution of Tris with HCl. A freshly prepared solution of $NADP^+$ was used.

(e) CP assay.

The enzyme, creatine kinase catalyses the reaction:

creatine phosphate + ADP $\rightleftharpoons$ creatine + ATP.

Since CP has a much higher free energy of hydrolysis than ATP, the formation of ATP from CP is favoured. The ATP formed was measured as described in section (d).

CP and ATP were measured in the same sample. When the ATP reaction was complete, 0.1 ml. of a mixture of creatine kinase and ADP was added, giving a final concentration of creatine kinase, 80 μg/ml. (18 U/mg protein) and ADP, 0.03 mM. Incubation at 27°C was continued for a further 20 minutes. The final reading was taken. The mixture of creatine kinase and ADP was prepared at 0°C within one hour of use.

(f) ADP assay.

The enzyme, pyruvate kinase catalyses the phosphorylation of ADP by phosphoenolpyruvate according to the equation:

ADP + phosphoenolpyruvate $\xrightleftharpoons{Mg^{++},\ K^+}$ ATP + pyruvate.

The pyruvate formed is reduced to lactate by NADH in the presence of the enzyme lactate dehydrogenase according to the equation:

Pyruvate + NADH + H^+ $\longrightarrow$ lactate + NAD^+.

The reaction is followed by recording the disappearance of NADH

fluorimetrically.

The ADP assay mixture contained phosphoenolpyruvate, 0.02 mM; NADH, 0.003 mM; $MgCl_2$, 2 mM; phosphate buffer pH 7, 50 mM; lactate dehydrogenase, 8 μg/ml. (200 U/mg) and the sample (0.1 ml.) in a final volume of 1 ml. The initial reading was taken. The reaction was started by the addition of pyruvate kinase, 0.5 μg/ml. (150 U/mg) and the mixture was incubated at 27°C for 5 minutes. The final reading was taken.

The phosphate buffer was prepared by adjusting the pH of a solution of KH_2PO_4 with NaOH. Freshly prepared solutions of phosphoenolpyruvate and NADH were used.

(vi) Microscopy and the eosin exclusion test.

The foetal gastric mucosal cell suspensions were prepared as described in Section B(v), part a of this chapter. They were incubated with oxygenation and shaking for 0, 5 and 10 hours. The cell suspensions were centrifuged at 1700 x g for 10 minutes and the cell pellet was resuspended in a small volume of supernatant. Smears of the cells were made, air dried and stained with haematoxylin and eosin. The smears were observed using a Patholette microscope. A drop of cell suspension was put on a glass slide and covered with a cover-slip. The cells were then observed under a phase contrast microscope.

The eosin exclusion test (Blum, Shah, Wiebelhaus, Brennan, Helander, Ceballos & Sachs, 1971) was used as an indication of whether the cells were alive. Cell counts were not possible because of the clumping of cells due to the presence of mucus. Equal volumes of cell suspension and glucose-free medium, containing eosin, 0.05 mg/100 ml. were incubated for 1 minute at 35°C. A haemocytometer was used to determine

whether the cells had taken up eosin or excluded it. Exclusion of eosin was taken as indication that the cells were alive.

(vii)Statistical methods.

The data was analysed statistically using the Control Data Corporation 6600 computer (Fortran, computer language) of the University of London. Standard programmes were used to obtain the following:

(a) the mean of a set of scores and the standard error of the mean;

(b) the t-statistic of the difference between the means of two sets of scores ($n<30$).

CHAPTER 3

RESULTS

A. MICROSCOPY AND THE EOSIN EXCLUSION TEST.

Smears of foetal gastric mucosal cells which had been incubated for 0, 5 and 10 hours, did not stain strongly with haematoxylin and eosin. This seemed to be due to the presence of silicone antifoam emulsion. Xylene, which dissolves wax from histological sections, did not remove the silicone antifoam emulsion. However, the staining was strong enough to be able to distinguish epithelial cells in all the smears. Phase contrast microscopy confirmed the presence of epithelial cells at 0, 5 and 10 hours of incubation. The cells appeared to be intact.

The eosin exclusion test showed that foetal gastric mucosal cells did exclude eosin. Cell suspensions incubated for 5 hours did not show a marked difference from time 0 cell suspensions. However, both time 0 and 5 hour cell suspensions contained a higher proportion of cells which excluded eosin, compared with cell suspensions which had been incubated for 10 hours.

B. INCUBATION IN GLUCOSE-FREE CONDITIONS, IN THE PRESENCE AND ABSENCE OF Na^+.

The maintenance of active ion transport (total s.c.c.) in the foetal stomach has been shown to be dependent on the presence of exogenous glucose (Kendall, 1968). The total s.c.c. declined slowly when incubated in glucose-free conditions. Addition of glucose to the serosal side, to give a final concentration of 24 mM, caused a return of the total s.c.c. to its initial value within 1 hour. Incubation in glucose-free conditions was therefore chosen as a way in which to alter active ion transport slowly and to study its relationship to foetal gastric mucosal cell oxygen consumption, ATP and ADP concentrations, ATP/ADP ratio and CP concentration.

(i) The short-circuit current.

The foetal stomach was set up from time 0 with glucose-free medium on both sides of the membrane and with continuous oxygenation. The s.c.c. in the presence of Na^+(total s.c.c.) and the s.c.c. in the absence of Na^+(Na^+ independent s.c.c.) were measured in these conditions over a period of 10 hours. The results are shown in Table 1, Fig.3.

The total s.c.c. increased to a maximum in the first hour and then slowly declined during the following 9 hours. At 10 hours, 36.80% of the total s.c.c. was still present. The Na^+ independent s.c.c. remained constant for 6¼ hours and then slowly declined. By 10 hours, it was virtually at zero.

(ii) Oxygen consumption of foetal gastric mucosal cells.

Foetal gastric mucosal cells were incubated in glucose-free medium with and without Na^+ for 10 hours. In the presence of Na^+, the oxygen consumption was always greater than in the absence of Na^+ (Table 2, Fig.4). In both cases, the oxygen consumption decreased slowly. By 10 hours, it had decreased by 73% ($P < 0.05$) in the presence of Na^+ and by 82% ($P < 0.02$) in the absence of Na^+.

(iii)ATP, ADP and CP concentrations in foetal gastric mucosal cells.

Foetal gastric mucosal cells were incubated in continuously oxygenated, glucose-free medium with and without Na^+ over a period of 10 hours. Both in the presence and absence of Na^+, there was an initial decrease ($P < 0.002$) in the ATP concentration (Table 3, Fig.5). Following this decrease, the ATP concentration remained constant during 10 hours in the presence of Na^+, while it decreased slowly in the absence of Na^+ ($P < 0.001$). The ADP concentration in foetal gastric mucosal cells (Table 4, Fig.6) followed approximately similar changes

Table 1

The short-circuit current of the foetal stomach (0.6 cm^2) incubated in oxygenated, glucose-free medium with and without Na^+. The values given are means ± S.E. of mean (number of experiments).

TIME (hours)	SHORT-CIRCUIT CURRENT (μA) + Na^+
0	44.50 ± 2.03 (6)
½	77.29 ± 6.98 (7)
1	105.86 ± 7.78 (7)
1½	107.57 ± 8.51 (7)
2	99.21 ± 8.62 (7)
3	86.50 ± 11.51 (7)
4	79.14 ± 9.57 (7)
5	69.43 ± 7.73 (7)
6	61.00 ± 8.29 (6)
7	53.58 ± 8.95 (6)
8	44.58 ± 10.30 (6)
9	45.20 ± 10.24 (5)
10	39.60 ± 10.47 (5)

TIME (hours)	SHORT-CIRCUIT CURRENT (μA) - Na^+
1	23.00 ± 6.76 (4)
2¼	21.50 ± 4.81 (4)
3¼	18.13 ± 5.63 (4)
4¼	19.13 ± 6.48 (4)
6¼	17.25 ± 5.89 (4)
7¼	11.38 ± 5.63 (4)
8¼	6.75 ± 5.12 (4)
10	3.00 ± 3.00 (4)

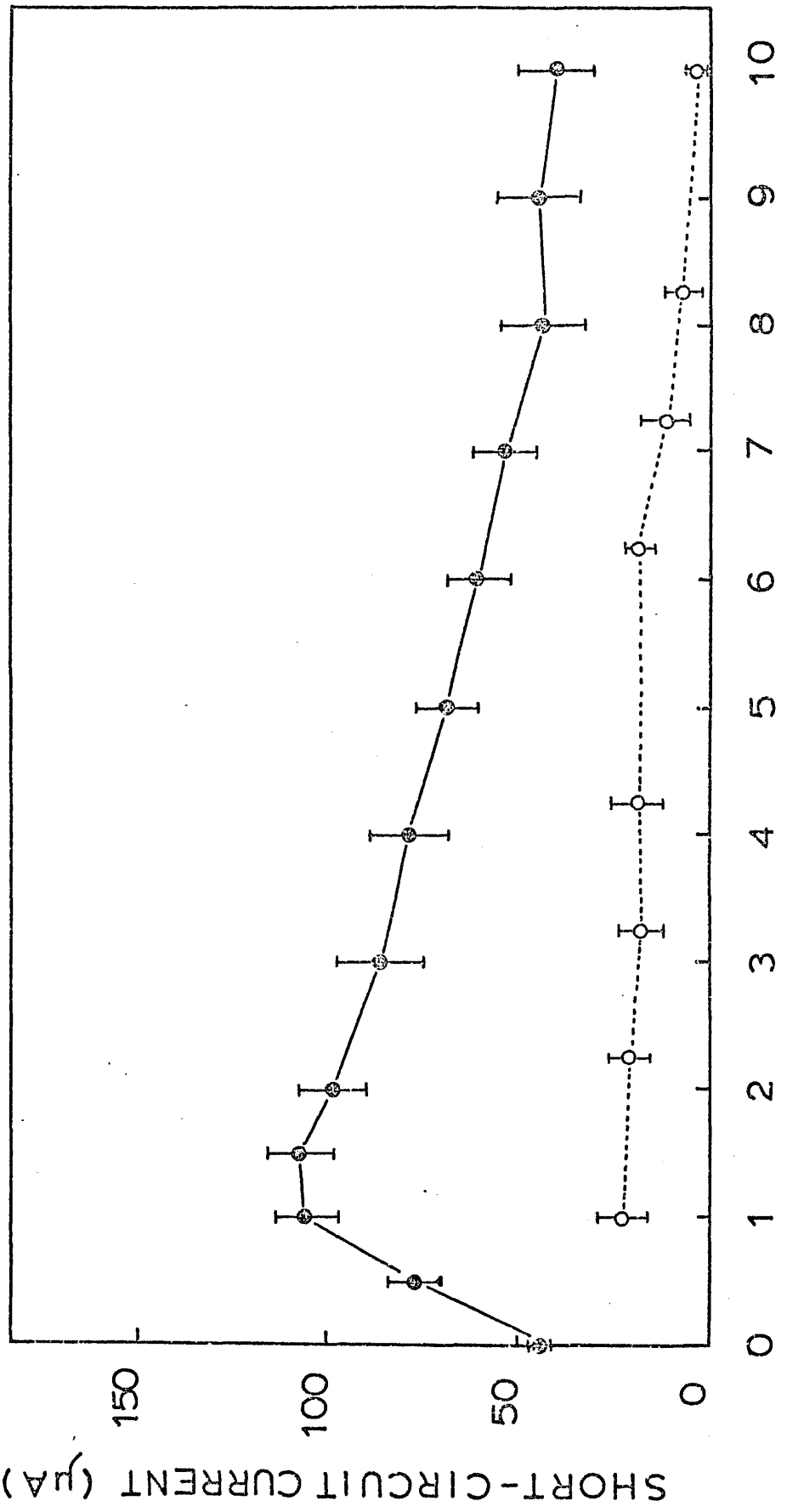


Fig.3 The short-circuit current of the foetal stomach (0.6 cm^2) incubated in oxygenated, glucose-free medium with and without Na^+. ● —— ● + Na^+; ○ ---- ○ - Na^+. Each point represents the mean ± S.E. of mean.

Table 2

Oxygen consumption of foetal gastric mucosal cells incubated in glucose-free medium with and without Na^+. The values given are means ± S.E. of mean (number of experiments).

TIME (hours)	OXYGEN CONSUMPTION (μ-moles O_2/mg dry wt./hr)	
	+ Na^+	- Na^+
1	0.27 ± 0.07 (5)	0.17 ± 0.04 (4)
2	0.24 ± 0.06 (5)	0.13 ± 0.02 (4)
3	0.18 ± 0.06 (4)	0.12 ± 0.02 (4)
4	0.18 ± 0.04 (4)	0.07 ± 0.01 (4)
5	0.16 ± 0.03 (4)	0.06 ± 0.001 (4)
6	0.13 ± 0.04 (4)	0.04 ± 0.01 (4)
$7\frac{1}{2}$	0.11 ± 0.02 (4)	0.04 ± 0.004 (4)
9	0.08 ± 0.01 (4)	0.03 ± 0.003 (4)
10	0.07 ± 0.02 (4)	0.03 ± 0.01 (4)

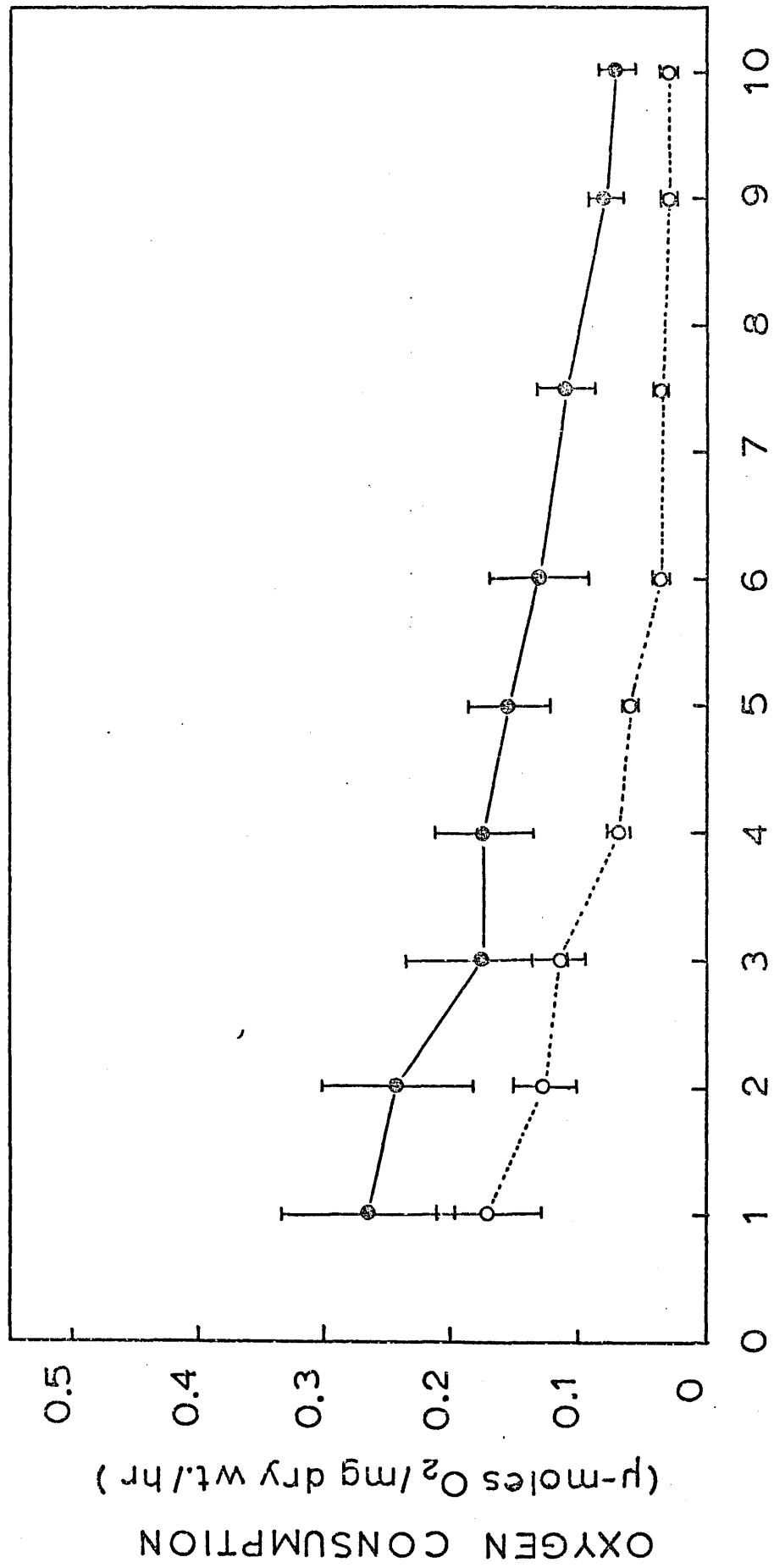


Fig.4 Oxygen consumption of foetal gastric mucosal cells incubated in glucose-free medium with and without Na^+.

● —— ● + Na^+; O ---- O - Na^+. Each point represents the mean ± S.E. of mean.

Table 3

ATP concentration in foetal gastric mucosal cells incubated in oxygenated, glucose-free medium with and without Na^+. The values given are means $\pm$ S.E. of mean (n = 6).

TIME (hours)	ATP CONCENTRATION (n-moles/g wet wt.)	
	+ Na^+	- Na^+
0	44.33 ± 6.18	44.33 ± 6.18
¼	16.18 ± 1.81	17.26 ± 1.66
½	17.16 ± 2.86	19.99 ± 3.44
¾	20.05 ± 5.89	20.81 ± 5.75
1	12.74 ± 1.50	19.52 ± 2.85
2	17.37 ± 4.14	15.88 ± 2.91
3	28.50 ± 5.48	7.62 ± 2.13
4	16.86 ± 2.58	13.66 ± 1.94
5	13.81 ± 3.75	5.76 ± 0.91
7½	17.49 ± 2.85	5.17 ± 1.70
10	14.27 ± 4.31	2.38 ± 1.13

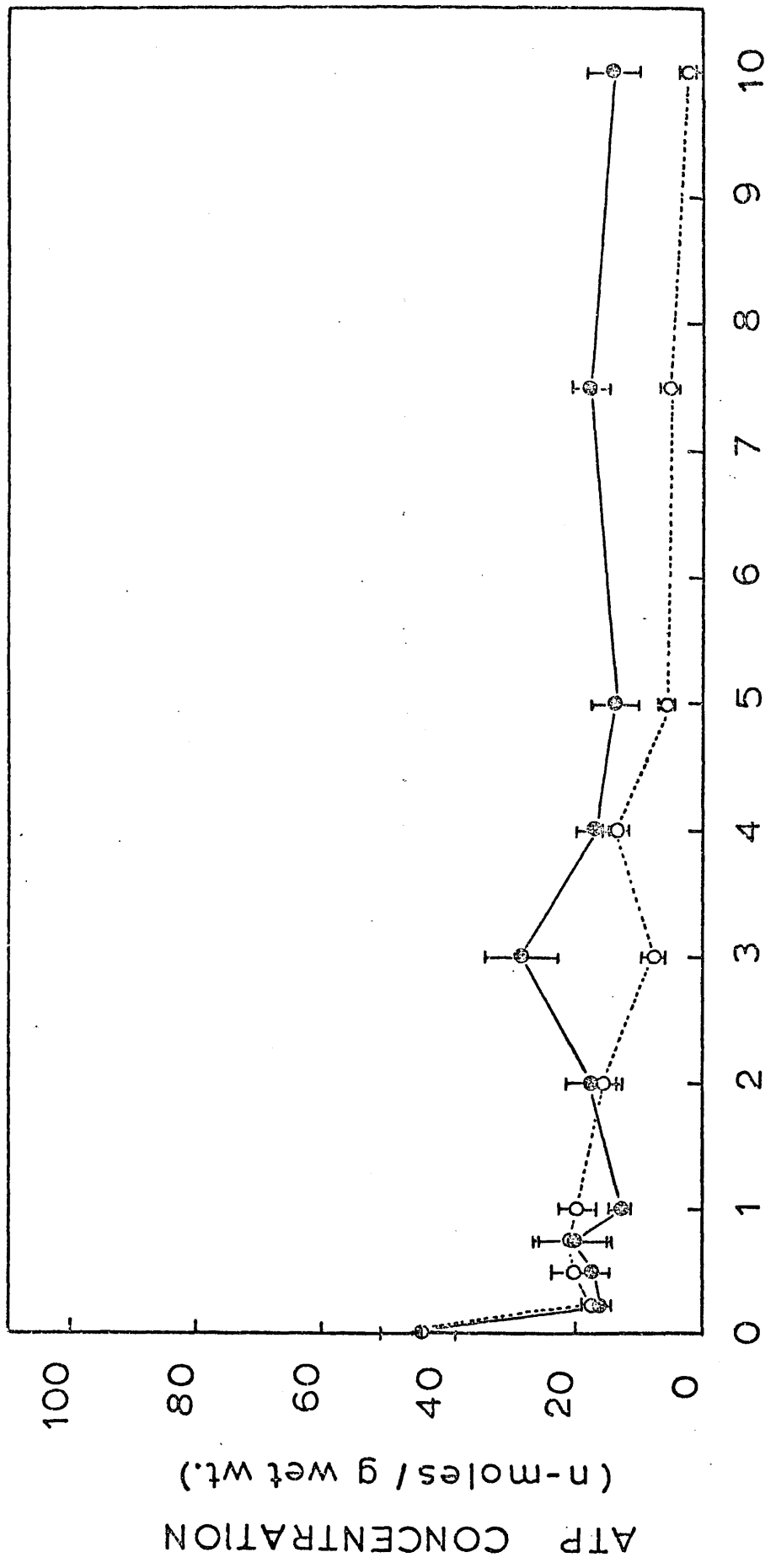


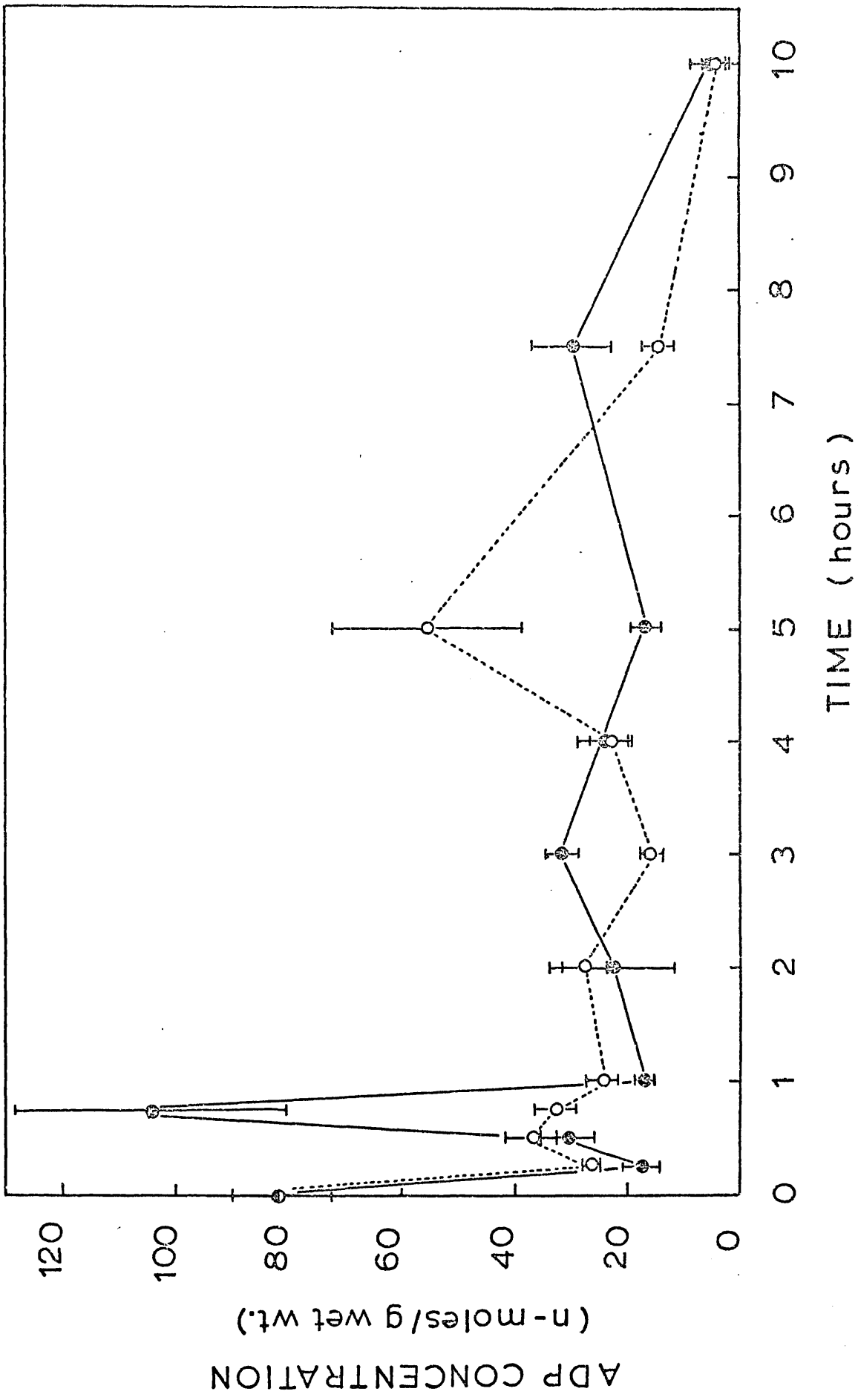
Fig.5 ATP concentration in foetal gastric mucosal cells incubated in oxygenated, glucose-free medium with and without Na^+. ● —— ● + Na^+; ○ ---- ○ - Na^+. Each point represents the mean ± S.E. of mean.

Table 4

ADP concentration in foetal gastric mucosal cells incubated in oxygenated, glucose-free medium with and without Na^+. The values given are means ± S.E. of mean (n = 6).

TIME (hours)	ADP CONCENTRATION (n-moles/g wet wt.)	
	+ Na^+	- Na^+
0	81.29 ± 8.61	81.29 ± 8.61
¼	17.56 ± 3.26	26.69 ± 1.56
½	30.74 ± 4.75	36.75 ± 4.72
¾	104.29 ± 24.00	32.90 ± 3.68
1	17.08 ± 1.61	24.48 ± 2.59
2	22.68 ± 10.82	27.77 ± 3.93
3	31.41 ± 2.85	15.76 ± 2.13
4	24.15 ± 4.23	22.95 ± 3.44
5	16.68 ± 2.73	55.34 ± 16.68
7½	29.59 ± 6.87	14.36 ± 2.68
10	5.57 ± 3.26	4.17 ± 2.22

Fig.6 ADP concentration in foetal gastric mucosal cells incubated in oxygenated, glucose-free medium with and without Na^+. ● —— ● + Na^+; O ---- O - Na^+. Each point represents the mean ± S.E. of mean.


ADP CONCENTRATION
(n-moles/g wet wt.)
120
100
80
60
40
20
0
0
1
2
3
4
5
6
7
8
9
10
TIME (hours)

to the ATP concentration in the presence and absence of Na^+. The ATP/ADP ratio (Table 5, Fig.7) did not change significantly during $7\frac{1}{2}$ hours either in the presence or absence of Na^+. In both cases, it was maintained at approximately 0.70. Incubation for 10 hours in glucose-free conditions did not exhaust endogenous CP (Table 6, Fig.8). There were no distinct differences between the rate of utilization of CP in the presence and absence of Na^+. In both cases, there was an overall decrease of 65%($P < 0.01$) in the CP concentration in 10 hours.

C. INCUBATION WITH 24 mM GLUCOSE, IN THE PRESENCE AND ABSENCE OF Na^+.

Experiments with 24 mM glucose present were performed to confirm the maintenance of active ion transport in the foetal stomach (Kendall, 1968) and to investigate the oxygen consumption, ATP and ADP concentrations, ATP/ADP ratio and CP concentration of foetal gastric mucosal cells, in these conditions.

(i) The short-circuit current.

The total s.c.c. was measured with continuously oxygenated medium on both sides of the foetal stomach membrane. This solution contained 24 mM glucose. For measurements of the Na^+ independent s.c.c., the choline chloride solution also contained 24 mM glucose. The results are shown in Table 7, Fig.9. The total s.c.c. was constant during the first 5 hours, increasing slightly during the following 5 hours. The Na^+ independent s.c.c. increased steadily during 10 hours ($P < 0.02$). At $1\frac{1}{2}$ hours, it was 10.67% of the total s.c.c.; at 10 hours, it was 30.94% of the total s.c.c.

(ii) Oxygen consumption of foetal gastric mucosal cells.

Foetal gastric mucosal cells were incubated for 10 hours in medium with and without Na^+. This solution contained 24 mM glucose. Oxygen consumption in the presence of Na^+ was always greater than in

Table 5

ATP/ADP ratio in foetal gastric mucosal cells incubated in oxygenated, glucose-free medium with and without Na^+. The values given are means $\pm$ S.E. of mean (n = 6). At 10 hours, the ATP/ADP ratio was calculated from the mean concentration of ATP and ADP.

TIME (hours)	ATP/ADP RATIO	
	+ Na^+	- Na^+
0	0.61 $\pm$ 0.12	0.61 $\pm$ 0.12
$\frac{1}{4}$	1.02 $\pm$ 0.15	0.64 $\pm$ 0.03
$\frac{1}{2}$	0.60 $\pm$ 0.13	0.57 $\pm$ 0.12
$\frac{3}{4}$	0.40 $\pm$ 0.20	0.60 $\pm$ 0.11
1	0.76 $\pm$ 0.09	0.88 $\pm$ 0.18
2	1.12 $\pm$ 0.37	0.60 $\pm$ 0.13
3	0.94 $\pm$ 0.19	0.54 $\pm$ 0.20
4	0.74 $\pm$ 0.14	0.64 $\pm$ 0.11
5	0.77 $\pm$ 0.18	0.18 $\pm$ 0.06
$7\frac{1}{2}$	0.97 $\pm$ 0.36	0.56 $\pm$ 0.28
10	2.56	0.57

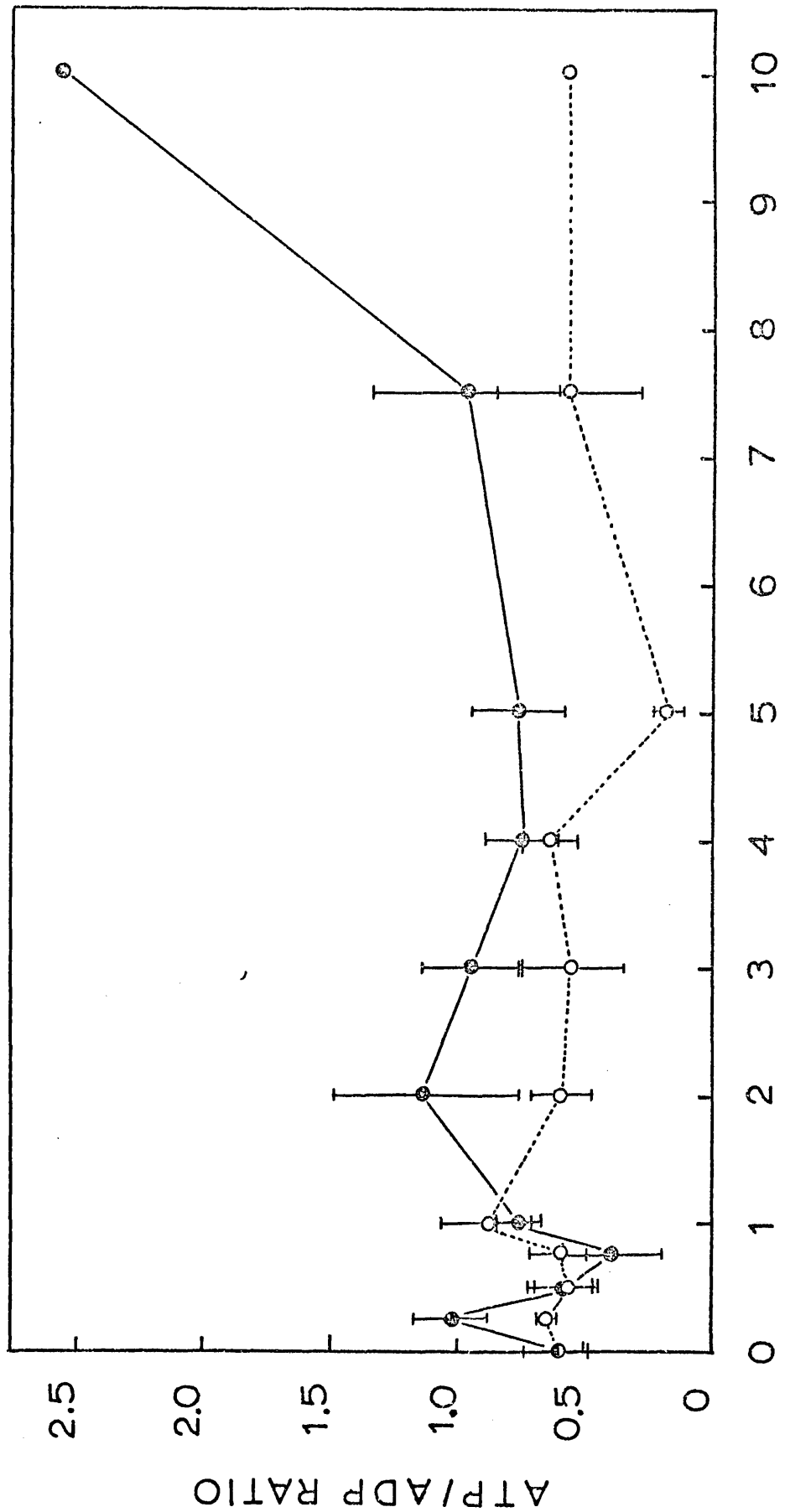


Fig.7 ATP/ADP ratio in foetal gastric mucosal cells incubated in oxygenated, glucose-free medium with and without Na^+. ● —— ● + Na^+; O ---- O - Na^+. Each point represents the mean ± S.E. of mean. (At 10 hours, the ATP/ADP ratio was calculated from the mean concentrations of ATP and ADP).

Table 6

CP concentration in foetal gastric mucosal cells incubated in oxygenated, glucose-free medium with and without Na^+. The values given are means ± S.E. of mean (n = 6).

TIME (hours)	CP CONCENTRATION (n-moles/g wet wt.)	
	+ Na^+	- Na^+
0	400.33 ± 72.08	400.33 ± 72.08
¼	248.34 ± 32.97	409.35 ± 19.16
½	452.50 ± 72.59	560.18 ± 93.97
¾	353.57 ± 71.24	291.91 ± 43.89
1	537.99 ± 76.19	311.88 ± 52.01
2	335.28 ± 41.16	325.29 ± 84.33
3	393.22 ± 143.77	166.05 ± 118.23
4	229.81 ± 31.71	72.78 ± 31.20
5	208.40 ± 50.08	314.16 ± 37.31
7½	319.17 ± 33.01	205.71 ± 22.23
10	160.78 ± 22.17	122.04 ± 18.45

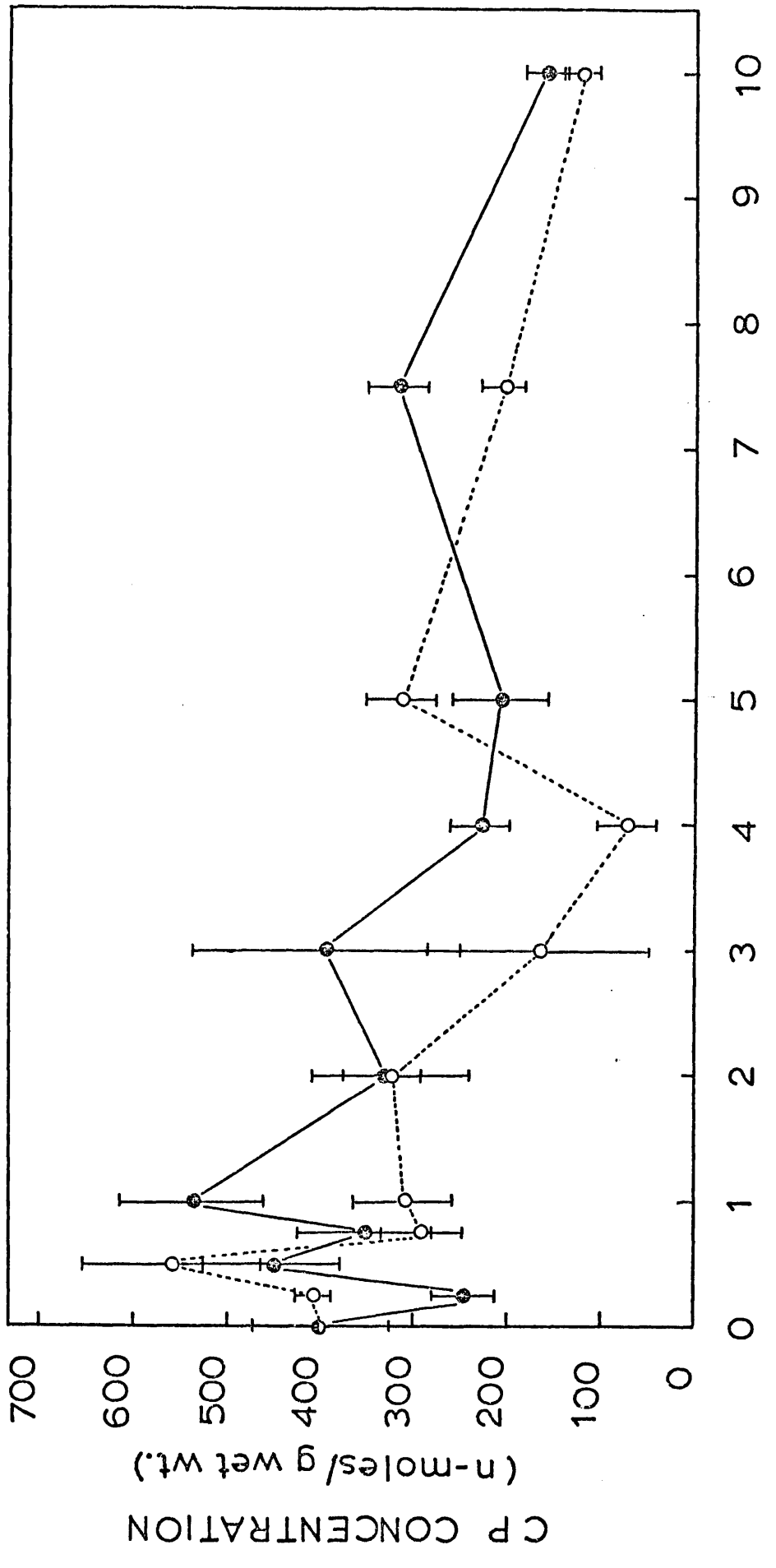


Fig.8 CP concentration in foetal gastric mucosal cells incubated in oxygenated, glucose-free medium with and without Na^+. ● —— ● + Na^+; ○ ---- ○ - Na^+. Each point represents the mean ± S.E. of mean.

Table 7

The short-circuit current of the foetal stomach (0.6 cm^2) incubated in oxygenated medium with and without Na^+. This solution contained 24 mM glucose. The values given are means ± S.E. of mean (number of experiments).

TIME (hours)	SHORT-CIRCUIT CURRENT (μA) + Na^+
½	88.25 ± 15.91 (4)
1	104.25 ± 16.96 (4)
2	97.38 ± 13.12 (4)
3	101.38 ± 11.31 (4)
4	105.00 ± 10.34 (4)
5	106.00 ± 9.17 (4)
6	112.50 ± 13.20 (4)
7	118.25 ± 13.50 (4)
8	120.00 ± 15.96 (4)
9	122.13 ± 16.07 (4)
10	121.75 ± 18.94 (4)

TIME (hours)	SHORT-CIRCUIT CURRENT (μA) - Na^+
1½	10.67 ± 4.33 (3)
2¼	14.33 ± 4.06 (3)
3	17.33 ± 3.76 (3)
4	21.00 ± 2.89 (3)
5¼	24.67 ± 1.45 (3)
6¼	25.33 ± 2.03 (3)
7¼	31.67 ± 1.86 (3)
8¼	34.33 ± 1.67 (3)
9¼	36.83 ± 2.80 (3)
10	37.67 ± 4.06 (3)

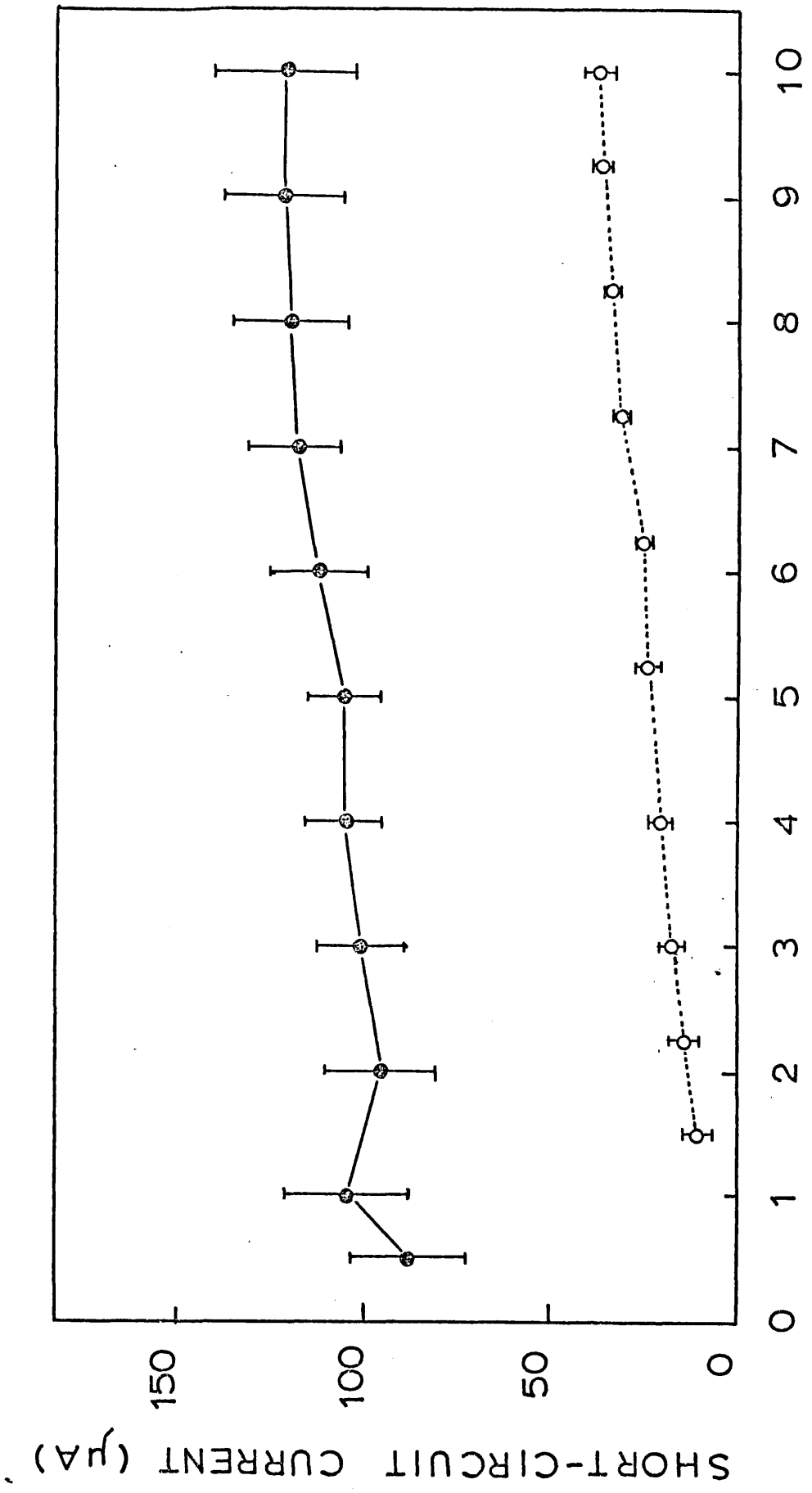


Fig.9 The short-circuit current of the foetal stomach (0.6 cm^2) incubated in oxygenated medium with and without Na^+. This solution contained 24 mM glucose. ● —— ● + Na^+; O ---- O - Na^+. Each point represents the mean ± S.E. of mean.

the absence of Na^+ (Table 8, Fig.10). During 10 hours of incubation, the oxygen consumption both in the presence and absence of Na^+ slowly decreased to 50% of its initial value ($P < 0.01$ in the presence of Na^+; $P < 0.05$ in the absence of Na^+). The decrease in both cases was less marked than in the absence of glucose (Table 2, Fig.4).

(iii)ATP, ADP and CP concentrations in foetal gastric mucosal cells.

Foetal gastric mucosal cells were incubated in continuously oxygenated medium with and without Na^+. This solution contained 24 mM glucose. The ATP concentration (Table 9, Fig.11) decreased during $7\frac{1}{2}$ hours incubation both in the presence and absence of Na^+. However, in the first hour, in the presence of Na^+, the ATP concentration was maintained, while in the absence of Na^+, there was a large decrease. In the following 2 hours, the ATP concentration decreased in the presence of Na^+ and was maintained in the absence of Na^+. Subsequently, from 3 hours to $7\frac{1}{2}$ hours of incubation, the ATP concentration slowly decreased in both cases to a similar level. The ADP concentration (Table 9, Fig.12) decreased in the first hour both in the presence and absence of Na^+. However, the decrease in the absence of Na^+ was greater than in the presence of Na^+. Subsequently, in the absence of Na^+, the ADP concentration was maintained during incubation from 1 hour to $7\frac{1}{2}$ hours. In the presence of Na^+, after remaining constant from 1 hour to 3 hours of incubation, it decreased to a value at $7\frac{1}{2}$ hours which was similar to that in the absence of Na^+. The ATP/ADP ratio (Table 10, Fig.13) was maintained for 1 hour in the presence of Na^+ and then it slowly decreased. In the absence of Na^+, the ATP/ADP ratio decreased in the first hour, was maintained for the following 2 hours and then slowly decreased. The CP concentration (Table 10, Fig.14) decreased steadily during $7\frac{1}{2}$ hours of incubation both in the presence

Table 8

Oxygen consumption of foetal gastric mucosal cells incubated in medium with and without Na^+. This solution contained 24 mM glucose. The values given are means ± S.E. of mean (number of experiments).

TIME (hours)	OXYGEN CONSUMPTION (μ-moles O_2/mg dry wt./hr)	
	+ Na^+	- Na^+
1 - 2	0.25 ± 0.02 (4)	0.08 ± 0.01 (4)
3 - 4	0.19 ± 0.02 (4)	0.06 ± 0.01 (4)
5 - 6	0.19 ± 0.01 (4)	0.05 ± 0.01 (4)
$7\frac{1}{2}$ - 10	0.12 ± 0.02 (6)	0.04 ± 0.01 (6)

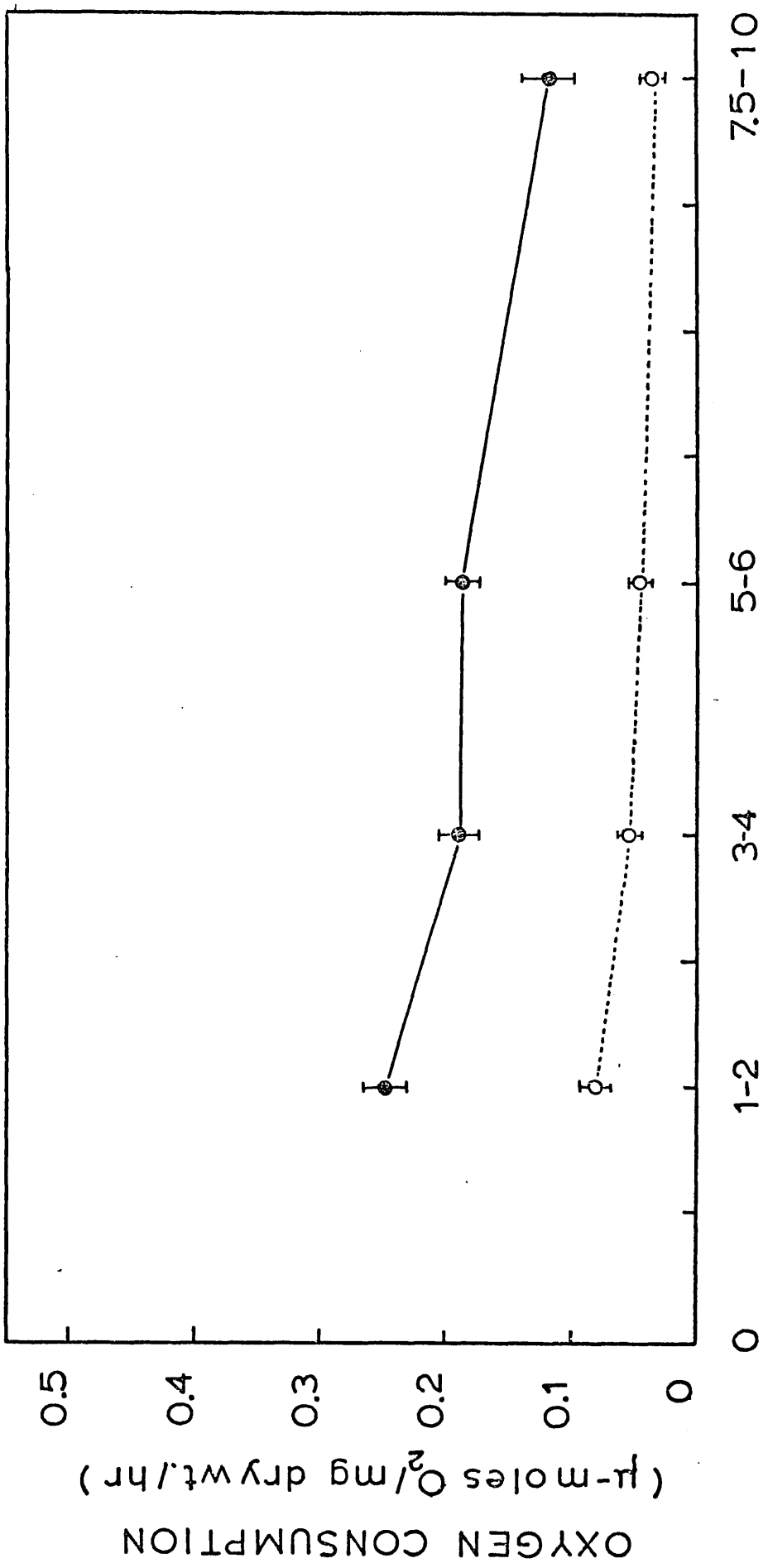


Fig.10 Oxygen consumption of foetal gastric mucosal cells incubated in medium with and without Na^+. This solution contained 24 mM glucose. ● —— ● + Na^+; ○ ---- ○ - Na^+. Each point represents the mean ± S.E. of mean.

Table 9

ATP and ADP concentrations in foetal gastric mucosal cells incubated in oxygenated medium with and without Na^+. This solution contained 24 mM glucose. The values given are the means of 2 experiments (individual values).

TIME (hours)	ATP CONCENTRATION (n-moles/g wet wt.)	
	+ Na^+	- Na^+
0	47.14 (45.36, 48.91)	55.72 (57.04, 54.40)
1	41.98 (39.01, 44.95)	16.82 (16.58, 17.05)
3	22.70 (24.51, 20.89)	16.26 (18.83, 13.68)
$7\frac{1}{2}$	8.68 (6.86, 10.50)	5.36 (7.11, 3.60)

TIME (hours)	ADP CONCENTRATION (n-moles/g wet wt.)	
	+ Na^+	- Na^+
0	75.41 (68.05, 82.77)	75.64 (82.92, 68.35)
1	56.57 (54.46, 58.67)	39.21 (37.29, 41.13)
3	52.36 (50.50, 54.21)	33.56 (32.43, 34.69)
$7\frac{1}{2}$	29.91 (29.91, 29.90)	32.69 (35.25, 30.13)

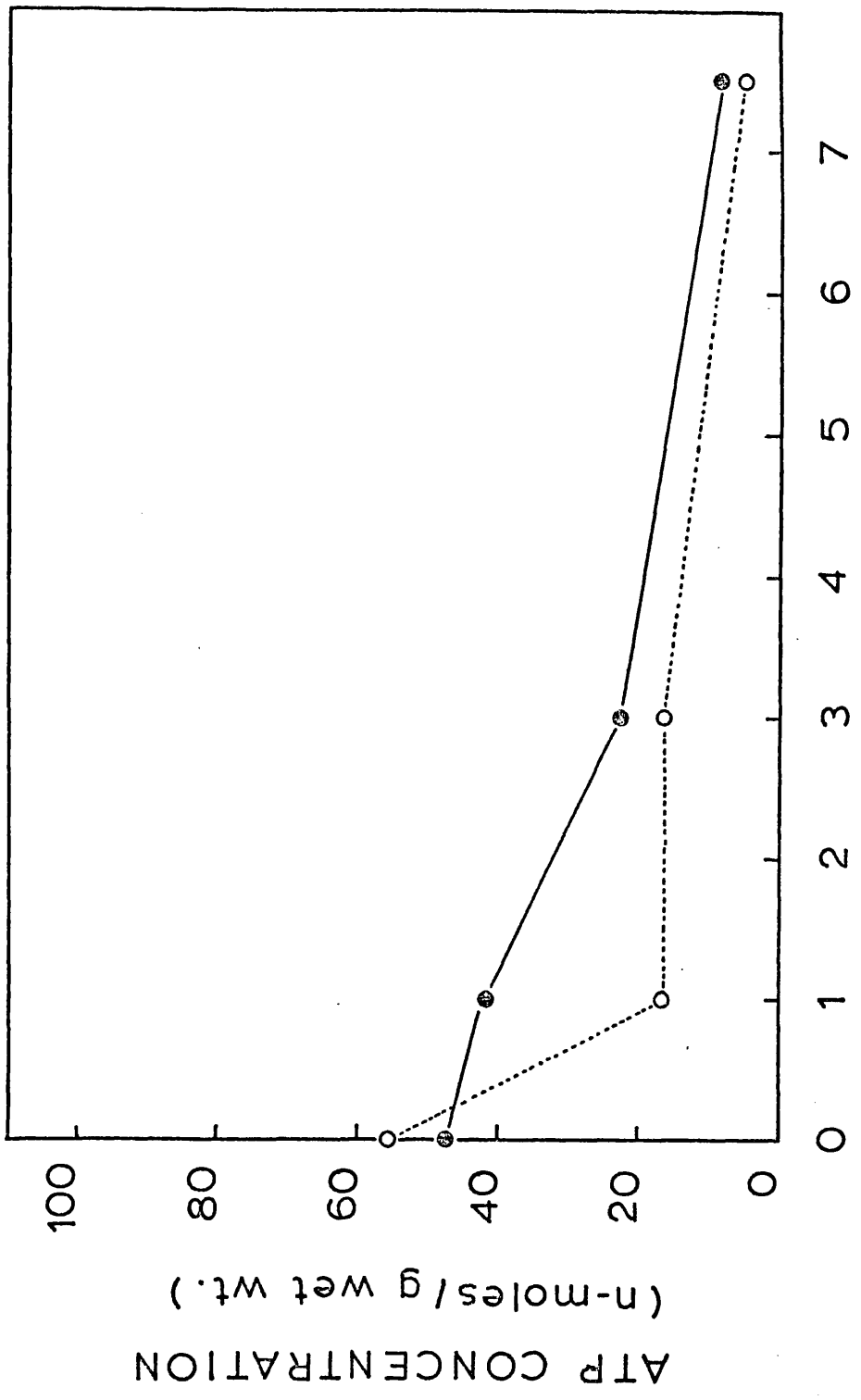


Fig.11 ATP concentration in foetal gastric mucosal cells incubated in oxygenated medium with and without Na^+. This solution contained 24 mM glucose. ● —— ● + Na^+; O ---- O - Na^+. Each point represents the mean of 2 experiments.

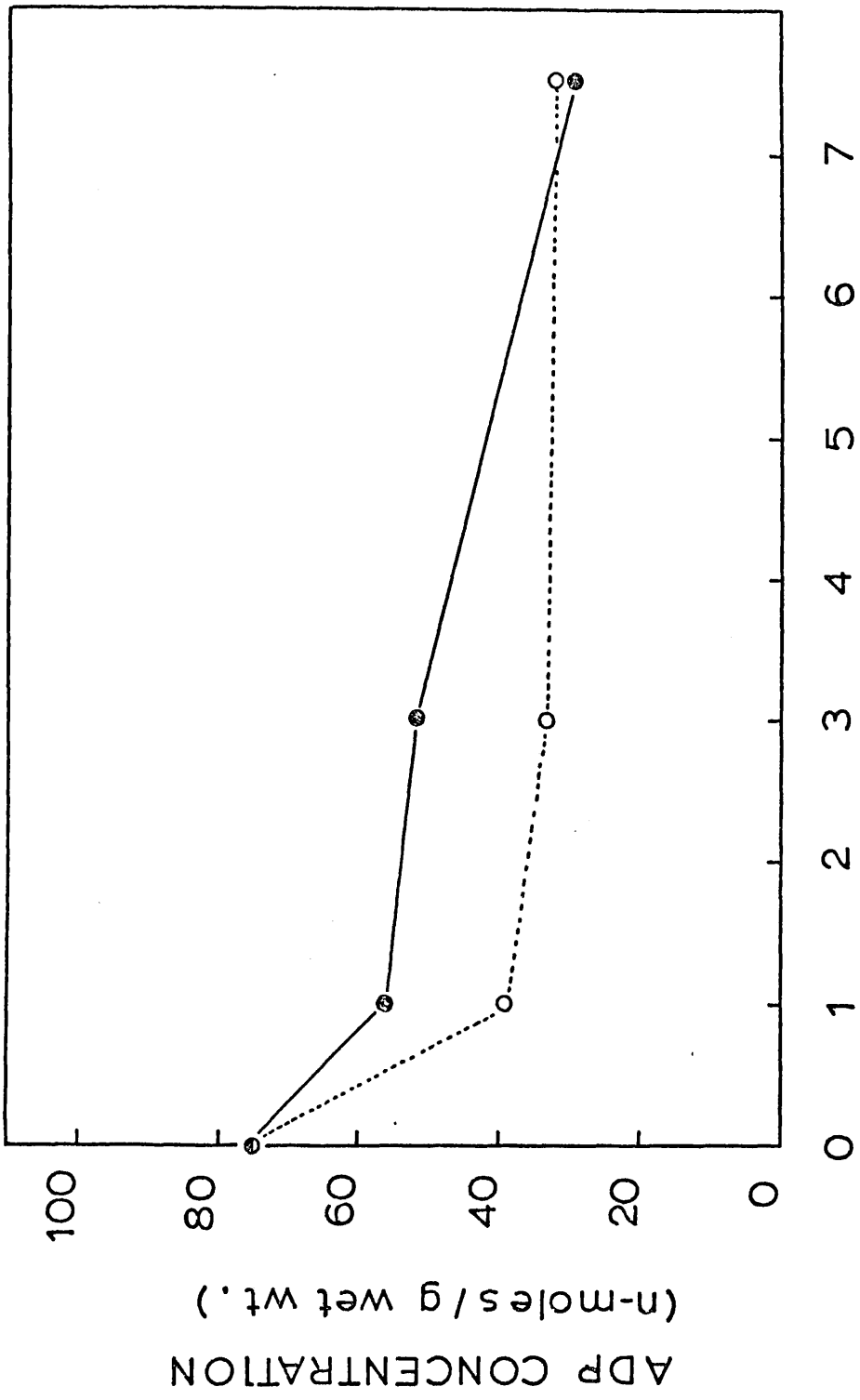


Fig.12 ADP concentration in foetal gastric mucosal cells incubated in oxygenated medium with and without Na^+. This solution contained 24 mM glucose. ● —— ● + Na^+; O ---- O - Na^+. Each point represents the mean of 2 experiments.

Table 10

ATP/ADP ratio and CP concentration in foetal gastric mucosal cells incubated in oxygenated medium with and without Na^+. This solution contained 24 mM glucose. The values given are the means of 2 experiments (individual values).

TIME (hours)	ATP/ADP RATIO	
	+ Na^+	- Na^+
0	0.63 (0.67, 0.59)	0.74 (0.69, 0.79)
1	0.74 (0.72, 0.76)	0.43 (0.44, 0.42)
3	0.44 (0.49, 0.39)	0.49 (0.58, 0.40)
7½	0.29 (0.23, 0.35)	0.16 (0.20, 0.12)

TIME (hours)	CP CONCENTRATION (n-moles/g wet wt.)	
	+ Na^+	- Na^+
0	575.38 (565.86, 584.89)	562.11 (563.55, 560.66)
1	363.17 (309.52, 416.82)	351.32 (335.35, 367.28)
3	280.51 (272.11, 288.90)	251.21 (279.27, 223.14)
7½	115.42 (106.22, 124.61)	120.01 (118.09, 121.93)

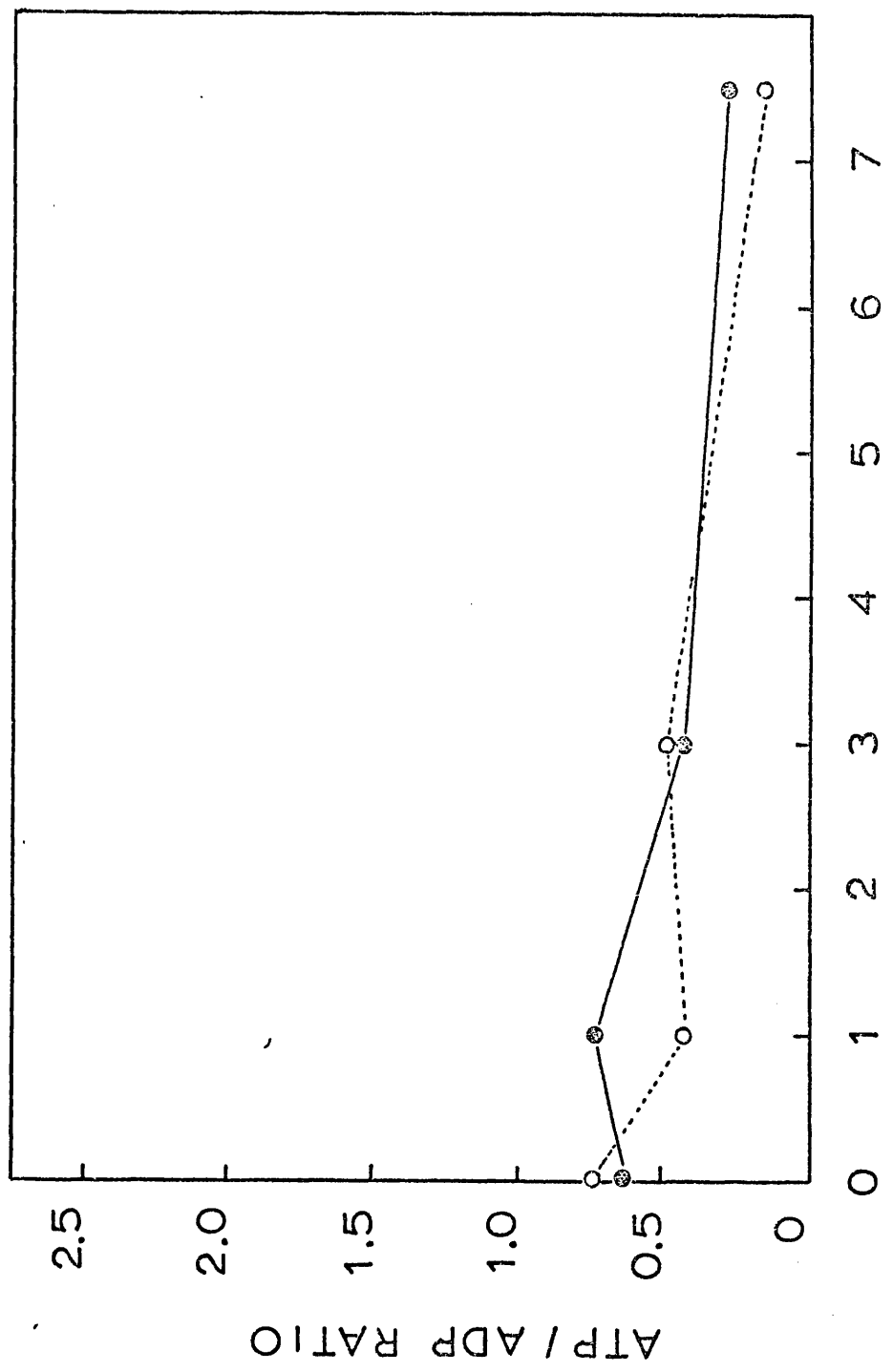


Fig.13 ATP/ADP ratio in foetal gastric mucosal cells incubated in oxygenated medium with and without Na^+. This solution contained 24 mM glucose. ● —— ● + Na^+; O ---- O - Na^+. Each point represents the mean of 2 experiments.

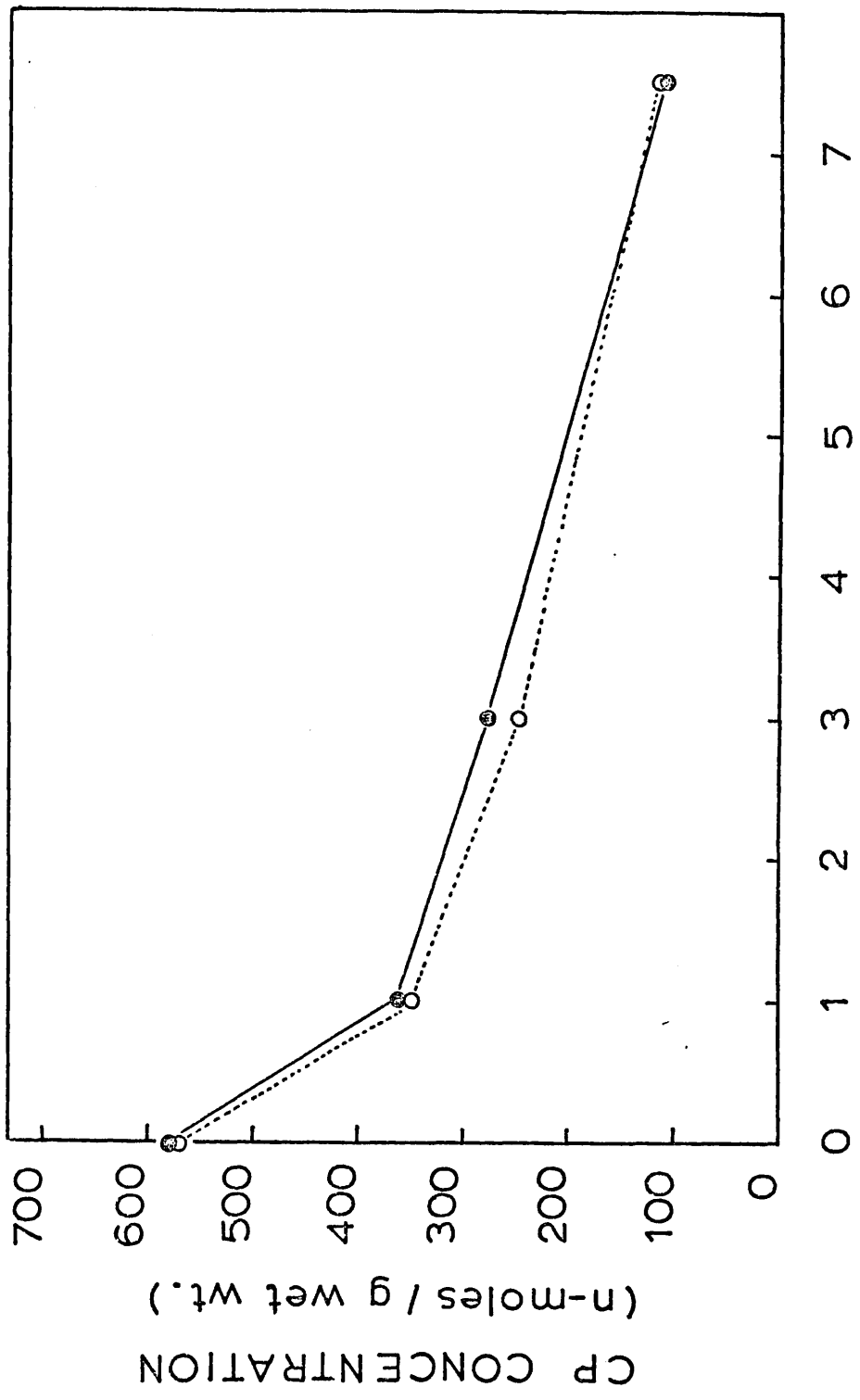


Fig.14 CP concentration in foetal gastric mucosal cells incubated in oxygenated medium with and without Na^+. This solution contained 24 mM glucose. ● —— ● + Na^+; O ----- O - Na^+. Each point represents the mean of 2 experiments.

and absence of Na^+. The rate of utilization of CP was similar in both cases.

D. THE EFFECT OF ANOXIA AND REOXYGENATION, IN THE PRESENCE AND ABSENCE OF Na^+.

(i) The short-circuit current.

Kendall (1968) investigated the effect of anoxia on the total and Na^+ independent s.c.c. of the foetal stomach in the presence of 24 mM glucose. Complete inhibition of the Na^+ independent s.c.c. was obtained within 10 minutes. The total s.c.c. decreased rapidly, but 15% was still present after 50 minutes of anoxia. The experiment was repeated imposing anoxia for a longer period of time in order to investigate whether complete inhibition of the total s.c.c. occurred. The experiment was carried out with medium containing Na^+ on both sides of the membrane. This solution contained 24 mM glucose. Anoxic conditions were imposed by gassing with 95% N_2 + 5% CO_2. The results are shown in Table 11, Fig.15. In the first 30 minutes, the total s.c.c. decreased rapidly and after 1 hour and 20 minutes, only 4% of the total s.c.c. was present. This cannot be considered to be significantly greater than zero. Reoxygenation with 95% O_2 + 5% CO_2 caused an immediate increase in the total s.c.c. In 15 minutes, it was 31.90% of the initial total s.c.c.; in 45 minutes, it was 58% of the initial total s.c.c. In most of the experiments, 95-100% recovery of the total s.c.c. was obtained within 2 hours of reoxygenation.

(ii) ATP, ADP and CP concentrations in foetal gastric mucosal cells.

(a) The effect of anoxia.

Foetal gastric mucosal cells were incubated in glucose-free medium with and without Na^+. Anoxic conditions were imposed from

Table 11

The effect of anoxia and reoxygenation on the short-circuit current of the foetal stomach (0.6 cm^2) incubated in medium containing Na^+. This solution contained 24 mM glucose. The values given are means ± S.E. of mean (number of experiments).

TIME (min)	SHORT-CIRCUIT CURRENT (μA) + Na^+
5	136.50 ± 3.95 (4)
10	136.50 ± 3.95 (4)
11	Anoxia begun
15	118.00 ± 8.16 (4)
20	88.00 ± 8.22 (4)
25	60.25 ± 3.75 (4)
30	46.13 ± 2.98 (4)
35	33.75 ± 3.73 (4)
40	25.00 ± 1.96 (4)
45	20.00 ± 2.61 (4)
50	16.13 ± 1.74 (4)
55	13.38 ± 1.21 (4)

TIME (hr)(min)	SHORT-CIRCUIT CURRENT (μA) + Na^+
1	11.88 ± 1.13 (4)
1 5	9.88 ± 0.97 (4)
1 10	8.50 ± 0.87 (4)
1 15	7.75 ± 0.72 (4)
1 20	6.38 ± 0.80 (4)
1 25	5.88 ± 0.77 (4)
1 30	5.75 ± 0.85 (4)
1 31	Preparation reoxygenated
1 45	43.50 ± 7.41 (4)
2	61.33 ± 7.06 (3)
2 15	79.33 ± 8.74 (3)

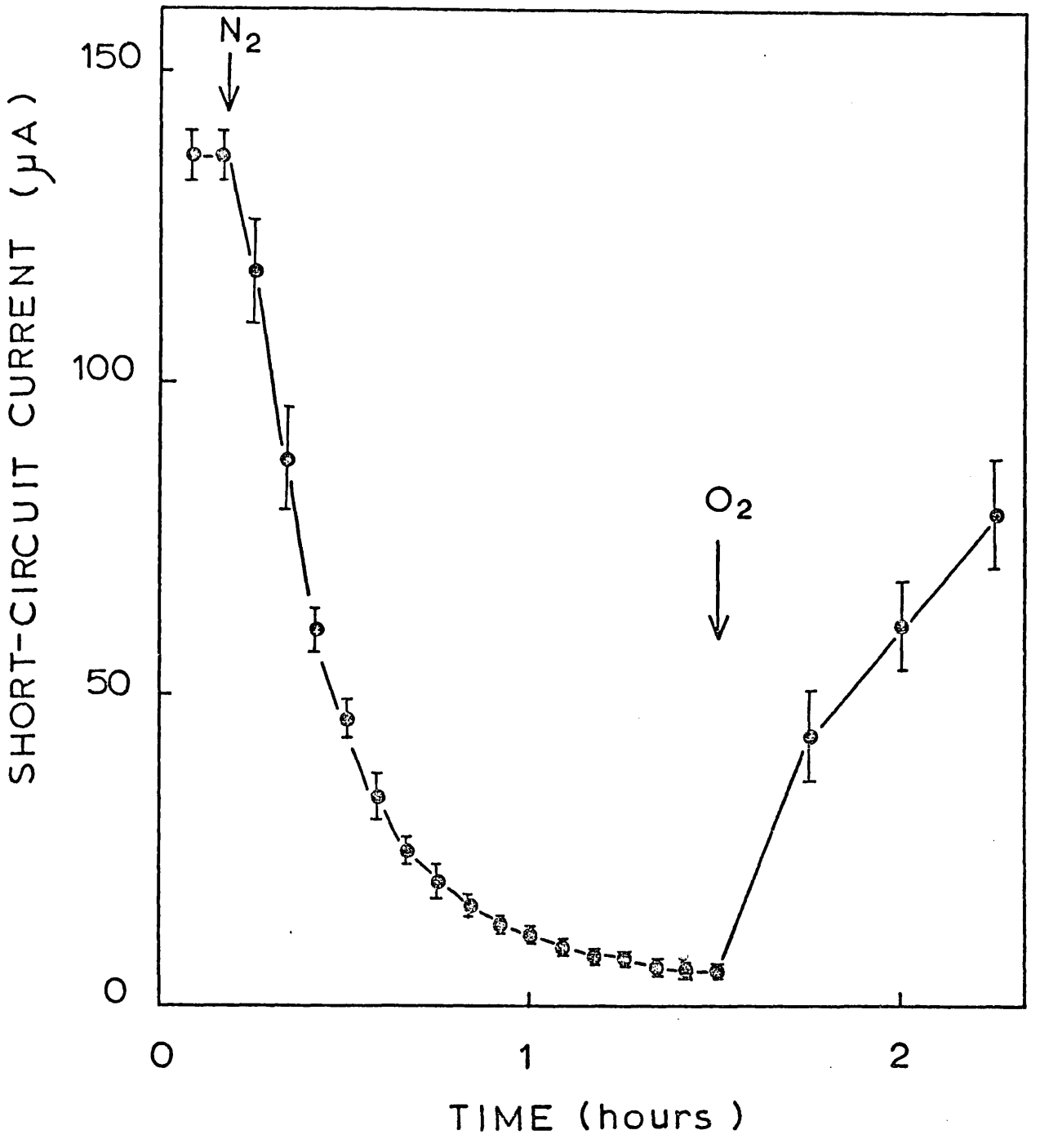


Fig.15 The effect of anoxia and reoxygenation on the short-circuit current of the foetal stomach (0.6 cm^2) incubated in medium containing Na^+. This solution contained 24 mM glucose. Each point represents the mean ± S.E. of mean.

time 0 by gassing continuously with 95% N_2 + 5% CO_2. The effect of anoxia on the ATP and ADP concentrations, ATP/ADP ratio and CP concentration was not influenced by the presence or absence of Na^+.

One hour of anoxia caused a large decrease (87% in the presence of Na^+, $P < 0.01$; 72% in the absence of Na^+, $P < 0.02$) in the ATP concentration, which then remained constant during the following 2 hours of anoxia (Table 12, Fig.16A). Similar changes occurred in the ADP concentration (Table 12, Fig.16B). A large decrease (74% in the presence of Na^+, $P < 0.002$; 52% in the absence of Na^+, $P < 0.01$) occurred in the first hour. In the following 2 hours of continued anoxia, the ADP concentration did not change significantly. In the first hour of anoxia, the ATP/ADP ratio (Table 13, Fig.17A) decreased from 0.51 to 0.26 ($P < 0.05$) in the presence of Na^+ and from 0.66 to 0.43 ($P > 0.05$) in the absence of Na^+. By 3 hours, the ratio in both cases returned to its initial value. Anoxia caused a 50% decrease ($P < 0.002$ in the presence of Na^+; $P < 0.01$ in the absence of Na^+) in the CP concentration in 3 hours (Table 13, Fig.17B).

(b) The effect of reoxygenation.

Foetal gastric mucosal cells were incubated in glucose-free medium with and without Na^+. After the imposition of anoxic conditions for 1 hour from time 0 by gassing continuously with 95% N_2 + 5% CO_2, the cells were reoxygenated by gassing with 95% O_2 + 5% CO_2 for 15 minutes. The effect of reoxygenation on the ATP and ADP concentrations, ATP/ADP ratio and CP concentration was not influenced by the presence or absence of Na^+.

Reoxygenation caused a large increase ($P < 0.001$) in the ATP concentration (Fig.18A) to a value which was similar to the initial concentration. The ADP concentration (Fig.18B) did not change

Table 12

The effect of anoxia on the ATP and ADP concentrations in foetal gastric mucosal cells incubated in glucose-free medium with and without Na^+. Anoxic conditions were imposed from time 0. The values given are means ± S.E. of mean (number of experiments).

TIME (hours)	ATP CONCENTRATION (n-moles/g wet wt.)	
	+ Na^+	- Na^+
0	29.98 ± 6.63 (7)	29.02 ± 6.69 (7)
1	3.94 ± 1.44 (6)	8.10 ± 1.71 (6)
3	3.17 ± 0.81 (6)	5.31 ± 1.01 (6)

TIME (hours)	ADP CONCENTRATION (n-moles/g wet wt.)	
	+ Na^+	- Na^+
0	58.63 ± 9.34 (7)	45.23 ± 5.76 (7)
1	15.29 ± 1.81 (6)	21.68 ± 3.72 (6)
3	7.76 ± 1.35 (6)	13.78 ± 4.10 (6)

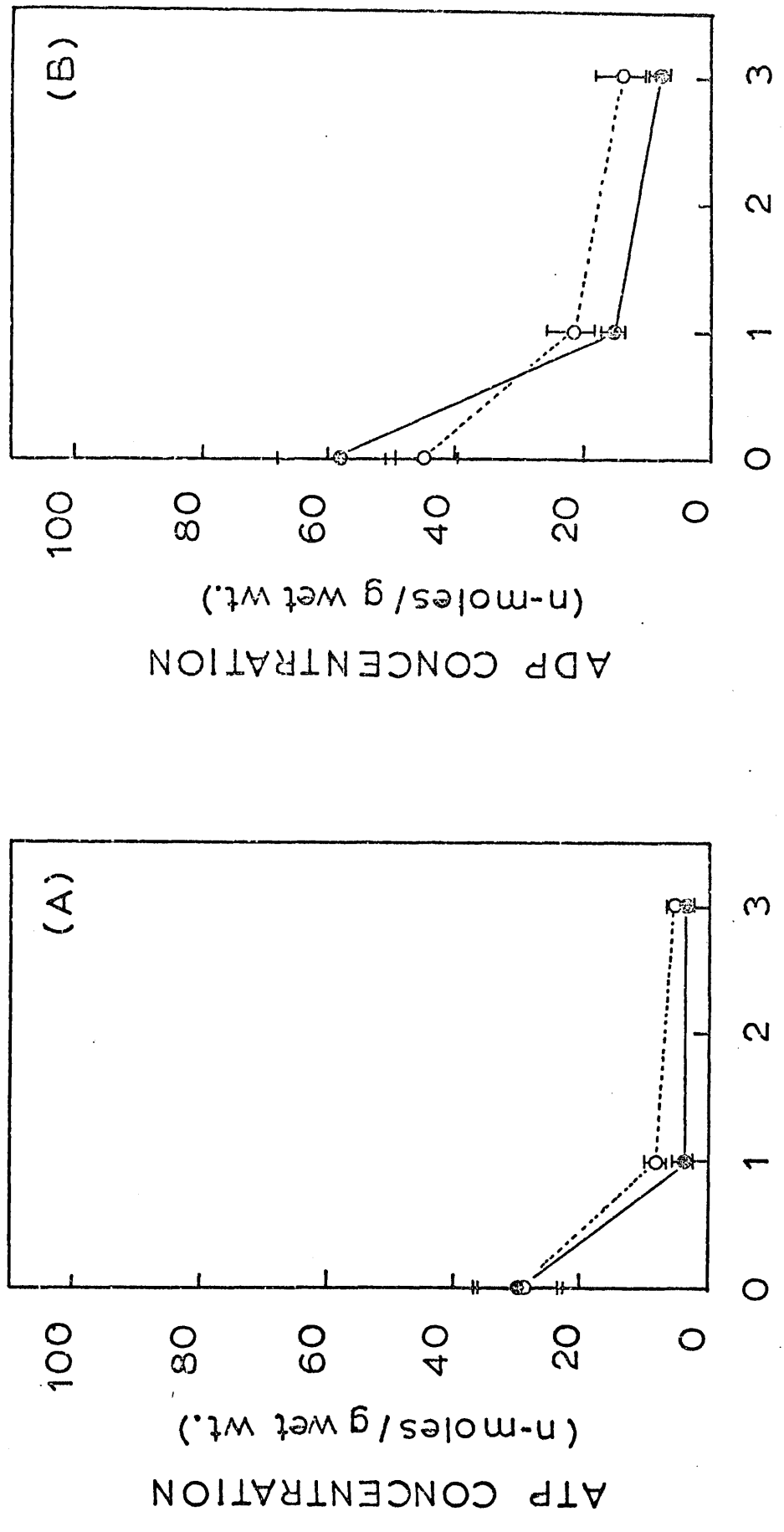


Fig.16 The effect of anoxia on the ATP concentration (A) and ADP concentration (B) in foetal gastric mucosal cells incubated in glucose-free medium with and without Na^+. Anoxic conditions were imposed from time 0. ● —— ● + Na^+; ○ ---- ○ - Na^+. Each point represents the mean ± S.E. of mean.

Table 13

The effect of anoxia on the ATP/ADP ratio and CP concentration in foetal gastric mucosal cells incubated in glucose-free medium with and without Na^+. Anoxic conditions were imposed from time 0. The values given are means ± S.E. of mean (number of experiments).

TIME (hours)	ATP/ADP RATIO	
	+ Na^+	- Na^+
0	0.51 ± 0.08 (7)	0.66 ± 0.16 (7)
1	0.26 ± 0.08 (6)	0.43 ± 0.12 (6)
3	0.43 ± 0.11 (6)	0.57 ± 0.16 (6)

TIME (hours)	CP CONCENTRATION (n-moles/g wet wt.)	
	+ Na^+	- Na^+
0	423.06 ± 41.41 (7)	401.17 ± 54.24 (7)
1	251.50 ± 24.07 (6)	302.45 ± 26.87 (6)
3	224.61 ± 18.91 (6)	192.25 ± 15.34 (6)

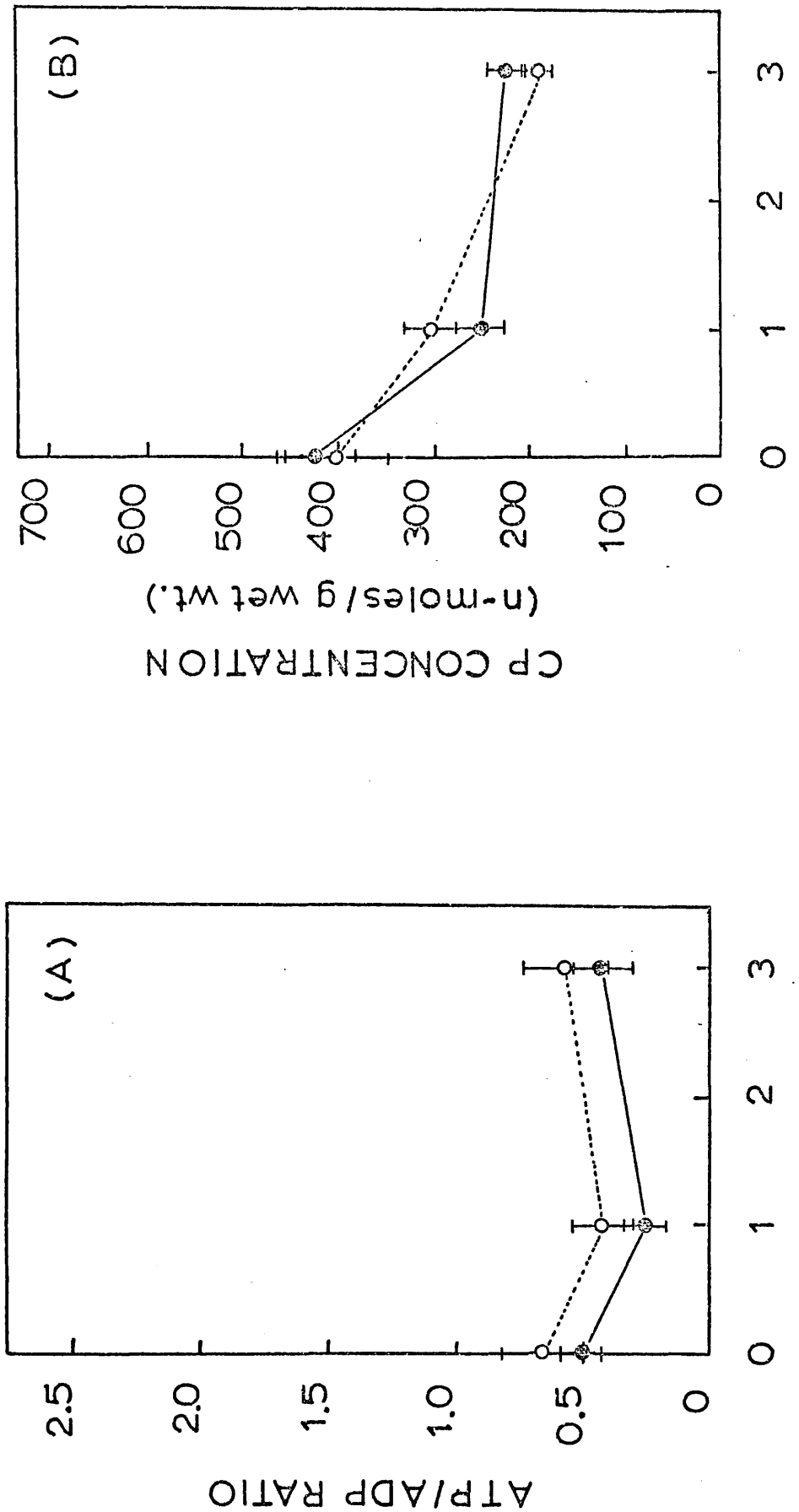


Fig.17 The effect of anoxia on the ATP/ADP ratio (A) and CP concentration (B) in foetal gastric mucosal cells incubated in glucose-free medium with and without Na^+. Anoxic conditions were imposed from time 0.

● —— ● + Na^+; ○ ---- ○ - Na^+. Each point represents the mean ± S.E. of mean.

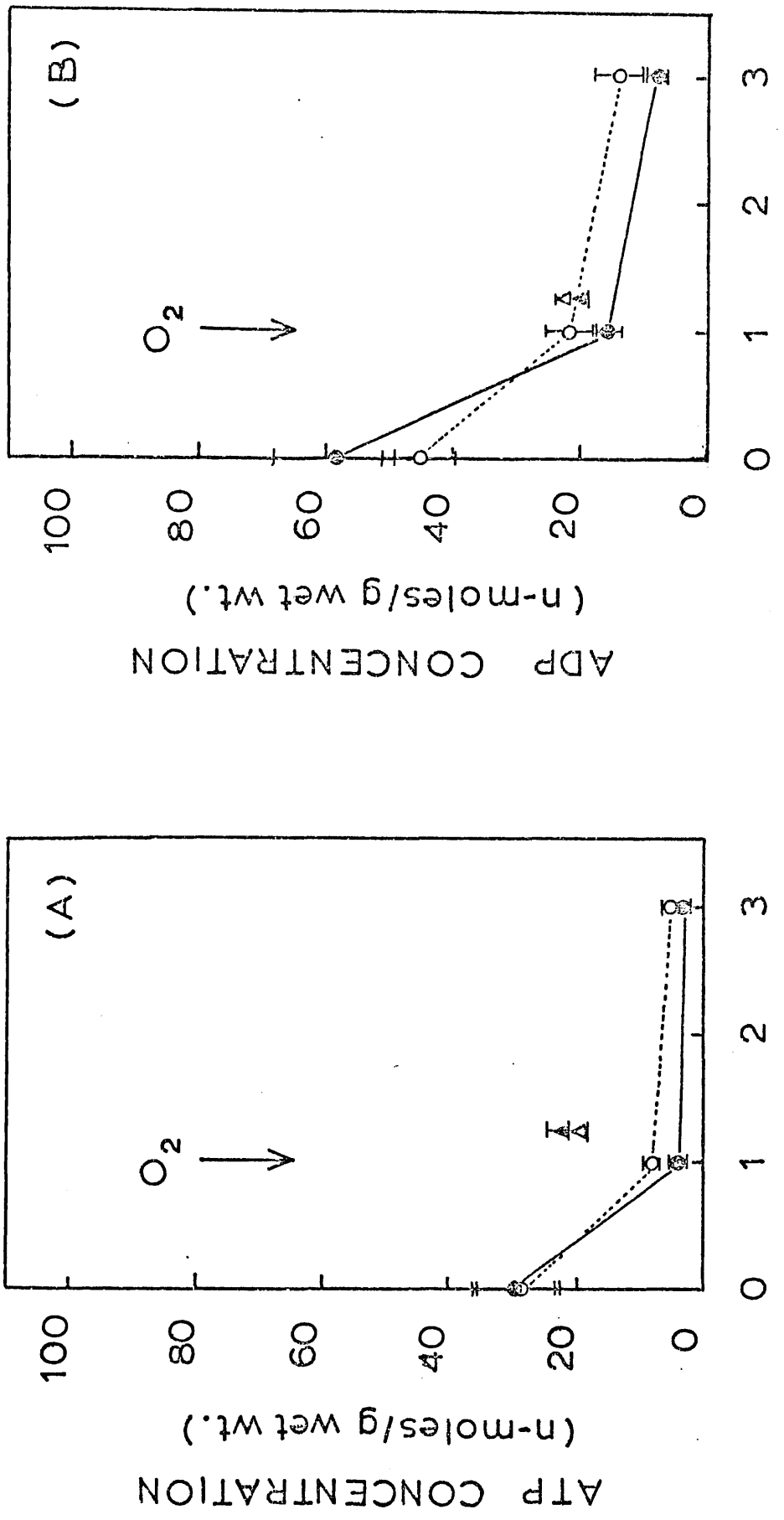


Fig.18 The effect of reoxygenation after 1 hour of anoxia imposed from time 0, on the ATP concentration (A) and ADP concentration (B) in foetal gastric mucosal cells incubated in glucose-free medium with and without Na^+. The effect of anoxia: ● —— ● + Na^+; O ---- O - Na^+. The effect of reoxygenation: ▲ + Na^+; Δ - Na^+. Each point represents the mean ± S.E. of mean.

significantly. The ATP/ADP ratio (Fig.19A) increased ($P < 0.01$) on reoxygenation, returning to a value similar to its initial value. The CP concentration (Fig.19B) did not change significantly.

E. THE EFFECT OF OUABAIN, IN THE PRESENCE AND ABSENCE OF Na^+.

Kendall (1968) investigated the effect of 10^{-5}M ouabain on the total s.c.c. and Na^+ independent s.c.c. of the foetal stomach. In the presence of 24 mM glucose, 10^{-5}M ouabain caused an immediate rapid decrease of the total s.c.c. After 15 minutes, the rate of decrease became more gradual and after 1 hour and 40 minutes, 20-25% of the total s.c.c. was still present. This residual s.c.c. was inhibited by anoxia. The Na^+ independent s.c.c. was completely inhibited by 10^{-5}M ouabain within 1 hour. In the absence of glucose, 10^{-5}M ouabain caused complete inhibition of the total s.c.c. in 1 hour and 40 minutes.

(i) Oxygen consumption of foetal gastric mucosal cells.

Foetal gastric mucosal cells were incubated in glucose-free medium with and without Na^+. When the preparation had stabilized and the oxygen consumption had been measured at 1 hour, 10^{-5}M ouabain was added. Ouabain caused a decrease of the oxygen consumption both in the presence ($P < 0.02$) and absence ($P < 0.05$) of Na^+ in one hour (Table 14, Fig.20). Incubation for a further 2 hours with 10^{-5}M ouabain caused no significant change in the oxygen consumption in the presence or absence of Na^+. In one hour, ouabain inhibited the oxygen consumption associated with the presence of Na^+ in the medium. Two hours after the addition of ouabain, the mean value of the oxygen consumption in the presence of Na^+ was similar to that in the absence of Na^+.

(ii) ATP, ADP and CP concentrations in foetal gastric mucosal cells.

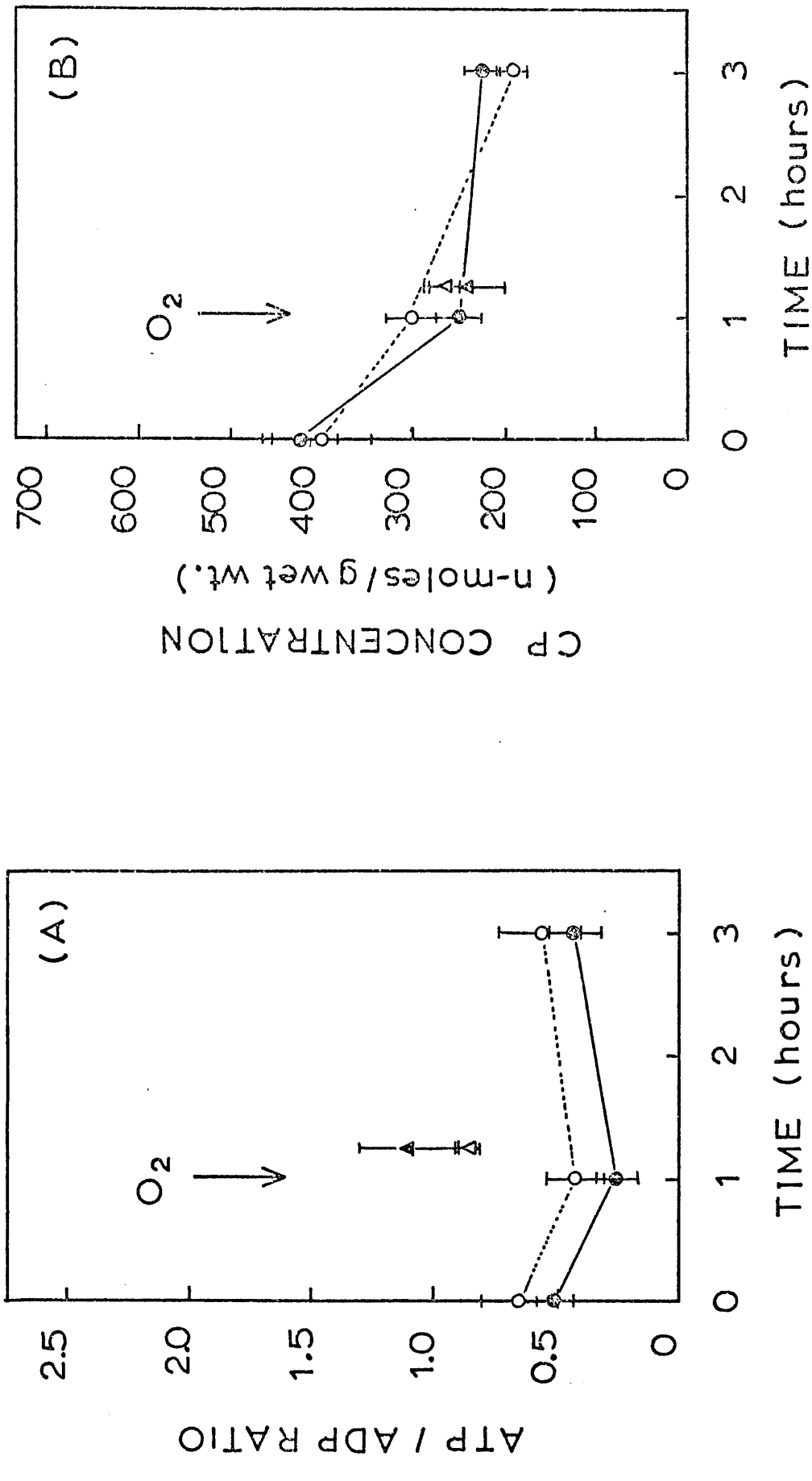


Fig.19 The effect of reoxygenation after 1 hour of anoxia imposed from time 0, on the ATP/ADP ratio (A) and CP concentration (B) in foetal gastric mucosal cells incubated in glucose-free medium with and without Na^+.

The effect of anoxia: ● —— ● + Na^+; ○ ---- ○ - Na^+. The effect of reoxygenation: ▲ + Na^+; △ - Na^+.

Each point represents the mean ± S.E. of mean.

Table 14

The effect of 10^{-5}M ouabain on the oxygen consumption of foetal gastric mucosal cells incubated in glucose-free medium with and without Na^+. Ouabain was added at 1 hour. The values given are means ± S.E. of mean (number of experiments).

TIME (hours)	OXYGEN CONSUMPTION (μ-moles O_2/mg dry wt./hr)	
	+ Na^+	- Na^+
1	0.24 ± 0.01 (4)	0.16 ± 0.03 (5)
	Ouabain added	Ouabain added
2	0.13 ± 0.03 (4)	0.08 ± 0.02 (5)
3	0.09 ± 0.03 (4)	0.08 ± 0.01 (4)
4	0.07 ± 0.03 (4)	0.06 ± 0.004 (4)

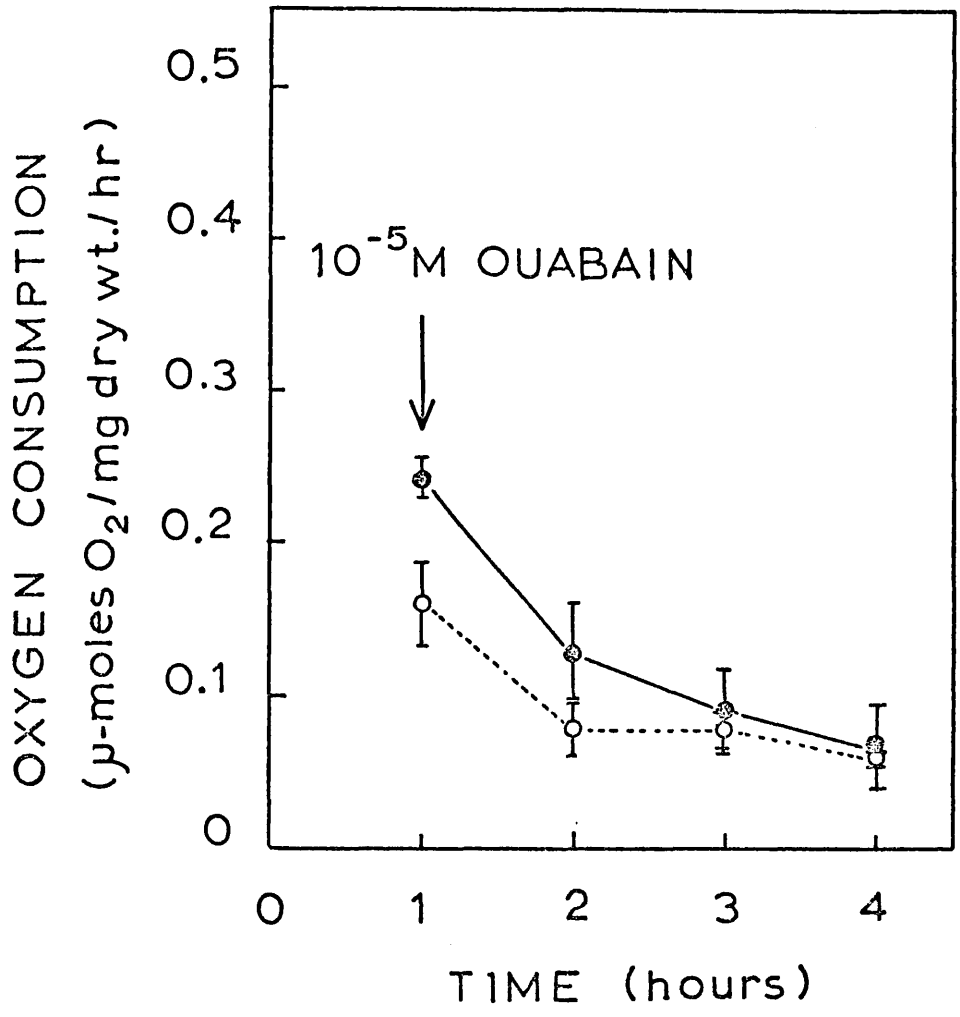


Fig.20 The effect of 10^{-5}M ouabain on the oxygen consumption of foetal gastric mucosal cells incubated in glucose-free medium with and without Na^+. ● —— ● + Na^+; O ---- O - Na^+. Each point represents the mean ± S.E. of mean.

Foetal gastric mucosal cells were incubated in continuously oxygenated, glucose-free medium with and without Na^+. At time 0, 10^{-5}M ouabain was added. The effect of ouabain on the ATP and ADP concentrations, ATP/ADP ratio and CP concentration was not influenced by the presence or absence of Na^+.

The ATP concentration (Table 15, Fig.21A) did not change significantly during 3 hours of incubation with ouabain. The ADP concentration (Table 15, Fig.21B) decreased in the first hour ($P < 0.01$ in the presence of Na^+; $P < 0.001$ in the absence of Na^+) and remained constant during the following 2 hours. Ouabain caused an increase in the ATP/ADP ratio in the first hour ($P < 0.01$ in the presence of Na^+; $P < 0.001$ in the absence of Na^+) as shown in Table 16, Fig.22A. The ratio was then maintained at the increased value for the following 2 hours. No significant change occurred in the CP concentration during 3 hours of incubation with ouabain (Table 16, Fig.22B).

F. THE EFFECT OF OLIGOMYCIN, IN THE PRESENCE AND ABSENCE OF Na^+.

Lardy, Johnson & McMurray (1958) first showed that oligomycin is a specific inhibitor of phosphorylations coupled to the respiratory chain. It inhibits tightly-coupled respiration and the inhibition is released by uncouplers such as DNP. In loosely coupled mitochondria, it abolished the phosphorylation without an effect on respiration (Huijing & Slater, 1961).

(i) The short-circuit current.

Kendall (1968) investigated the effect of oligomycin (10 μg/ml.) in alcoholic solution on the total and Na^+ independent s.c.c. of the foetal stomach in the presence of 24 mM glucose. The addition of oligomycin to the serosal chamber caused no inhibition in 40 minutes.

Table 15

The effect of 10^{-5}M ouabain on the ATP and ADP concentrations in foetal gastric mucosal cells incubated in oxygenated, glucose-free medium with and without Na^+. Ouabain was added at time 0. The values given are means ± S.E. of mean (number of experiments).

TIME (hours)	ATP CONCENTRATION (n-moles/g wet wt.)	
	+ Na^+	- Na^+
0	23.88 ± 3.96 (7)	30.71 ± 4.72 (7)
1	25.30 ± 3.03 (6)	21.05 ± 2.07 (6)
3	17.21 ± 2.29 (6)	12.45 ± 2.87 (6)

TIME (hours)	ADP CONCENTRATION (n-moles/g wet wt.)	
	+ Na^+	- Na^+
0	57.52 ± 6.03 (7)	73.70 ± 7.93 (7)
1	29.53 ± 4.53 (6)	29.57 ± 5.09 (6)
3	19.32 ± 2.74 (6)	20.07 ± 5.31 (6)

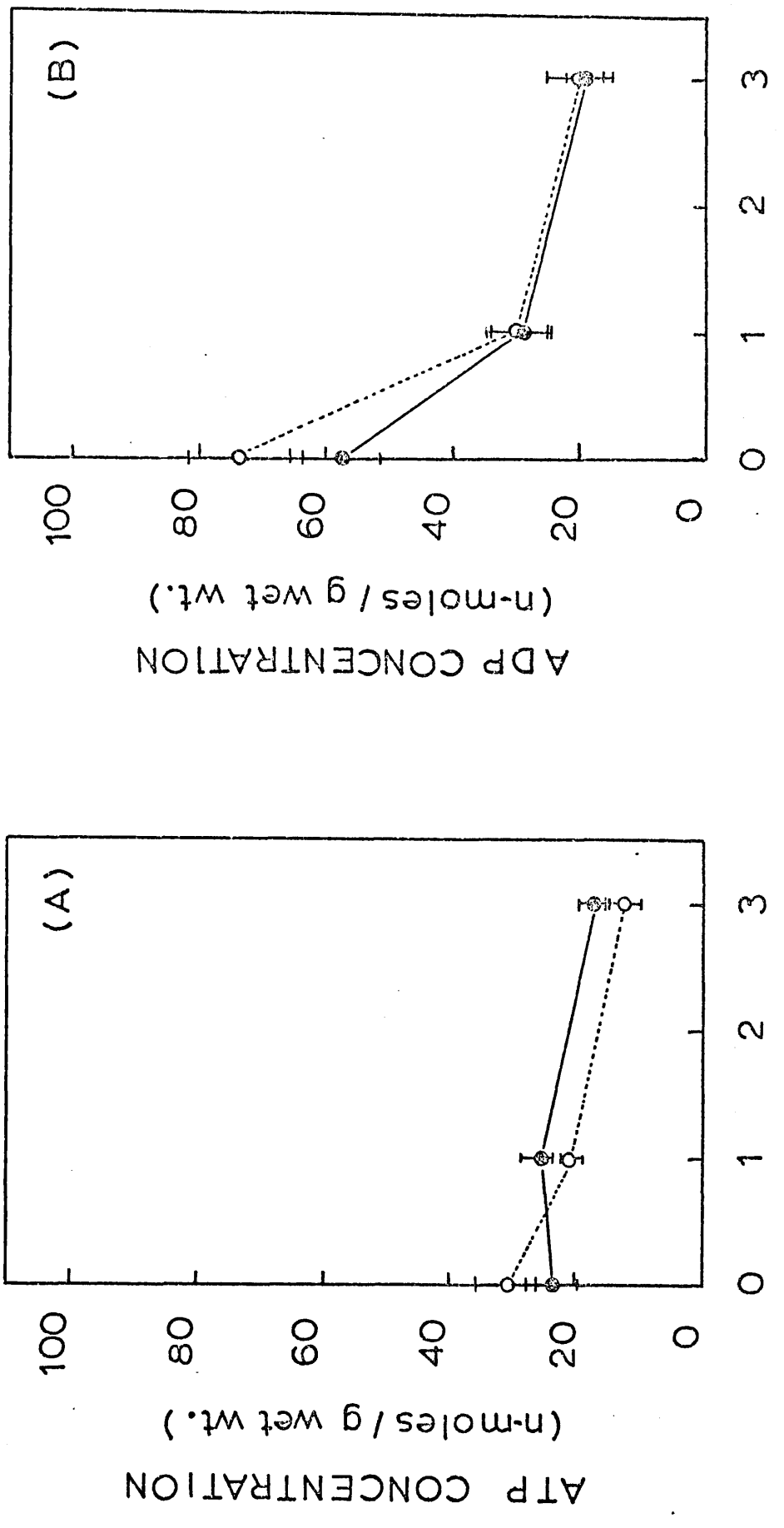


Fig.21 The effect of 10^{-5}M ouabain on the ATP concentration (A) and ADP concentration (B) in foetal gastric mucosal cells incubated in oxygenated, glucose-free medium with and without Na^+. Ouabain was added at time 0. ● —— ● + Na^+; ○ ---- ○ - Na^+. Each point represents the mean ± S.E. of mean.

Table 16

The effect of 10^{-5}M ouabain on the ATP/ADP ratio and CP concentration in foetal gastric mucosal cells incubated in oxygenated, glucose-free medium with and without Na^+. Ouabain was added at time 0. The values given are means ± S.E. of mean (number of experiments).

TIME (hours)	ATP/ADP RATIO	
	+ Na^+	- Na^+
0	0.41 ± 0.04 (7)	0.42 ± 0.04 (7)
1	0.95 ± 0.16 (6)	0.75 ± 0.05 (6)
3	0.93 ± 0.13 (6)	0.74 ± 0.19 (6)

TIME (hours)	CP CONCENTRATION (n-moles/g wet wt.)	
	+ Na^+	- Na^+
0	396.75 ± 51.93 (7)	448.90 ± 60.72 (7)
1	369.37 ± 73.37 (6)	390.37 ± 52.20 (6)
3	286.13 ± 49.22 (6)	291.59 ± 55.07 (6)

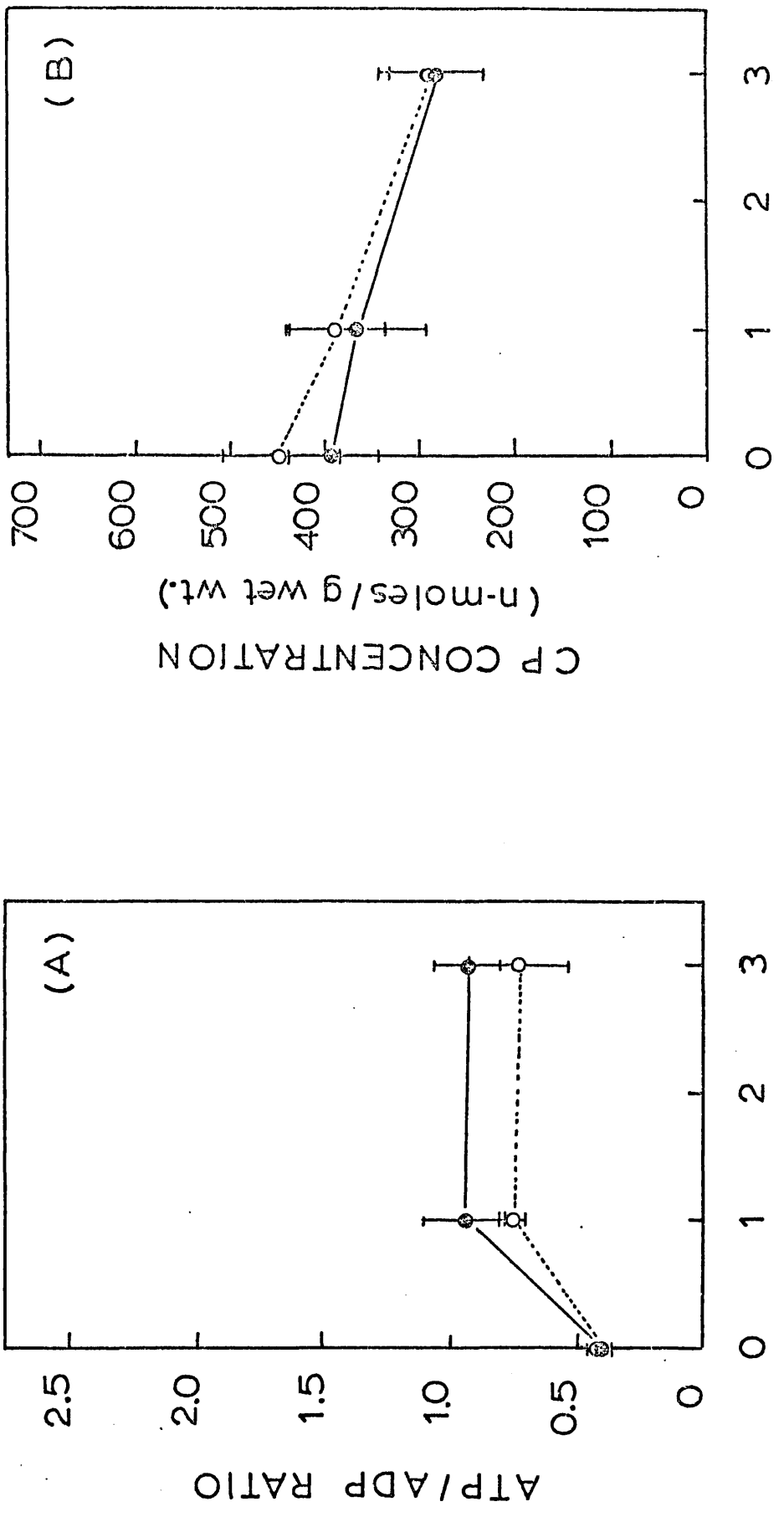


Fig.22 The effect of 10^{-5}M ouabain on the ATP/ADP ratio (A) and CP concentration (B) in foetal gastric mucosal cells incubated in oxygenated, glucose-free medium with and without Na^+. Ouabain was added at time 0. ● —— ● + Na^+; O ---- O - Na^+. Each point represents the mean ± S.E. of mean.

However, when added to the mucosal chamber, oligomycin caused 50% inhibition of the total s.c.c. in approximately 30 minutes and after 2 hours, 25-30% of the total s.c.c. was still present, which was unaffected by anoxia. Oligomycin added to the mucosal chamber caused 50% inhibition of the Na^+ independent s.c.c. in 15-20 minutes and usually complete inhibition within one hour. The effect of glucose-free conditions on the inhibition of the total s.c.c. by oligomycin was investigated in the present work.

The foetal stomach was set up from time 0 with glucose-free medium containing Na^+ on both sides of the membrane and with continuous oxygenation. When the total s.c.c. had increased to its maximum and was just beginning to decrease, oligomycin (10 μg/ml.) in alcoholic solution was added to the mucosal chamber. Absolute alcohol alone had no effect on the s.c.c. Oligomycin caused a 50% decrease in the total s.c.c. in 50 minutes (Table 17, Fig.23) and after 2 hours, 27% of the total s.c.c. was still present, as found by Kendall (1968) in the presence of 24 mM glucose. However, complete inhibition of the total s.c.c. was obtained after $3\frac{1}{2}$ hours of incubation with oligomycin.

(ii) Oxygen consumption of foetal gastric mucosal cells.

Foetal gastric mucosal cells were incubated in glucose-free medium with and without Na^+. The oxygen consumption was measured at 1 hour and oligomycin (10 μg/ml.) in alcoholic solution was then added. Absolute alcohol alone had no effect on the oxygen consumption. The effect of oligomycin is shown in Table 18, Fig.24. In the presence of Na^+, oligomycin had an inhibitory effect ($P < 0.05$) on the oxygen consumption in the first 5 minutes, reducing it to a value similar to that in the absence of Na^+. During the following 55 minutes, the

Table 17

The effect of oligomycin, 10 μg/ml., on the short-circuit current of the foetal stomach (0.6 cm^2) incubated in oxygenated, glucose-free medium containing Na^+. Oligomycin was added in alcoholic solution to the mucosal side only. The values given are means ± S.E. of mean (number of experiments).

TIME (hr)(min)	SHORT-CIRCUIT CURRENT (μA) + Na^+	TIME (hr)(min)	SHORT-CIRCUIT CURRENT (μA) + Na^+
5	79.63 ± 5.14 (4)	1 55	25.68 ± 3.88 (4)
14	76.00 ± 6.31 (4)	2 5	23.20 ± 4.41 (4)
15	Oligomycin added	2 15	21.13 ± 4.19 (4)
25	68.50 ± 3.48 (4)	2 25	19.38 ± 4.34 (4)
35	59.38 ± 3.22 (4)	2 35	18.25 ± 3.95 (4)
45	50.00 ± 3.51 (4)	2 45	16.50 ± 4.31 (4)
55	44.00 ± 2.68 (4)	2 55	15.65 ± 3.83 (4)
1 5	38.88 ± 2.38 (4)	3 5	12.67 ± 4.65 (3)
1 15	36.08 ± 2.25 (4)	3 15	8.67 ± 3.33 (3)
1 25	32.45 ± 2.50 (4)	3 25	6.17 ± 2.13 (3)
1 35	29.88 ± 2.82 (4)	3 35	3.60 ± 1.14 (3)
1 45	28.03 ± 3.02 (4)	3 45	2.00 ± 0.58 (3)

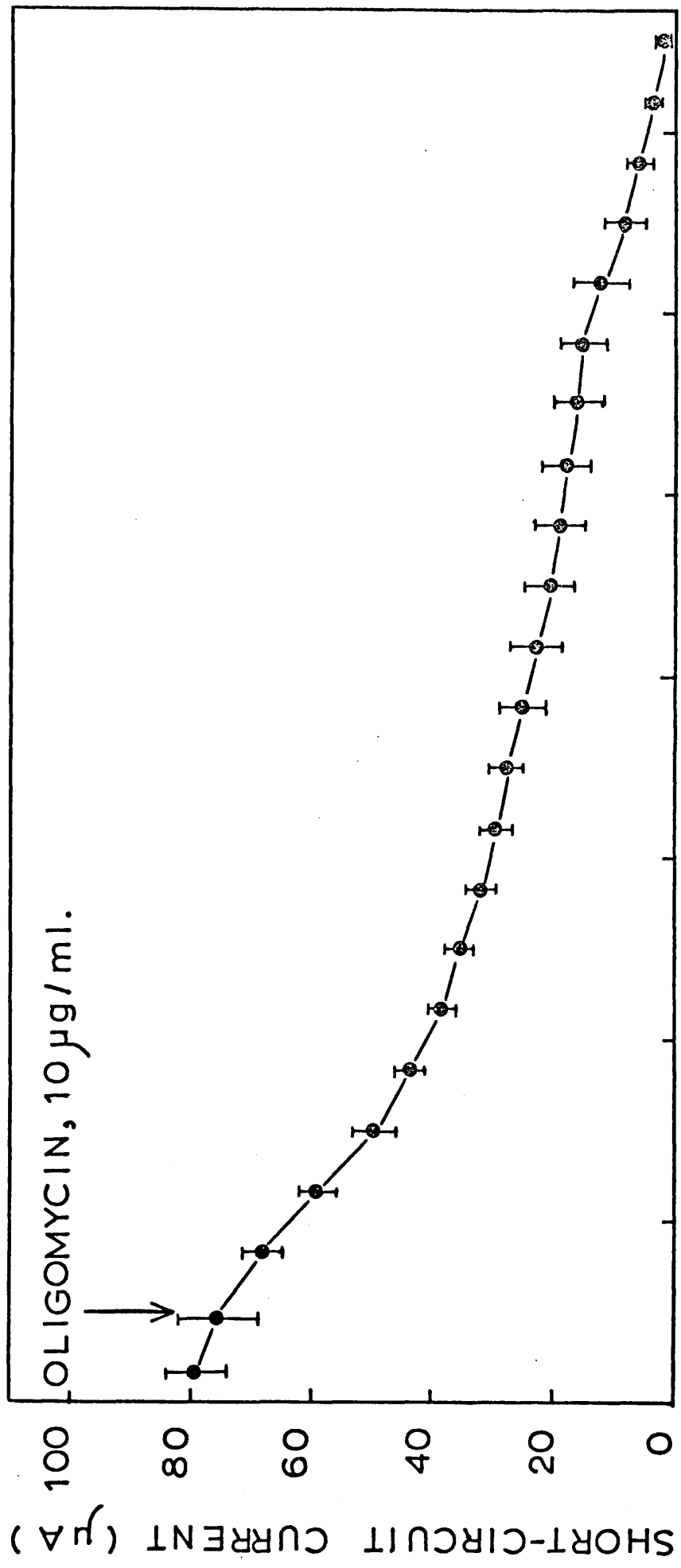


Fig.23 The effect of oligomycin, 10 μg/ml., on the short-circuit current of the foetal stomach (0.6 cm^2) incubated in oxygenated, glucose-free medium containing Na^+. Oligomycin in alcoholic solution was added to the mucosal side only. Each point represents the mean ± S.E. of mean.

Table 18

The effect of oligomycin, 10 μg/ml., on the oxygen consumption of foetal gastric mucosal cells incubated in glucose-free medium with and without Na^+. Oligomycin in alcoholic solution was added at 1 hour. The values given are means ± S.E. of mean (number of experiments).

TIME (hr)(min)	OXYGEN CONSUMPTION (μ-moles O_2/mg dry wt./hr)	
	+ Na^+	- Na^+
1	0.22 ± 0.06 (4)	0.14 ± 0.03 (4)
	Oligomycin added	Oligomycin added
1 5	0.10 ± 0.01 (4)	0.11 ± 0.03 (4)
1½	0.06 ± 0.01 (4)	0.10 ± 0.02 (3)
2	0.05 ± 0.02 (4)	0.08 ± 0.02 (4)

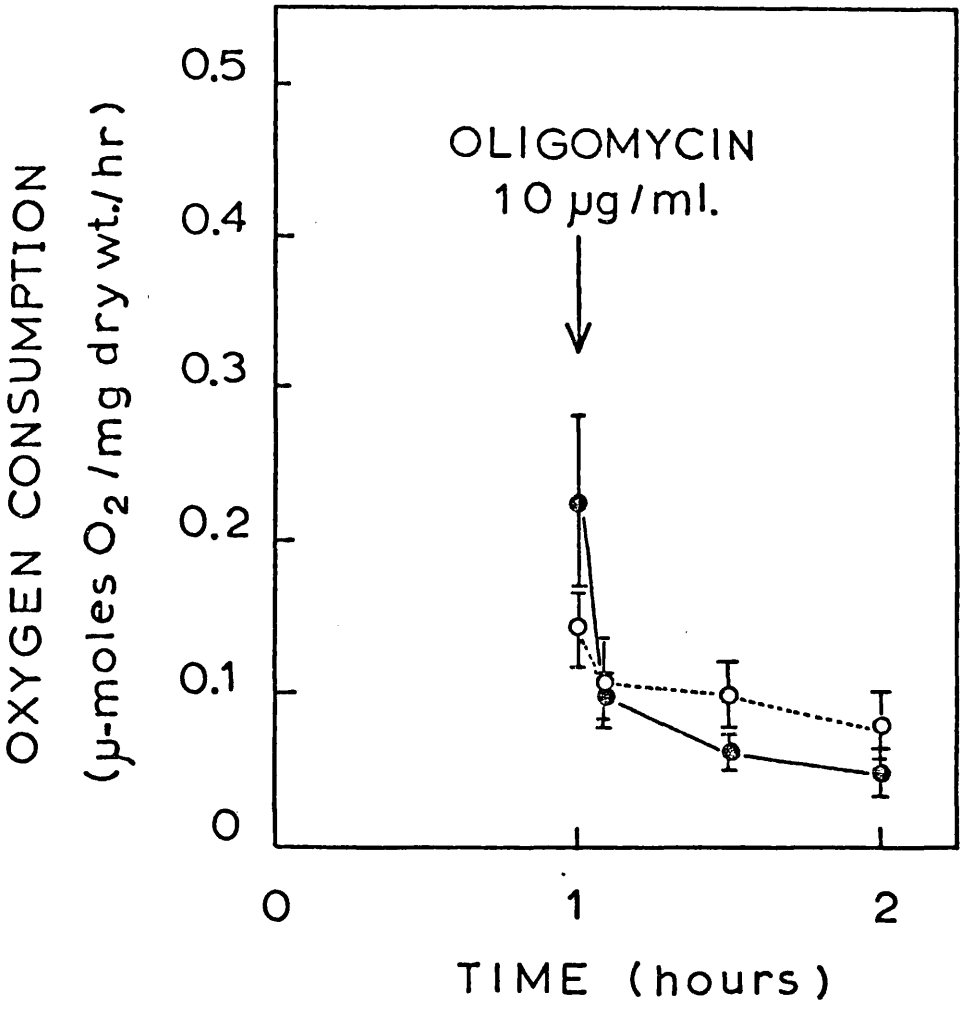


Fig.24 The effect of oligomycin, 10 μg/ml., on the oxygen consumption of foetal gastric mucosal cells incubated in glucose-free medium with and without Na^+. Oligomycin was added in alcoholic solution. ● —— ● + Na^+; O ---- O - Na^+. Each point represents the mean ± S.E. of mean.

mean oxygen consumption in the presence of Na^+ decreased slowly. Oligomycin had no significant effect on the oxygen consumption in the absence of Na^+.

(iii) ATP, ADP and CP concentrations in foetal gastric mucosal cells.

Foetal gastric mucosal cells were incubated in continuously oxygenated, glucose-free medium with and without Na^+. Oligomycin (10 μg/ml.) in alcoholic solution was added at time 0. The effect of oligomycin on the ATP and ADP concentrations, ATP/ADP ratio and CP concentration was not influenced by the presence or absence of Na^+.

The ATP concentration (Table 19, Fig.25A) decreased by approximately 80% ($P < 0.001$) in 1 hour. The ADP concentration (Table 19, Fig.25B) decreased also by approximately 80% ($P < 0.001$) in 1 hour. The ATP/ADP ratio (Table 20, Fig.26A) and the CP concentration (Table 20, Fig.26B) did not change significantly in 1 hour.

G. THE EFFECT OF 2,4-DINITROPHENOL.

2,4-dinitrophenol is a classic uncoupler of oxidative phosphorylation. It permits oxidation to proceed, usually at an increased rate, without net phosphorylation (Loomis & Lipmann, 1948; Cross, Taggart, Covo & Green, 1949).

(i) Oxygen consumption of foetal gastric mucosal cells, in the presence of Na^+.

Kendall (1968) showed that 4×10^{-4}M DNP in acetone solution caused initially a slight increase in the total s.c.c. of the foetal stomach, which was maintained for 10-15 minutes. The total s.c.c. then decreased rapidly. Maximum inhibition occurred in 1 hour, but 20-30% of the total s.c.c. remained. Anoxia then caused a further small reduction of the total s.c.c., leaving approximately 15-20% of

Table 19

The effect of oligomycin, 10 μg/ml., on the ATP and ADP concentrations in foetal gastric mucosal cells incubated in oxygenated, glucose-free medium with and without Na^+. Oligomycin in alcoholic solution was added at time 0. The values given are means ± S.E. of mean (number of experiments).

TIME (hours)	ATP CONCENTRATION (n-moles/g wet wt.)	
	+ Na^+	- Na^+
0	44.33 ± 6.18 (6)	44.33 ± 6.18 (6)
1	10.25 ± 1.76 (4)	13.80 ± 2.00 (3)

TIME (hours)	ADP CONCENTRATION (n-moles/g wet wt.)	
	+ Na^+	- Na^+
0	81.29 ± 8.61 (6)	81.29 ± 8.61 (6)
1	11.94 ± 2.97 (4)	18.66 ± 2.27 (3)

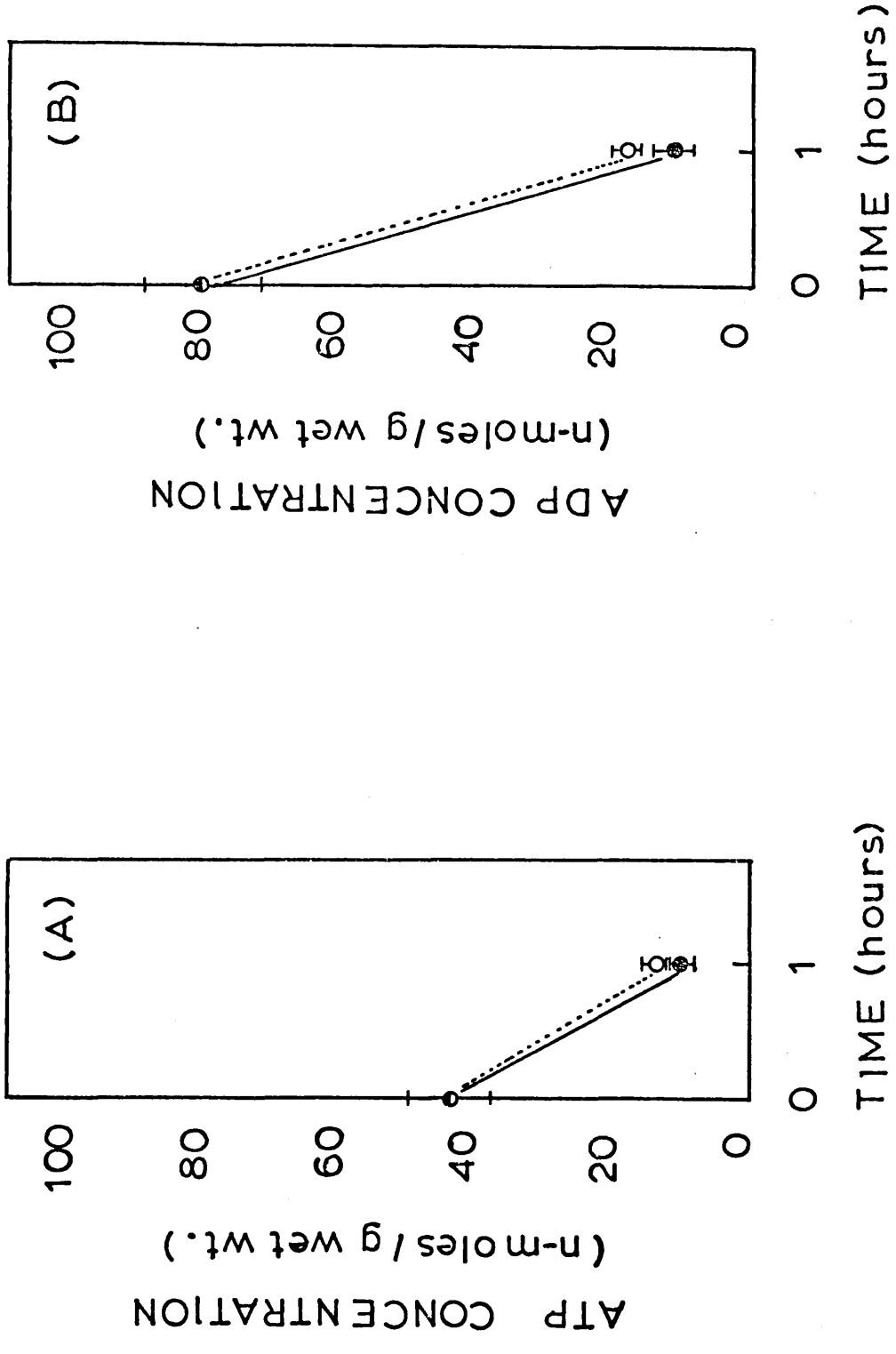


Fig.25 The effect of oligomycin, 10 μg/ml., on the ATP concentration (A) and ADP concentration (B) in foetal gastric mucosal cells incubated in oxygenated, glucose-free medium with and without Na^+. Oligomycin in alcoholic solution was added at time 0. ● —— ● + Na^+; 0 ---- 0 - Na^+. Each point represents the mean ± S.E. of mean.

Table 20

The effect of oligomycin, 10 μg/ml., on the ATP/ADP ratio and CP concentration in foetal gastric mucosal cells incubated in oxygenated, glucose-free medium with and without Na^+. Oligomycin in alcoholic solution was added at time 0. The values given are means ± S.E. of mean (number of experiments).

TIME (hours)	ATP/ADP RATIO	
	+ Na^+	- Na^+
0	0.61 ± 0.12 (6)	0.61 ± 0.12 (6)
1	0.92 ± 0.11 (4)	0.74 ± 0.09 (3)

TIME (hours)	CP CONCENTRATION (n-moles/g wet wt.)	
	+ Na^+	- Na^+
0	400.33 ± 72.08 (6)	400.33 ± 72.08 (6)
1	352.76 ± 26.75 (4)	347.36 ± 37.42 (3)

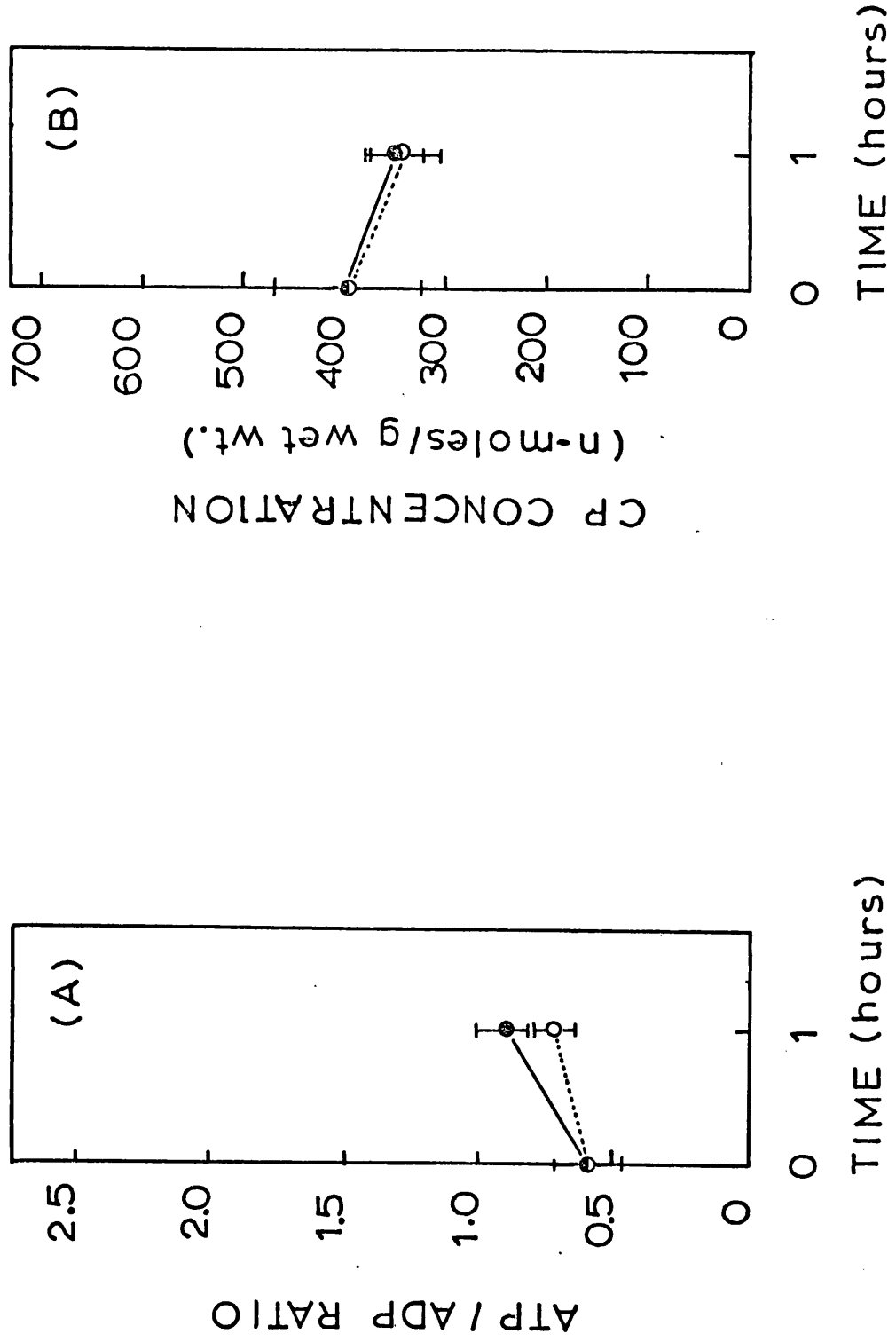


Fig.26 The effect of oligomycin, 10 μg/ml., on the ATP/ADP ratio (A) and CP concentration (B) in foetal gastric mucosal cells incubated in oxygenated, glucose-free medium with and without Na^+. Oligomycin in alcoholic solution was added at time 0.

● —— ● + Na^+; O ---- O - Na^+. Each point represents the mean ± S.E. of mean.

the initial current. The effect of 4×10^{-4}M DNP in acetone solution on the oxygen consumption of foetal gastric mucosal cells in the presence of Na^+, was therefore studied. After 1 hour of incubation with DNP, oligomycin (10 μg/ml.) in alcoholic solution was added to investigate whether uncoupling had in fact occurred.

Foetal gastric mucosal cells were incubated in glucose-free medium containing Na^+. When the oxygen consumption was measured at 1 hour, DNP (4×10^{-4}M) in acetone solution was added. Since DNP is relatively insoluble in aqueous solution, it was first dissolved in a small volume of acetone and then diluted 1:9(v/v) with glucose-free medium containing Na^+. After 1 hour of incubation with DNP, oligomycin (10 μg/ml.) in alcoholic solution was added. The immediate effect of oligomycin was measured. DNP caused a small increase in the mean oxygen consumption in the first 5 minutes, and then a rapid decrease of 75% ($P < 0.05$) during the following 55 minutes (Table 21, Fig.27). Oligomycin did not cause any significant further change in the oxygen consumption.

(ii) Oxygen consumption of foetal gastric mucosal cells, in the absence of Na^+.

Kendall (1968) showed that 1×10^{-4}M DNP in acetone solution caused a rapid, total inhibition of the Na^+ independent s.c.c. of the foetal stomach within 20 minutes. The effect of 1×10^{-4}M DNP in acetone solution on the oxygen consumption of foetal gastric mucosal cells in the absence of Na^+ was therefore studied, and after 1 hour of incubation with DNP, oligomycin (10 μg/ml.) in alcoholic solution was added to investigate whether uncoupling had in fact occurred.

Foetal gastric mucosal cells were incubated in glucose-free medium without Na^+. After the oxygen consumption was measured at 1 hour,

Table 21

The effect of 4×10^{-4}M DNP on the oxygen consumption of foetal gastric mucosal cells incubated in glucose-free medium containing Na^+. DNP was added at 1 hour. One hour after the addition of DNP, oligomycin, 10 μg/ml., in alcoholic solution was added. The values given are means $\pm$ S.E. of mean (number of experiments).

TIME (hr)(min)	OXYGEN CONSUMPTION (μ-moles O_2/mg dry wt./hr) + Na^+	
1	0.26 $\pm$ 0.06	(4)
	DNP added	
1 5	0.36 $\pm$ 0.08	(4)
$1\frac{1}{2}$	0.18 $\pm$ 0.03	(4)
2	0.09 $\pm$ 0.02	(4)
	Oligomycin added	
2 5	0.07 $\pm$ 0.02	(4)

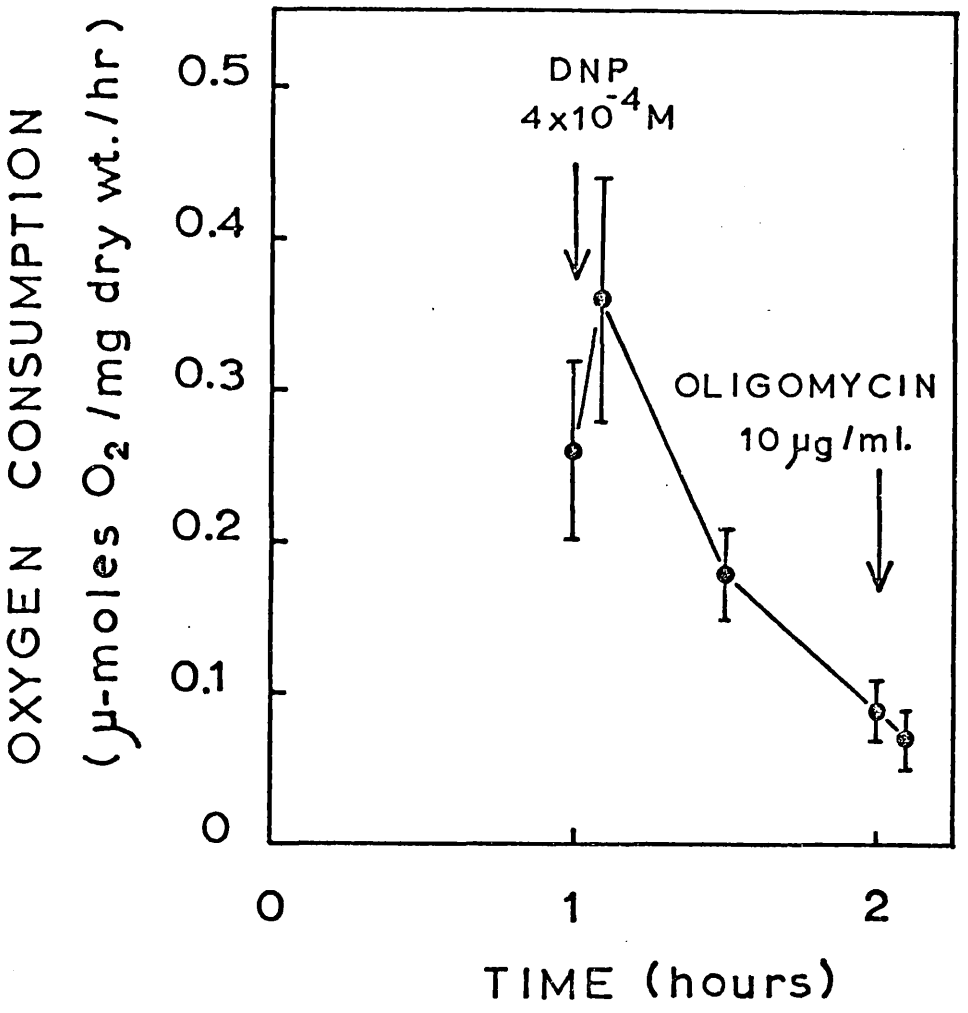


Fig.27 The effect of 4×10^{-4}M DNP on the oxygen consumption of foetal gastric mucosal cells incubated in glucose-free medium containing Na^+. One hour after the addition of DNP, oligomycin, 10 μg/ml., in alcoholic solution was added. Each point represents the mean ± S.E. of mean.

DNP (1×10^{-4}M) in acetone solution was added. DNP was first dissolved in a small volume of acetone and then diluted 1:9(v/v) with glucose-free medium without Na^+. After 1 hour of incubation with DNP, oligomycin (10 μg/ml.) in alcoholic solution was added and its immediate effect measured. The mean oxygen consumption increased by 47% in the first 5 minutes and was maintained at this value for the following 55 minutes (Table 22, Fig.28). Oligomycin then caused a 50% decrease ($P < 0.01$) in the oxygen consumption in 5 minutes to a value which was similar to the initial oxygen consumption measured at 1 hour, before the addition of DNP.

Table 22

The effect of 1×10^{-4} M DNP on the oxygen consumption of foetal gastric mucosal cells incubated in glucose-free medium without Na^+. DNP was added at 1 hour. One hour after the addition of DNP, oligomycin, 10 μg/ml., in alcoholic solution was added. The values given are means ± S.E. of mean (number of experiments).

TIME (hr)(min)	OXYGEN CONSUMPTION (μ-moles O_2/mg dry wt./hr) - Na^+	
1	0.14 ± 0.03	(4)
	DNP added	
1 5	0.20 ± 0.03	(4)
1½	0.15 ± 0.02	(4)
2	0.20 ± 0.02	(4)
	Oligomycin added	
2 5	0.10 ± 0.003	(3)

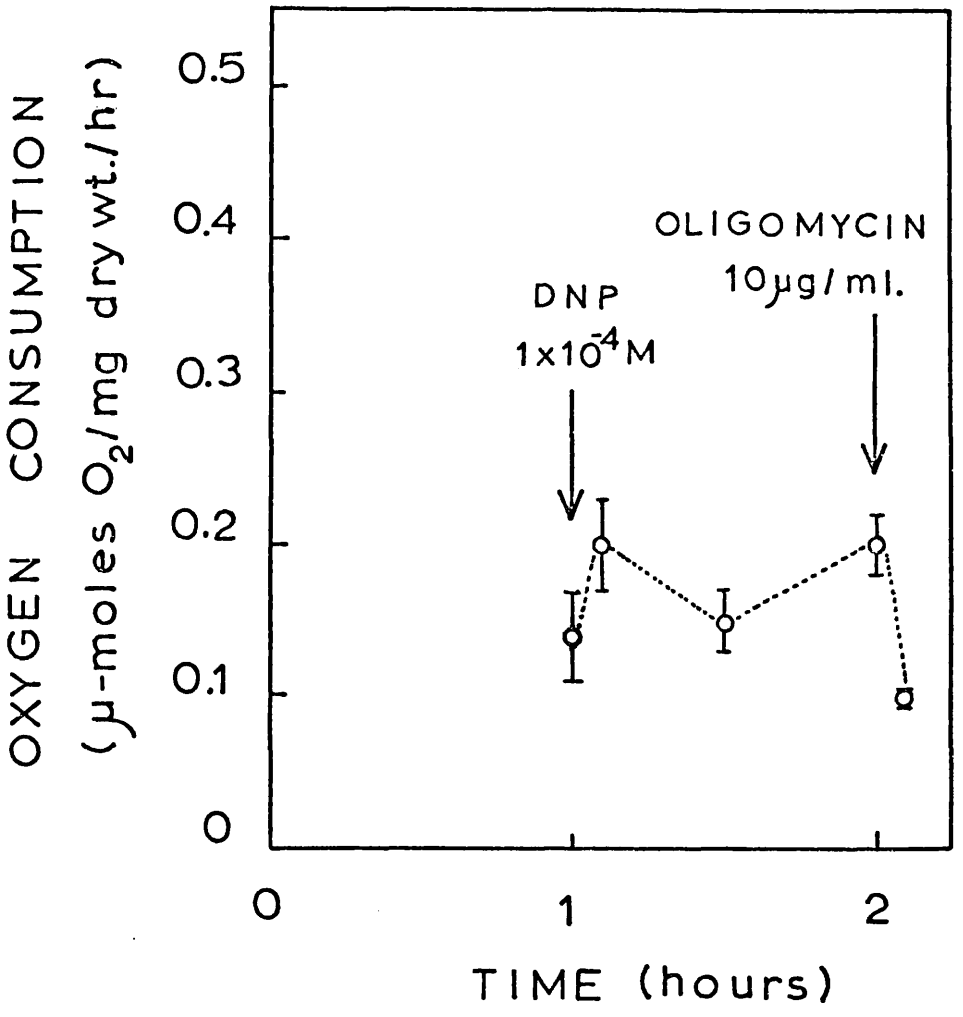


Fig.28 The effect of 1 x 10^{-4}M DNP on the oxygen consumption of foetal gastric mucosal cells incubated in glucose-free medium without Na^+. One hour after the addition of DNP, oligomycin, 10 μg/ml., in alcoholic solution was added. Each point represents the mean ± S.E. of mean.

CHAPTER 4

DISCUSSION

Initial plans for the experiments to determine foetal gastric mucosal cell concentrations of ATP, ADP and CP consisted of the measurement of the p.d. and s.c.c. of a stomach in controlled metabolic conditions, followed by the rapid scraping off of the mucosa at low temperature for ATP, ADP and CP extraction and estimation. However, this procedure could not be followed because the amount of tissue scraped from one stomach was insufficient for ATP, ADP and CP determination by the fluorimetric method used, even though assay methods based upon the measurement of fluorescence emission from solutions, are usually capable of very much greater sensitivity than the corresponding absorptiometric procedures. Therefore foetal gastric mucosal cell suspensions from three stomachs were used. For oxygen consumption measurements, foetal gastric mucosal cell suspensions were also used in order to eliminate the effect of the muscle coat and measure the oxygen consumption of mucosal cells only. Although during measurements of the s.c.c. the muscle coat was present, it does not contribute to the p.d. (Rehm, 1946; Wright, G.H., unpublished) or therefore to the s.c.c. Hence, oxygen consumption, ATP, ADP and CP concentrations and active ion transport were all measured in foetal gastric mucosal cells only. Nevertheless, some caution is necessary when comparing results obtained using the intact tissue with those obtained using isolated cell suspensions.

Since in the rabbit foetal stomach the mucosa is very thin, it could be easily removed by the simple, mechanical method used, to give a cell suspension. Foetal gastric mucosal cell suspensions prepared in this way had been shown to consume oxygen (Wright, G.H., unpublished), which gave a preliminary indication that the cells were viable. Therefore it was unnecessary to use techniques for the isolation of

cells, which involved the use of enzymes such as collagenase, pronase, hyaluronidase, and trypsin or the use of EDTA (Walder & Lunseth, 1963; Gatzy & Berndt, 1968; Croft & Ingelfinger, 1969; Blum et al., 1971). In some cases, these investigators were attempting to isolate a specific cell population from the mucosa, which was not necessary in the present work. Since it was possible to use the mechanical method, it was thought to be better to avoid introducing external factors which may affect the cell membrane and therefore possibly the active ion transporting systems of the mucosal cells.

The use of silicone antifoam emulsion in all experiments, particularly when cell suspensions were used, was important to enable continuous and thorough gassing with oxygen or nitrogen. The gastric mucosal cells produce mucus, which froths on gassing. In the absence of silicone antifoam emulsion, this leads to loss of solution and cells out of the top of the flasks or chambers used for the experiments. However, active ion transport was unaffected by the silicone antifoam emulsion, since it had no effect on the s.c.c. of foetal stomachs. Better and more thorough oxygenation of the cell suspensions was possible because of the absence of the muscle coat.

The foetal gastric mucosal cells appeared intact when observed by phase-contrast microscopy and in smears stained with haematoxylin and eosin. The simplest test for viability of the foetal gastric mucosal cell suspensions was the eosin exclusion test. The cells distinctly excluded eosin even after 10 hours of incubation. There was some cell death during this time, occurring mainly after the first 5 hours. However, this test is only a measure of the passive permeability properties of the cell membrane, which may persist even after cell damage. On the other hand, isolated foetal gastric mucosal cells

incubated for 1 hour in control conditions (i.e. with 24 mM glucose present) consumed oxygen at the rate of 0.25 μ-moles O_2/mg dry wt./hr in the presence of Na^+ and 0.08 μ-moles O_2/mg dry wt./hr in the absence of Na^+. During 10 hours of incubation, the cells, both with and without Na^+, continued to consume oxygen at a slowly decreasing rate. These observations indicate not only that the cells were viable, but also that their ability to transport Na^+ actively was not destroyed during the isolation procedure. Wright (1970) using the intact rabbit foetal stomach membrane set up in sealed chambers, also found that oxygen was consumed at a higher rate when Na^+ was present than when it was absent from the mucosal side.

Further evidence that isolated foetal gastric mucosal cells were viable, was that anoxia both in the presence and absence of Na^+ caused a sharp decrease in the ATP concentration in the cells. Reoxygenation for 15 minutes caused the return of the ATP concentration to its initial value. Therefore the cells were capable of resynthesizing ATP, and it would seem that mitochondrial oxidative phosphorylation was not uncoupled in the cells, as could occur if they had been damaged. The changes observed in the oxygen consumption and in the cellular concentrations of ATP and ADP when inhibitors (ouabain, oligomycin and DNP) were added, further indicated that the cells were reactive to their metabolic conditions, which would not occur if the cells were dead.

Some evidence, however, has been put forward that isolated mitochondria from sheep kidney cortex and rat liver can accumulate Na^+ and K^+ against adverse concentration gradients and that this process is dependent on metabolism. It was also found that small quantities of Na^+ and K^+ can be retained by the mitochondria without the expenditure of energy (Bartley & Davies, 1952, 1954; MacFarlane &

Spencer, 1953; Christie, Ahmed, McLean & Judah, 1965). Measurements of ionic gradients across the mitochondrial membrane are, however, subject to several sources of error such as the distribution of intramitochondrial water; trapped intermitochondrial water and its solutes, and the difference between ions in solution and bound ions. Nevertheless, the possibility arises that just respiring mitochondria and not intact mucosal cells were present in the present experiments. However, Bartley & Davies (1954) found that sheep kidney cortex mitochondria lost this ability slowly on storing at $0^{\circ}C$ and rapidly at $20^{\circ}C$. The endogenous respiration remained stable for a few hours at $0^{\circ}C$, but decreased to zero in 20 minutes at $20^{\circ}C$. The addition of ATP, Mg^{++}, and phosphate to the mitochondria did not prolong the maintenance of respiration at $20^{\circ}C$ and the addition of substrate (α-oxoglutarate) prolonged it for up to 2 hours only. In the present work, in the absence of exogenous substrate, the oxygen consumption of foetal gastric mucosal cells both in the presence and absence of Na^{+} was maintained, although at a slowly decreasing rate, for 10 hours at $35^{\circ}C$. In the presence of glucose, the decrease in the oxygen consumption was smaller both in the presence and absence of Na^{+}. These results eliminate the possibility that just respiring mitochondria and not intact mucosal cells were present for two reasons. First of all, the oxygen consumption was maintained for a much longer period of time. Secondly, the effect of glucose suggests that glycolysis was occurring and since glycolytic mechanisms are extramitochondrial (Kennedy & Lehninger, 1949), intact viable foetal gastric mucosal cells must be present.

Subsequent work with mitochondria suggests that the spontaneous, energy-dependent accumulation of K^{+} is slow and limited (Rottenberg &

Solomon, 1965), but can be stimulated by the antibiotics, valinomycin or gramicidin and more importantly for the present work, that there is no energy-dependent uptake of Na^+, unless stimulated by gramicidin (Chappell & Crofts, 1965, 1966; Pressman, 1965; Harris, 1968). Blond & Whittam (1964) investigated the effect of Na^+, K^+ and ouabain on the respiration and ATPase activity of mitochondria isolated from rabbit kidney cortex. Ouabain, Na^+ and K^+ had no effect on the ATPase activity. However, although K^+ stimulated mitochondrial respiration, ouabain and the lack of Na^+ were without effect. In contrast, the ATPase activity and respiration of kidney cortex and brain cortex slices are inhibited by removal of Na^+ from the medium or by the addition of ouabain (Whittam, 1961a, 1962; Whittam & Willis, 1963; Blond & Whittam, 1964; Whittam & Blond, 1964). Inhibition of the Na^+ dependent oxygen consumption of rabbit foetal gastric mucosal cells by ouabain, further indicates that viable, intact cells and not just respiring mitochondria were present. Considering all the evidence obtained, one can conclude that the isolated gastric mucosal cells were viable and that in these cells, glycolysis, oxidative phosphorylation and active ion transport were functioning.

It is difficult to compare oxygen consumption rates in various tissues or in gastric mucosae from various animals not only because of tissue and species differences, but also because of differences in the experimental conditions used such as temperature. However, one comparison can be cautiously made. The rat stomach transports Na^+ actively (Cummins & Vaughan, 1965a) and secretes acid (Sernka & Hogben, 1969; Sernka & Harris, 1972) and is therefore similar to the rabbit foetal stomach. Sernka & Harris (1972) found that rat gastric mucosal slices consumed oxygen at the rate of 0.37 μ-moles O_2/mg dry wt./hr at

36°C when Na^+ was present, which is only slightly higher than that measured in similar conditions, but at 35°C, in the present work. Higher oxygen consumption rates in the presence of Na^+ have been measured in other Na^+ transporting tissues such as frog skin and toad bladder (Zerahn, 1956; Leaf & Renshaw, 1957a; Leaf, Page & Anderson, 1959; Gatzy & Berndt, 1968; Parisi & Bentley, 1970; Singer, Civan & Sharp, 1970). The rate of oxygen consumption above a basal level (in the absence of Na^+) has been directly related to net sodium transport (Leaf & Renshaw, 1957a; Leaf, Page & Anderson, 1959).

Difficulties are also encountered with comparisons of ATP, ADP and CP concentrations in various tissues and in the same tissue from different species. In fact, it was found that the concentrations of ATP, ADP and CP measured in rabbit foetal gastric mucosal cells are low compared to those measured in other tissues, such as frog gastric mucosa (Forte, Adams & Davies, 1965; Sachs, Shoemaker & Hirschowitz, 1967; Durbin, 1968; Durbin & Michelangeli, 1972); turtle bladder (Klahr & Bricker, 1965; Bricker & Klahr, 1966; Nakagawa, Klahr & Bricker, 1967); toad bladder (Croker, Saladino & Trump, 1970); canine kidney (Urbaitis & Kessler, 1969; Ross & Weiner, 1972) and rat kidney (Needleman, Passonneau & Lowry, 1968). One factor which greatly affects these comparisons is the difference in the water content of the tissues and in the size of the extracellular space where fluid is trapped, since the concentrations are expressed per gram wet weight of tissue. Nevertheless, the optimum concentration of ATP, ADP, and CP for the reactions taking place is likely to differ from tissue to tissue and from species to species. In the present work, it was found in nearly all experiments that the ATP and ADP concentrations, ATP/ADP ratio and CP concentration measured in foetal gastric mucosal

cells were not influenced by the presence or absence of Na^+. This finding was at first surprising, particularly since distinct differences in the oxygen consumption rates with and without Na^+ were observed. However, in a coupled system, the rate of oxygen consumption by cells is an indication of the cellular turnover rate of metabolites. Since the turnover rate of a metabolite may change without alteration of the tissue concentration of the metabolite, interpretation of the tissue concentrations of ATP, ADP and CP, measured in the present work is difficult.

Although the Na^+ independent s.c.c. of the rabbit foetal stomach is associated with acid secretion (Wright, 1962, 1964; Kendall & Wright, 1967), it must be pointed out once more, that the Na^+ independent s.c.c. has not been precisely characterized due to technical difficulties. The Na^+ dependent s.c.c., however, has been shown to be an exact measure of the active transport of Na^+ from mucosa to serosa (Kendall & Wright, 1967).

A. <u>THE EFFECT OF INCUBATION IN THE PRESENCE AND ABSENCE OF GLUCOSE.</u>

The observations of Kendall (1968) that exogenous glucose seemed necessary for the maintenance of active ion transport over a long period by the foetal stomach were confirmed. Kendall (1968) also showed that the addition of glucose to the serosal side after 6 hours of incubation in glucose-free conditions when the total s.c.c. had declined to less than 50% of the maximum value obtained, caused a reversal of the decline after only a short time lag. Within one hour, the s.c.c. returned to its optimum value. In the present work, active Na^+ transport clearly remained constant during 10 hours of incubation when 24 mM glucose was present in the medium. However in the absence of glucose, it decreased slowly. Nevertheless, after 10 hours

approximately 45% of the active Na^+ transport was still present (Fig.29). Acid secretion, which increased steadily in the presence of glucose, was maintained for $6\frac{1}{4}$ hours in the absence of glucose and then decreased to zero by 10 hours. Since in both cases there was not an immediate, rapid effect upon incubation in glucose-free conditions, the tissue must contain an energy source (possibly glycogen, see Appendix), which can be utilized to support active ion transport. Since the decrease in the tissue concentration of CP in the presence and absence of glucose was similar during 10 hours of incubation, both in the presence and absence of Na^+, it is unlikely that CP was the energy source used in the absence of glucose.

Considering that there was some cell death during 10 hours of incubation of cell suspensions, as indicated by the eosin exclusion test, decreasing oxygen consumption of foetal gastric mucosal cells in the presence of glucose, both in the presence and absence of Na^+, was not surprising. However, in the absence of glucose, the decrease in the oxygen consumption both with and without Na^+ was greater over the 10 hour period of incubation. In both cases, there was a greater decrease in the s.c.c. than in the oxygen consumption. Similar results were found in the turtle bladder upon incubation in substrate-free Ringer solution (LeFevre, Dox & Brodsky, 1972). Although in the presence of Na^+ oxygen consumption changes did not mirror changes in the total s.c.c., both the s.c.c. and oxygen consumption decreased gradually in the absence of glucose. However, in glucose-free conditions in the absence of Na^+, the Na^+ independent s.c.c. was maintained in the first 6 hours when oxygen consumption was decreasing most rapidly and subsequently from 6 to 10 hours of incubation when the oxygen consumption was maintained at the low level reached, the

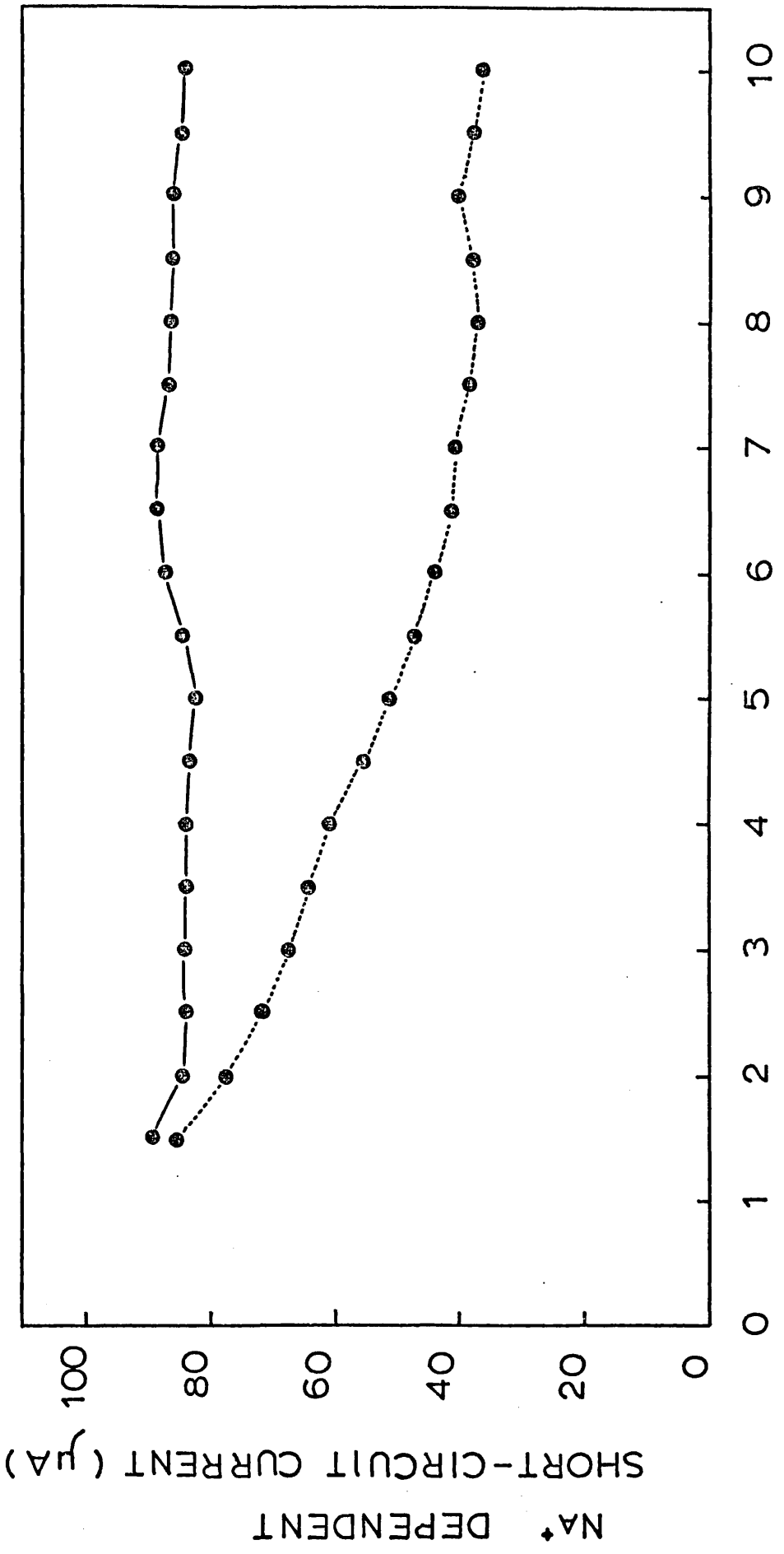


Fig.29 The Na^+ dependent short-circuit current of the foetal stomach (0.6 cm^2) incubated in oxygenated medium in the presence and absence of 24 mM glucose. ● —— ● + 24 mM glucose; ● ---- ● - glucose. Each point represents the difference between the mean total s.c.c. (+ Na^+) and the mean Na^+ independent s.c.c., calculated from Fig.3 (- glucose) and Fig.9 (+ glucose).

Na^+ independent s.c.c. rapidly declined to zero. The difference could be interpreted as indicating that different mechanisms are involved in the coupling of Na^+ transport and acid secretion to metabolism. It seems that oxygen consumption above a critical level can maintain total acid secretion, but with Na^+ transport, as oxygen consumption decreases, so active Na^+ transport decreases, or vice versa.

Maintenance, in the absence of glucose, of the ATP/ADP ratio both in the presence and absence of Na^+ when both oxygen consumption and active ion transport were decreasing, indicates that the ratio alone is not the controlling factor of either active Na^+ transport or acid secretion. This contradicts the view put forward from work with giant squid axons (Keynes, 1960; De Weer, 1968) that a high ATP/ADP ratio is required for optimum operation of the Na pump. In the present work, maintenance of the ATP/ADP ratio was due to the fact that the ATP and ADP concentrations followed similar changes. In glucose-free conditions, after initial decreases in the presence of Na^+ both concentrations were maintained; while in the absence of Na^+, both concentrations decreased. Although the presence of glucose inhibited transiently the initial decrease in the ATP concentration in the presence of Na^+ and inhibited partly the large decrease in the ADP concentration both in the presence and absence of Na^+, ATP and ADP concentrations still both decreased. A number of investigators have reported loss of total adenine nucleotides (ATP, ADP and AMP) in various tissues under the influence of inhibitors (Forte, Adams & Davies, 1965; Daniel & Robinson, 1971; Urbaitis & Kessler, 1971). Although AMP concentrations were not measured in the present work, it would seem that there is breakdown of adenine nucleotides in the rabbit foetal gastric mucosa, since ATP and ADP concentrations both

decrease. These findings, as well as the fact that it is not possible to isolate the part of the ATP, ADP and CP concentration changes that may be related to active ion transfer alone, make interpretation of the observed changes more difficult. The continued presence of CP in the cells after 10 hours of incubation in glucose-free conditions further complicates the interpretation of ATP and ADP concentration changes. However, in the case of Na^+ transport, it is evident that the decrease in the transport in the absence of glucose was not due to a decrease of tissue ATP, since its concentration was maintained. In fact, maintenance of the ATP concentration could be observed if both the production and utilization of energy in the cells decreased by similar amounts. This is supported by the finding that both oxygen consumption and active Na^+ transport decrease in the absence of glucose. In the case of acid secretion, initially when the oxygen consumption and the tissue ATP concentration were decreasing most rapidly in the absence of glucose, acid secretion was maintained. Subsequently, when oxygen consumption and the ATP concentration were at very low levels and were decreasing more slowly, acid secretion declined to zero. These results could support a role for both oxygen consumption and ATP, or one or the other, in acid secretion. Kendall (1968) showed that the decline in the total s.c.c. observed in the absence of glucose was not prevented by the presence of lactate or β-hydroxybutyrate in the incubation medium. Therefore it seems that glucose is specifically needed to maintain active ion transport in this tissue. In light of this finding and of the results obtained in the present work, it would be of interest to investigate more thoroughly glucose metabolism in the foetal gastric mucosa.

B. THE EFFECT OF ANOXIA AND REOXYGENATION

Anoxia inhibited acid secretion rapidly and completely (Kendall, 1968), suggesting a direct dependence of acid secretion on oxidation processes. However, after 50 minutes of anoxia Kendall (1968) found that 15% of the total s.c.c. remained and interpreted this as an ability by the tissue to utilize energy derived from glycolysis to maintain a fraction of the Na^+ transport. In the present work, it was found that after 50 minutes of anoxia, only approximately 8% of the total s.c.c. remained which was not maintained. It declined to zero after a further 30 minutes of anoxia. Therefore it seems that anaerobic glycolysis cannot maintain Na^+ transport although it possibly contributes during part of the anoxic period. This would have to be confirmed by experiments in which the effect of anoxia on the production of lactate is measured. These observations are in contrast with the effect of anoxia in some other tissues. Anaerobic glycolysis maintained 20-50% of the active Na^+ transport in the isolated frog skin for 1 to 2 hours (Leaf & Renshaw, 1957b). In the isolated turtle bladder, 84% of the net Na^+ flux was maintained in anaerobic conditions: a linear relationship being observed between lactate formation (a measure of anaerobic glycolysis) and net anaerobic Na^+ transport (Klahr & Bricker, 1965). Although one hour of anoxia caused a 60% decrease in the s.c.c. of the toad bladder (a measure of active Na^+ transport in this tissue), after 3 hours less than 10% was still present, which was maintained during a further 3 hours of anoxia (Croker et al., 1970).

Acid secretion in the frog gastric mucosa was inhibited more slowly than in the rabbit foetal stomach, and a small, distinct fraction was maintained, indicating that anaerobic glycolysis which

increased (since lactate production increased) could support acid secretion at a greatly reduced rate (Forte, Adams & Davies, 1965; Durbin, 1968). Inhibition of anaerobic glycolysis by iodoacetate reduced acid secretion to zero (Forte, Adams & Davies, 1965). However, acid secretion is the main function of the frog gastric mucosa, whilst in the rabbit foetal stomach, it is probably not of prime importance.

The sharp decrease caused by 1 hour of anoxia, of the ATP concentration in foetal gastric mucosal cells both in the presence and absence of Na^+, to levels lower than in control aerobic incubations, both in the absence of glucose, was as expected, since anoxia inhibits oxidative phosphorylation. Although Durbin (1968) observed a similar decrease in the frog gastric mucosa, Forte, Adams & Davies (1965) found that 60-90 minutes of anoxia caused only a 35% decrease in the ATP concentration in the bullfrog gastric mucosa. The CP concentration in the frog gastric mucosa decreased sharply during anoxia and was virtually zero after 1 hour (Durbin, 1968). Therefore in the frog gastric mucosa, it seems that CP can be utilized. The 50% decrease in the CP concentration observed in rabbit foetal gastric mucosal cells both in the presence and absence of Na^+ after 3 hours of anoxia, indicates that CP possibly can be used to some extent in adverse conditions such as during anoxia, but not as efficiently as seen in the frog gastric mucosa. Since no oxidative phosphorylation was occurring, the maintenance of the ATP concentration at a very low level during the second and third hours of anoxia, both in the presence and absence of Na^+, could be due to glycolysis and/or the formation of ATP from CP. However, during this time, no active ion transport was maintained.

As found in the presence and absence of glucose, both ATP and ADP concentrations decreased with and without Na^+. However, since in both cases the decrease in the ATP concentration in the first hour was greater than the decrease in the ADP concentration, the ATP/ADP ratio decreased. Although the ATP/ADP ratio returned to its initial value at 3 hours of anoxia, there continued to be no active ion transport. However, it must be pointed out that the tissue ATP and ADP concentrations after 3 hours of anoxia are very low. Nevertheless, applying a degree of caution, these findings seem to further support the conclusion that the ATP/ADP ratio alone does not control active ion transport in this tissue.

Reoxygenation of foetal gastric mucosal cells for 15 minutes after 1 hour of anoxia caused an increase in the ATP concentration, which returned to its initial value, both in the presence and absence of Na^+. Therefore, although both ATP and ADP concentrations decreased during anoxia, resynthesis of ATP does occur. However, after 15 minutes of reoxygenation, the total s.c.c. increased to only 32% of its initial value. It would appear, therefore, that not only does active ion transport in this tissue not depend on the ATP/ADP ratio, but it also does not seem to depend on the absolute value of the tissue ATP concentration. In the frog gastric mucosa, the increase of the s.c.c. (acid secretion) paralleled the restoration of tissue ATP and CP concentrations on reoxygenation after anoxia (Durbin, 1968). In the present work, since the total s.c.c. of the foetal stomach recovered within 2 hours of reoxygenation, the long period of anoxia did not permanently damage the cells. On reoxygenation, it is possible that the energy produced by oxidative phosphorylation is utilized to restore the functioning of cellular

processes in order of importance to the cells. The restoration of part of the s.c.c. in 15 minutes of reoxygenation indicates that it is an important function of the cells. However, total restoration of this function rapidly may not be of prime importance. Forte, Adams & Davies (1965) found that acid secretion and the ATP concentration in the bullfrog gastric mucosa were restored on reoxygenation after short (30 minute) periods of anoxia, but not after 60 minutes of anoxia.

Foetal animals have been shown to be more resistant than adult animals to anoxia. The explanation of this phenomenon is thought to be that foetal tissues, particularly the liver and cardiac muscle contain large amounts of glycogen at certain stages in their development, which can be metabolized anaerobically if required (Shelley, 1961; Dawes & Shelley, 1968). Higher rates of anaerobic glycolysis, which decrease towards term, have been reported in foetal tissues as compared with adult tissues (Villée & Hagerman, 1958; van Rossum, 1963a). Kidney cortex slices of the developing rabbit and the liver in the developing rat have the ability to utilize energy derived from anaerobic glycolysis to maintain active transport of Na^+ and K^+ (Whittam, 1960, 1961b; van Rossum, 1963b). In the present work, however, it was found that the rabbit foetal stomach cannot use anaerobic glycolysis to maintain any active ion transport. Nevertheless, a degree of protection from irreversible changes due to anoxia was shown by the observation that all active ion transport, both active Na^+ transport and acid secretion, recovered during reoxygenation following 1 hour of anoxia.

C. THE EFFECT OF OUABAIN

Since the report by Schatzman (1953) that ouabain, a cardiac glycoside, inhibited active Na^+ and K^+ transport by red blood cells, it has been shown that cardiac glycosides inhibit Na^+ and K^+ transport by a number of tissues (Whittam, 1962; Elshove & van Rossum, 1963; Glynn, 1964; Herrera, 1968a,b; Solinger et al., 1968). Schatzman (1953) and Glynn (1957) presented evidence that the effect of the glycosides is on the ion transport mechanism itself rather than on its energy supply; and Skou (1965, 1969) demonstrated that the effect may occur through a specific and highly selective inhibition of (Na^+ + K^+)-stimulated ATPase. Therefore, initially, the results of Kendall (1968) that 10^{-5}M ouabain caused total inhibition within one hour of the Na^+ independent s.c.c. (acid secretion) of the foetal stomach was somewhat surprising. Similar results have been reported in the frog gastric mucosa (Cooperstein, 1959; Davenport, 1962) and in the lizard gastric mucosa (Hansen, Bonting, Slegers & de Pont, 1972) when ouabain concentrations greater than 10^{-7}M were used. However 10^{-7}M and lower concentrations of ouabain did not inhibit gastric acid secretion in the lizard (Hansen et al., 1972) or in the frog(Flemström & Öbrink, 1972). When concentrations of ouabain that inhibit acid secretion are used, it has been found that the tissue concentration of Na^+ increases and of K^+ decreases (Davenport, 1962; Hansen et al., 1972). These results suggest that ouabain does not have a specific inhibitory effect on acid secretion, but that it primarily interferes with the maintenance of the normal intracellular Na^+ and K^+ concentrations, which if substantially altered, cause inhibition of acid secretion. As

discussed in Chapter 1, section D, K^+ seems to play an important role in gastric acid secretion and Na^+ probably also plays a role. Therefore, this is a possible explanation of the inhibition of acid secretion by high concentrations of ouabain, as found by Kendall (1968) in the rabbit foetal stomach. It must be pointed out that when foetal gastric mucosal cells are incubated in Na^+-free medium, the suspension is not absolutely free of Na^+ due to the large extracellular space which traps medium (Wright & Woods, 1971). Measurements were made of the Na^+ concentration in the supernatant medium after incubation with cells, and it was found to be of the order of 5-8 mM. This concentration is low enough to abolish transepithelial Na^+ transport (Wright, 1964), but, since the Na^+ independent s.c.c. is maintained in these conditions, can be assumed to be sufficient to maintain the necessary intracellular concentration of Na^+, remembering that foetal gastric mucosal cells are easily permeable to Na^+.

Within one hour, 10^{-5}M ouabain caused maximum inhibition (50%) of the oxygen consumption of foetal gastric mucosal cells incubated in Na^+-free, glucose-free medium. Subsequently, it remained constant. This inhibition followed closely the total inhibition of the acid secretion (Na^+ independent s.c.c.) within 1 hour. Concurrently, the ATP and CP concentrations remained constant, which would be expected if production and utilization of energy (ATP) decreased by similar amounts. The ADP concentration, however, decreased as occurred in cells incubated in the absence of ouabain, causing the ATP/ADP ratio to increase. Although ouabain is inhibiting acid secretion probably indirectly, the changes in the oxygen consumption and in the ATP and ADP concentrations, ATP/ADP ratio and CP concentration, can be

associated for the most part with inhibition of acid secretion. However, this would need confirmation by a more thorough investigation of the action of ouabain on both acid secretion and intracellular Na^+ and K^+ concentrations in foetal gastric mucosal cells.

Inhibition of the active transport of Na^+ by the foetal stomach by 10^{-5}M ouabain (Kendall, 1968) suggests the direct involvement of a (Na^+ + K^+)-stimulated ATPase. Similar inhibitory effects by ouabain on the s.c.c. of other Na^+ transporting epithelial tissues such as frog skin (Koefoed-Johnsen, 1957; Aceves & Erlij, 1971), toad bladder (Bonting & Canady, 1964; Herrera, 1966, 1968a,b; Coplon & Maffly, 1972) and turtle bladder (Solinger et al., 1968) have also been observed. However, there does seem to be rather wide variation in the sensitivity to cardiac glycosides of Na^+ transport and the (Na^+ + K^+)-stimulated ATPase of different tissues and species (Bonting & Caravaggio, 1963; Glynn, 1964; Repke, Est & Portius, 1965). Inhibition of the enzyme has been shown to be time- and temperature-dependent (Allen, Lindenmayer & Schwartz, 1970; Allen & Schwartz, 1970). Solinger et al. (1968) showed clearly that the time for complete inhibition by ouabain of the s.c.c. of the turtle bladder (active Na^+ transport) was directly dependent on the concentration of ouabain used. Complete inhibition of the s.c.c. occurred after 50, 85, 140 and 320 minutes for ouabain concentrations of 10^{-3}M, 10^{-4}M, 10^{-5}M and 10^{-6}M respectively. Therefore, although 10^{-5}M ouabain did not completely inhibit active Na^+ transport by the rabbit foetal stomach in 1 hour and 40 minutes, in the presence of glucose (Kendall, 1968), it seems probable that complete inhibition could be obtained either if a higher concentration of ouabain had been used, or if inhibition by 10^{-5}M ouabain had been followed over a longer period

of time. However, this point must be confirmed by further work.

In glucose-free conditions, ouabain inhibited the Na^+ dependent oxygen consumption of foetal gastric mucosal cells in 1 hour. At this time, in glucose-free medium, there was a small fraction of Na^+ transport present which declined to zero within the following 40 minutes (Kendall, 1968), which suggests that part of the Na^+ independent oxygen consumption can be used to support active Na^+ transport. As in the absence of Na^+, the ATP and CP concentrations remained constant during three hours of incubation with 10^{-5}M ouabain, while the ADP concentration decreased in the first hour and then remained constant. Therefore the ATP/ADP ratio increased in the first hour and remained constant at the increased value. These results are in good agreement with the effect of ouabain on ATP and ADP concentrations and the ATP/ADP ratio of renal cortex (Urbaitis & Kessler, 1971) and would be expected if changes in the utilization and production of ATP were similar. Since ouabain does not act on the mechanisms of energy production, the decrease in the Na^+ dependent oxygen consumption of foetal gastric mucosal cells must result from the inhibition of active Na^+ transport. The regulation of the rate of respiration by active ion transport has been studied extensively by Whittam and his collaborators (Whittam, 1961a, 1962, 1964; Whittam & Willis, 1963). Decreases in the rate of K^+ uptake by brain and kidney cortex induced by means believed not to inhibit respiration directly (e.g. the absence of external Na^+ or K^+, high external Ca^{++} or the presence of ouabain) were nevertheless accompanied by parallel decreases in respiration. Since active Na^+ and K^+ transport is thought to occur via the $(Na^+ + K^+)$-stimulated ATPase, it has been suggested that the activity of this enzyme

regulates part of the tissue respiration (Whittam & Willis, 1963; Blond & Whittam, 1964; Whittam & Blond, 1964). A similar inter-dependence between acid secretion and respiration can only be postulated from the effect of ouabain, since inhibition by ouabain of acid secretion is thought to be indirect. However, investigation of the effect of thiocyanate, which inhibits gastric acid secretion and the gastric HCO_3^--stimulated ATPase, on respiration would be of great interest.

Kendall (1968) was unable to demonstrate the presence of a $(Na^+ + K^+)$-stimulated ATPase in rabbit foetal gastric microsomes, although a HCO_3^--stimulated, SCN^--inhibited Mg^{++}-ATPase was found. A similar enzyme was found in bullfrog and adult rabbit microsomes (Kasbekar & Durbin, 1965; Sachs, Mitch & Hirschowitz, 1965; Tanisawa & Forte, 1971; Ganser & Forte, 1973); but a $(Na^+ + K^+)$-stimulated ATPase was found in the rat gastric mucosa (Cummins & Vaughan, 1965a); cat gastric mucosa (Bonting, Simon & Hawkins, 1961); lizard gastric mucosa (Hansen et al., 1972); human gastric mucosa (Mózsik & Øye, 1969; Mózsik, 1970) and dog gastric mucosa (Sachs, Shah, Strych, Cline & Hirschowitz, 1972). However, Kendall (1968) did find 25% inhibition with ouabain of the total Mg^{++}-ATPase activity found in foetal gastric mucosal cells after treatment with 1M urea. This probably indicated the presence of $(Na^+ + K^+)$-stimulated ATPase. Similarly, Limlomwongse & Forte (1970) found inhibitions of 12% and 7% of the total ATPase activity by ouabain in tadpole and bullfrog gastric mucosae respectively, which they did not investigate further.

Kendall (1968) also did not find $(Na^+ + K^+)$-stimulated ATPase activity in the adult rabbit gastric mucosa. However, Nakao, Tashima, Nagano & Nakao (1965) demonstrated $(Na^+ + K^+)$-stimulated ATPase activity

in this tissue after treatment of the tissue homogenate with sodium iodide. Therefore, it would seem that the inability to show the presence of this enzyme in foetal gastric mucosal cells may be due to inadequate preparation of the tissue homogenate rather than to species differences. In several other tissues, such as the human gastric mucosa (Mózsik & Øye, 1969) and the lizard gastric mucosa (Hansen et al., 1972), the sodium iodide treatment has been found to be more successful than treatment with urea, in unmasking $(Na^+ + K^+)$-stimulated ATPase activity. After sodium iodide treatment, Hansen et al. (1972) showed the Mg^{++}-ATPase and $(Na^+ + K^+)$-stimulated ATPase of the lizard gastric mucosa had pH optimums of 9.0 and 7.3 respectively, with $(Na^+ + K^+)$-stimulated ATPase activity decreasing rapidly at a pH of 6.0 or 8.0. Kendall (1968) found that the pH optimum of the Mg^{++}-ATPase activity of foetal gastric mucosal cells was between 8 and 8.3, and then assayed $(Na^+ + K^+)$-stimulated ATPase at this pH, which is another reason possibly, why Kendall (1968) failed to demonstrate $(Na^+ + K^+)$-stimulated ATPase activity, whose optimum pH is around 7.4 for mammalian tissues (Skou, 1965).

In conclusion, the presence of $(Na^+ + K^+)$-stimulated ATPase activity in rabbit foetal gastric mucosal cells should be re-investigated using more refined unmasking and assay techniques. However, the evidence obtained from the experiments with ouabain in the present work, coupled with the finding of 25% inhibition of the total Mg^{++}-ATPase activity by ouabain (Kendall, 1968) provide strong evidence that in this tissue, a $(Na^+ + K^+)$-stimulated ATPase is associated with the active transport of Na^+. It must be pointed out that in most, if not all, other Na^+-transporting tissues, such an enzyme has been demonstrated.

D. THE EFFECT OF OLIGOMYCIN AND 2,4-DINITROPHENOL

Experiments with isolated mitochondria have shown that oligomycin inhibits the synthesis of ATP (Lardy et al., 1958; Huijing & Slater, 1961) without affecting the direct utilization of the energy of respiration ('energy pressure') for energy-requiring processes in the mitochondria (Slater et al., 1964) or the dissipation of this energy by uncoupling agents such as DNP (Lardy et al., 1958; Huijing & Slater, 1961). Oligomycin inhibits tightly-coupled respiration and has no effect on non-phosphorylating or uncoupled respiration (Huijing & Slater, 1961). In isolated mitochondria, part of the respiration has been found to be loosely coupled to phosphorylation, depending on the substrate used and in this case, oligomycin abolishes phosphorylation without an effect on respiration (Lardy et al., 1958; Huijing & Slater, 1961). Therefore, it is clear that oligomycin is primarily an inhibitor of respiratory chain phosphorylation and that inhibition of respiration is secondary to inhibition of phosphorylation. DNP, a classic uncoupler of oxidative phosphorylation, permits oxidation to proceed, usually at an increased rate, without net phosphorylation (Loomis & Lipmann, 1948; Cross et al., 1949). According to the chemical hypothesis of oxidative phosphorylation described in Chapter 1, section B, DNP is thought to act by dissipating the first high-energy compound (A~I) and oligomycin is thought to act on the second high-energy compound (X~I), thereby in both cases preventing the formation of the phosphorylated high-energy intermediate (X~P) and ultimately ATP (Huijing & Slater, 1961; Ernster & Lee, 1964; Lee & Ernster, 1968; Cross & Wang, 1970). Since non-phosphorylating preparations respire, A~I must be dissipated or utilized. It has also been shown that oligomycin inhibits DNP-induced ATPase activity in

mitochondria (Lardy et al., 1958; Huijing & Slater, 1961).

In intact cells, oligomycin will not inhibit all ATP synthesis, since neither the phosphorylation linked with glycolysis nor substrate-linked phosphorylation is inhibited. However, oligomycin can be used to study the capacity of intact cells to use respiratory energy directly without converting it to the phosphorylated high-energy intermediate (X∼P) or ATP. If all or part of the tissue respiration is not inhibited by oligomycin, then the respiratory energy produced must be being used without its conversion to X∼P or ATP. Comparison of the effect of oligomycin on tissue respiration and energy-requiring processes (e.g. active ion transport) may thus give an indication whether the processes can utilize respiratory energy directly. The degree of inhibition of respiration by oligomycin is also a good measure of the tightness of the coupling of respiration to phosphorylation. Van Rossum (1962, 1964) found that the respiration of rat liver slices was inhibited only 20% by oligomycin, although almost total inhibition was found of the respiration of isolated liver mitochondria measured in the presence of ADP and inorganic phosphate (Lardy et al., 1958; Huijing & Slater, 1961). Similarly, little inhibition of respiration by oligomycin has been reported in various other tissues (Minakami, Kakinuma & Yoshikawa, 1963; Wu, 1964; Whittam, Wheeler & Blake, 1964; Tobin & Slater, 1965). In rat liver slices, active Na^+ extrusion and K^+ uptake have also been shown to be only partially inhibited (50%) by oligomycin and therefore it seems that in this tissue, part of the active cation transport may be driven directly by respiratory energy, possibly in the form of a high-energy intermediate (van Rossum, 1962, 1964). However, subsequently, van Rossum (1971) obtained results which

suggested that in liver slices, oligomycin was able effectively to inhibit oxidative phosphorylation; but it appeared that the persisting synthesis of ATP (presumably by substrate-level phosphorylations) was adequate to support oligomycin-resistant transport of Na^+. The partial inhibition of ion transport by oligomycin could however be adequately accounted for by the inhibition of oxidative phosphorylation. Therefore, although the 'energy pressure' has been shown to be used directly in some mitochondrial energy-requiring processes, it still remains to be shown whether this is possible for any extra-mitochondrial processes.

It has been shown that (Na^+ + K^+)-stimulated ATPase is inhibited by oligomycin (Järnefelt, 1962; Glynn, 1963; Jöbsis & Vreman, 1963; van Groningen & Slater, 1963; Whittam, Wheeler & Blake, 1964; Blake, Leader & Whittam, 1967). Whittam, Wheeler & Blake (1964) found that ouabain and oligomycin had similar effects on the respiration of kidney slices and on the (Na^+ + K^+)-stimulated ATPase in this tissue. Therefore they proposed that the inhibitory action of oligomycin on the respiration was due to its inhibition of the cell-membrane (Na^+ + K^+)-stimulated ATPase. However, the concentration at which (Na^+ + K^+)-stimulated ATPase inhibition is obtained (10 µg/ml.), is about 10 times greater than that needed to inhibit the mitochondrial phosphorylating respiratory chain (van Groningen & Slater, 1963). Therefore, it would still seem that the primary effect of oligomycin in respiring tissues is on mitochondrial oxidative phosphorylation.

Oligomycin (10 µg/ml.) inhibited both active Na^+ transport and acid secretion by the rabbit foetal stomach in the presence of glucose (Kendall, 1968). Using this concentration of oligomycin, it

is possible that the (Na^+ + K^+)-stimulated ATPase, if present in this tissue, was inhibited. However, careful comparison of the effects of ouabain and oligomycin tends to indicate that the primary effect of oligomycin is to inhibit the synthesis of ATP by oxidative phosphorylation rather than to inhibit (Na^+ + K^+)-stimulated ATPase only. In the present work, it was shown that the absence of glucose did not enhance the inhibition by oligomycin of the total s.c.c. of the foetal stomach. However, in contrast, inhibition of the total s.c.c. by ouabain was enhanced by the absence of glucose (Kendall, 1968). Furthermore, oligomycin caused a large decrease (80%) in the ATP concentration in foetal gastric mucosal cells, both in the presence and absence of Na^+, while with ouabain, the ATP concentration did not change both with and without Na^+. The ATP/ADP ratio was maintained with oligomycin, but increased with ouabain. Similar changes in the ATP and ADP concentrations, and ATP/ADP ratio both with oligomycin and ouabain were found in the dog renal cortex (Urbaitis & Kessler, 1971). CP could not be utilized to maintain the ATP concentration in foetal gastric mucosal cells, since it did not change significantly during one hour of incubation with oligomycin in the presence or absence of Na^+.

The lack of effect by oligomycin on the oxygen consumption of foetal gastric mucosal cells in the absence of Na^+ indicates that respiration in this case, is loosely coupled to phosphorylation. Conversely, since in the presence of Na^+, oligomycin rapidly inhibited the oxygen consumption of foetal gastric mucosal cells to a value similar to that in the absence of Na^+, the Na^+ dependent respiration seems to be tightly coupled to phosphorylation. The Na^+ dependent oxygen consumption was inhibited within the first 5 minutes of

incubation with oligomycin, while active Na^+ transport was totally inhibited in $3\frac{1}{2}$ hours, only a small inhibition occurring in the first 5 minutes. This indicates that respiratory energy is not being used directly for active Na^+ transport, which therefore must utilize energy from either the phosphorylated high-energy intermediate ($X\sim P$) or ATP. After 1 hour of incubation with oligomycin, there is still present 20% of the ATP concentration and about 50% of the total s.c.c. persists which, at this point, is mainly active Na^+ transport. It is not possible to conclude from these results whether ATP or $X\sim P$ is directly utilized for active Na^+ transport. Since oxidative phosphorylation is a process which can be reversed, it can always be argued that in the presence of oligomycin, $X\sim P$ can be formed from ATP. ATP or $X\sim P$ would also seem to be necessary for acid secretion, since it was totally inhibited by oligomycin within 1 hour and the ATP concentration had decreased, but the oxygen consumption was unchanged. Therefore, as with active Na^+ transport, respiratory energy directly could not support acid secretion.

The increase in the oxygen consumption of foetal gastric mucosal cells caused by DNP (1×10^{-4}M) when incubated in the absence of Na^+ suggests that uncoupling of phosphorylation from oxidation must have occurred, and at the same time, acid secretion has been observed to be rapidly and totally inhibited (Kendall, 1968). However, the increase in the respiration caused by DNP was inhibited by oligomycin, although in the absence of Na^+ it caused no inhibition of oxygen consumption. No adequate explanation of these results can be offered in terms of the known action of DNP. Tobin & Slater (1965) found that the degree of inhibition by oligomycin of the respiration of kidney slices is approximately the same in the absence or in the

presence of DNP. This result was interpreted as suggesting that the stimulation of the respiration by DNP is not only due directly to the dissipation of the energy of the high-energy intermediate (A~I) by the action of DNP, but also to an increased supply of ADP to the phosphorylating respiratory chain brought about by the DNP-induced ATPase. It would seem therefore that total uncoupling did not occur or that it did not occur immediately in the intact tissue. With DNP (4×10^{-4}M) in the presence of Na^+, the oxygen consumption of foetal gastric mucosal cells and the total s.c.c. of the foetal stomach followed similar changes, initially increasing slightly and then decreasing. After 1 hour, 20-30% of the total s.c.c. remained, although no Na^+ dependent respiration was present. Therefore it seems, as with previous experiments, that part of the Na^+ independent respiration can be used to support active Na^+ transport. The remaining s.c.c. was active Na^+ transport, since the Na^+ independent s.c.c. was totally inhibited in 20 minutes. The decrease in the oxygen consumption caused by DNP is difficult to explain. However, the lack of stimulation of respiration by DNP in the presence of Na^+ could be interpreted as being caused by the fact that with Na^+ present, the cells are already respiring maximally; while in the absence of Na^+, they are not respiring maximally and therefore can be stimulated by DNP. Considering the results of Tobin & Slater (1965) cited above, a more thorough investigation should be carried out of the concentration of DNP required by this system for complete uncoupling. This depends not only on the lipid solubility and pK of DNP and on the pH of the medium, but also on the nature and concentration of the mitochondria in the tissue. It must be pointed out that DNP has been shown to induce proton translocation in artificial lipid

systems (Chappell & Haarhoff, 1967) and in mitochondria (Mitchell, 1961b; Cunarro & Weiner, 1973), which could be important particularly with respect to the Na^+ independent s.c.c. in the foetal stomach which is associated with acid secretion. However, it is clear that without a more detailed investigation of the effect of DNP on the intact tissue and on mitochondria isolated from this tissue, it is not possible to explain more fully the results obtained with DNP in the present work. Nevertheless, inhibition by DNP of both acid secretion and active Na^+ transport supports the view that respiratory energy cannot be directly utilized for active ion transport in this tissue, but does not further distinguish between the two possibilities which resulted from the experiments with oligomycin: that X∿P or ATP is used for active ion transport.

E. GENERAL DISCUSSION

It is possible to conclude from all the experiments in the present work, that the ATP/ADP ratio does not seem to be a direct controlling factor of active ion transport in the rabbit foetal gastric mucosa. Experiments on the effect of the presence and absence of glucose suggested that an energy store (possibly glycogen, see Appendix) must be present in the tissue, which can be utilized in the absence of exogenous glucose to maintain the slowly decreasing active ion transport. The results of these experiments also emphasized the observation that the mechanisms by which active Na^+ transport and acid secretion are coupled to metabolism are different.

Experiments on the effect of anoxia and subsequent reoxygenation showed that the isolated foetal gastric mucosal cells are capable of resynthesizing ATP, although during anoxia both ATP and ADP concentrations decreased. Decreases in both ATP and ADP concentrations

were observed throughout the present experiments (except in the presence of ouabain) and were surprising results. Therefore, it was important to show that although the ADP concentration decreased as well, the cells were capable of resynthesizing ATP.

The results obtained in experiments with ouabain, strongly suggested that ATP is directly involved in active Na^+ transport through the action of a (Na^+ + K^+)-stimulated ATPase. Ouabain inhibition of acid secretion is thought to be indirect. However, further confirmation of both these points is necessary.

The experiments with oligomycin indicated clearly that the energy of respiration was not utilized directly to maintain active ion transport in the foetal gastric mucosa, and implied the direct involvement of either a phosphorylated high-energy intermediate ($X\sim P$) or ATP, without being able to distinguish definitely between these two possibilities. Nevertheless, in the case of active Na^+ transport, it seems more likely that ATP is involved on the basis of the ouabain experiments. Strong support for this point of view would be the definite identification of (Na^+ + K^+)-stimulated ATPase in this tissue. It has not been shown, as far as the author is aware, in any tissue that ouabain can inhibit the formation of ATP from $X\sim P$. However, the antibiotic, aurovertin is thought to inhibit mitochondrial oxidative phosphorylation by inhibiting precisely the formation of ATP from $X\sim P$ (Ernster, Lee & Janda, 1967). Therefore investigation of the action of aurovertin on active ion transport by the foetal stomach may provide more conclusive evidence on whether $X\sim P$ or ATP is directly involved in active Na^+ transport or acid secretion in this tissue.

In conclusion, it has been shown that respiratory energy cannot

be utilized directly for active Na^+ transport or acid secretion in the rabbit foetal stomach and in both cases, X~P or ATP seems necessary. However, it must be pointed out that for acid secretion, oxidative processes seem necessary as well, as shown by the rapid total inhibition of acid secretion by anoxia. On the other hand, for active Na^+ transport, most of the observed effects can be explained on the basis that only X~P or ATP is required, the ouabain experiments indicating that it is ATP rather than X~P that is more likely to be involved.

OUTLINE OF FUTURE WORK

The glycogen content of foetal gastric mucosal cells during incubation in glucose-free conditions over 10 hours should be measured to determine whether it is the energy source used in the absence of glucose to maintain slowly decreasing active ion transport. It would also be of interest to investigate more thoroughly glucose metabolism in the foetal gastric mucosa under different metabolic conditions, such as imposed in the present work. Measurement of lactate production would give an indication of the rate of glycolysis, and measurement of the evolution of $^{14}CO_2$ from [6-^{14}C] glucose and from [1-^{14}C] glucose would reflect the utilization of glucose via the Embden-Meyerhof pathway and from the Embden-Meyerhof pathway plus alternate pathways (the principal one of which is the hexose monophosphate shunt pathway) respectively.

The presence of $(Na^+ + K^+)$-stimulated ATPase in the foetal gastric mucosa should be re-investigated using the sodium iodide treatment which has been shown to be more successful in some cases in unmasking this enzyme. The optimum assay conditions for the enzyme (particularly the pH) should be more carefully chosen. The effect of ouabain and oligomycin on $(Na^+ + K^+)$-stimulated ATPase activity, if present, should be studied.

Mitochondria isolated from the foetal gastric mucosa should be investigated to see if they have any unusual properties specific to this tissue. The effect of oligomycin and DNP on the respiration of mitochondria should also be studied to investigate the mechanisms of energy production.

Investigation of the effect of aurovertin on active ion transport (s.c.c.) by the foetal stomach may provide more conclusive evidence on whether the phosphorylated high-energy intermediate or ATP is directly involved in active ion transport in this tissue.

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APPENDIX

Measurement of the glycogen concentration in rabbit foetal gastric mucosal cells.

The method of Lowry et al.(1964) was used. Gastric mucosal cells were prepared as described in Chapter 2, section B part v(a) using glucose-free medium, but they were not incubated at 37°C. After extraction of the frozen cell pellet with 3M-perchloric acid, the perchloric acid-insoluble residue was spun down. 1N- HCl (1 ml./100 mg tissue) was added to it and the tube was sealed and heated for 2 hours at 100°C. The supernatant was collected, neutralized with 2M-$KHCO_3$ and its volume measured. This treatment should release all the glucose from glycogen. Glucose was then estimated using the fluorimetric method used for the ATP assay (Chapter 2, section B part v(d)) with glucose omitted and 0.3 mM ATP present. The mixture, however, was incubated at 27°C for 20 minutes before the final reading was taken. Three glucose standards were assayed and a glucose standard line was plotted.

The glycogen content of gastric mucosal cells was 1.73 ± 0.16 µ-moles glucose equivalents/g wet weight (mean $\pm$ S.E. of mean, number of experiments = 8). There were two sources of error which were not corrected for: (a) the possibility of the presence of glucose in the residue before acid hydrolysis and (b) the possibility of the presence of glycogen in the original perchloric acid extract. Nevertheless, the result indicates that glycogen is present in rabbit foetal gastric mucosal cells.