TRANSPORT PHENOMENA IN THE FOETAL GASTRIC MUCOSA

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by

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ABSTRACT.

An outline of the history of gastric electrolyte physiology has been presented along with the development of general electrolyte physiology,.and these have been related to foetal gastric physiology. An outline of the present knowledge of A.T.P. and its formation and utilization related to carbohydrate metabolism, has been given.

By using isotopic tracers, 70% of the total short-circuit current has been found to be due to the active transport of sodium from mucosa to serosa. The relationship between the sodium independent fraction and the net chloride flux from serosa to mucosa in the foetal rabbit stomach, has been demonstrated by the use of isotopic tracers, and the latter found to be 166% of the former.

The metabolic dependence of both the sodium and the sodium independent current of the foetal rabbit stomach has been studied with their relationship to the normal functioning of the oxidative phosphorylation chain and the anaerobic glycolytic cycle, by the use of variousinhibitors of the metabolic cycle.

An A.T.P.-ase has been isolated from the foetal gastric mucosa and its activity studied in relation to the active transport of sodium and hydrochloric acid. No specific relationship was shown to exist.

It is concluded from these results that the sodium current

is most probably maintained by energy derived from the
hydrolysis of A.T.P., though adequate oxygenation is required
for maximum activity; and that the sodium independent current
seems to be wholly dependent on the presence of oxygen, provided
of course, that some energy source, such as glucose,be present.
The significance of these results has been discussed with
regard to foetal physiology, and to the secretion of acid. A
comparison between foetal and adult gastric physiology has
been made.

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CHAPTER 1. HISTORICAL

Before 1800

Prior to 1800 nearly all the views held on the process of
digestion were pure speculation. One concept, due originally
to Erastistratus, 300 B.C., and adopted later by Galen (circa
180 A.D.) was that "food was altered by a change called
'concoction' into a principle, which was absorbed and proceeded
to the liver, where it was converted into blood and endowed
with 'natural spirits'. This concept for the origin of natural
spirits from which animal and vital spirits finally derived,
held sway until the late 16th century.

A more realistic approach to the nature of digestion was
made by Van Helmont (1577-1644) who saw the course of digestion
as a series of fermentations, with the ferment of the stomach
prepared in the spleen and mixed with acid. He did however
believe that the tissues took up substances from the blood.
The notion of glands, ducts and secretions was put foreward
by Stensen, who discovered the parotid duct in 1662, and in
1663 Sylvius, following the views of Van Helmont, regarded
digestion as chemical in nature, with the digestive juices as
the active agents.

About this time the science of physics began to develop
and was regarded as the key to all the natural phenomena.

Borelli (1608-1679) led the iatro-physical school, which considered digestion as a progressively finer and finer tituration.

Digestion was regarded in 1708 by Boerhaave, as it is today, as a combination of physical and chemical processes; but at that time in the absence of chemical and experimental knowledge, speculation as regard to the chemical processes still continued.

Experimental work with regard to the process of digestion began with Réaumer (1772) teaching a tame kite to swallow perforated metal tubes containing various food stuffs; and then to regurgitate them after specific intervals. The meat and bone had not been subject to any mechanical assault, but were soaked in a liquid and partly dissolved; the starchy foods were unaffected.

Spallenzani (1772) confirmed his conclusion that the liquid had a solvent action, even in-vitro, when he squeezed the sponges, regurgitated by a kite, onto meat and kept it warm; but he failed to notice the acidity of the gastric juice. The first mention of gastric acidity was by Carminati a few years later when he observed that gastric juice was neutral before feeding but acidic afterwards. The acid was not identified until well into the next century, when Prout in 1834 finally recognised it as hydrochloric acid.

Early knowledge of foetal physiology was very limited.

Wolff about 1760 claimed that organs developed gradually, after study of the development of a chick; Hunter also made many anatomical observations and also some experimental ones such as when he showed that the stomach of the foetal calf did not contain acid. The next recorded observations on the foetal digestive system only appeared some 100 years later.

After 1800

The classical observations of gastric movements were made about 1830 by William Beaumont, who considered himself to be 'but a humble inquirer after truth and a simple experimenter.' His subject was a soldier who had received an abdominal gunshot wound which had healed to leave a gastric fistula. Bassow in 1842 made the first experimental gastric fistula: a technique which was to be exploited in the important researches of Pavlov and his school.

Identification of gastric 'ferments', later called enzymes by Kühne (1876), and the discovery of differences in the ratio of the inorganic constituents of gastric juice and blood were digestive phenomena needing explanation.

Microscopic examination of the stomach wall yielded the presence of a heterogeneous cell population. Langley and Swiecki demonstrated that pepsin was secreted, in the frog, by the large glands in the oesophagus, whilst the gastric mucosa secreted mainly acid. Study of the mucosal cells showed that they were similar to the parietal cells in the mammalian

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stomach, a large concentration being present in the fundic region where mainly acid was secreted. Various workers used dyes to try and locate the site of acid secretion, and acid secretion and parietal cells seemed to be linked. Langley in 1881 renamed these cells oxyntic.

Müller, with the sound morphological knowledge of the mid 19th century, held that the gastric secretion was formed by the walls of the acini, the constituents passing from the blood of the capilliary network; the amount and kind depending on the nature of the living acinar cells. Under the influence of physics, Ludwig advanced a mechanical theory of filtration, which was later opposed by Heidenhain who showed that physical causes alone provided an inadequate explanation.

The great advances in the fields of physics and chemistry, and in particular in physical chemistry, during the 19th century, enabled advances to be made in the field of gastric physiology. Hess's concepts of dynamic equilibrium and heat changes in reactions, led to the enunciation of the laws of thermodynamics in the 1860's. Helmholtz and Gibbs independently evolved the concept of free energy and with the Helmholtz-Gibbs equation a way of calculating its value in chemical cells. About the same time Faraday had defined the Laws of Electrolysis which formed the basis for extensions in electrochemistry by Arrhenius and Van't Hoff so that the idea that electrolytes in solution are permanently dissociated

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into pairs of oppositely charged ions became established. In 1855 Fick had discovered his laws of diffusion and Nernst (1889) combined the laws of thermodynamics and electro-chemistry to produce an equation, relating the electro-motive force (E.M.F.) of a system to electrolyte concentration gradients. These latter relations have been made use of by physiologists in their efforts to explain the ionic and potential differences between living cells and their environments.

Electrical potentials in living systems had been demonstrated at the turn of the 18th century by Galvani in frog muscle; and also in the electric eel. Donné (1834) had related the observed potential difference across the stomach wall to acid secretion and Du Bois Reymond (1848) had demonstrated that the frog skin was a site of E.M.F. and could produce a current flow. Galleotti (1904) extended these findings to show that the potential difference depended on the presence of sodium or lithium ions in the bathing solution; and it also persisted even with identical solutions bathing each side of the frog skin. Thus the potential difference was not due to diffusion, and could not be explained in simple physico-chemical terms; so must be dependent on forces characteristic of a living system. The same view was held about gastric acid secretion.

During the 19th century some further knowledge was

obtained about the physiology of the foetal mammal. Swallowing, whilst in the womb, was deducedd about 1850 from changes in the amniotic fluid volume and the presence of hair and souamous epithelial cells in the digestive tract of the foetus. Intestinal peristalsis in the foetal guinea-pig was observed at about one month gestation, and in the foetal rabbit, free acid was measured in the stomach; and related by Van Puteren to the length of the foetus. Acid was first detected at a foetal length of 7cm. and the amount increased from then onwards until birth. Acid in the stomach of the human foetus has been denied even at birth, (Schmidt, 1914).

The search for the actual site of acid secretion was continued amongst others, by Fitzgerald (1910), in rabbits, dogs and guinea-pigs; using the Prussian blue reaction. The blue stain occurred in the canaliculi of the parietal cells; and indicated the presence of free acid in these cells. Although her findings were rejected by Harvey they were confirmed by Collip (1920); and later by Dawson & Ivy (1926) who with more accurate methods, utilizing Pavlov pouches and neutral red; obtained stains both in-vivo and in-vitro, located in the parietal cell, and showing a pH of about 3.0. Canaliculi are present in yeast cells as a continuation of the cell wall and have been shown (Rothstein, 1950) to be the site of metabolic activity. A more recent study on the changes in the oxyntic cell before, during and after secretion, and

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under anoxic conditions has been made by Sedar (1965), who
showed that a change occurred in the fine structure of the
cell. Vial & Orrego (1963) demonstrated in the rat gastric
mucosa that dinitrophenol and iodoacetate, as well as
decreasing the acid secretion rate, caused an increase in the
number of cytoplasmic vesicles and a decrease in the number of
folds or villi in the intracellular canaliculi. Studies with
the protein synthesis inhibitor, puromycin,(Orrego, Navia &
Vial, 1966) showed that the membrane changes still occurred
and it was suggested that the vesicles represented a reserve
of membrane for use during secretion.

As the gastric mucosa contains three cell types, in the
mammal, the desirability of obtaining pure parietal secretion
has led to the use of indirect methods. The Pavlov pouches,
were, and still are, utilized to obtain knowledge of the
composition of, and control of, parietal secretion. Pavlov
(1910) noted that the acidity of gastric juices increased as
the rate of secretion increased. This he accounted for by the
hypothesis that the parietal secretion was high and constant
throughout and the acidity varied because of the varying
degree of neutralization and dilution from non-parietal cells.
Hollander (1931) confirmed Pavlov's findings and by plotting
acid against neutral chloride (ie. total chloride minus
hydrogen ion concentration), showed that the stomach secreted
170mM. hydrochloric acid when no neutral chloride was present.

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Extrapolation to 'Zero acidity' gave a value of 100meq/litre of neutral chloride. Hollander inferred from this finding that the remaining anion was buffer, probably bicarbonate, and stated that the non-parietal component contained 100meq of chloride and 70meq of bicarbonate per litre without specifying the cations involved. Similar views were taken by other workers in the field, although the different investigators were at a slight variance regarding the hydrogen ion concentration of the non-parietal component and the presence or absence of neutral chloride in the parietal component. Gray (1943) from a similar plot found that at zero acid secretion there was a neutral chloride concentration of 7meq/litre and also that the potassium concentration was constant at 7meq/litre. The composition of the parietal and non-parietal secretions was estimated, by Fisher & Hunt (1951), from data compiled by Ihre in 1939, to be:

$$Parietal \quad - \quad H^+ \quad 160meq/litre.$$
$$K^+ \quad 10meq/l.$$
$$Cl^- \quad 170meq/l.$$
$$Non-parietal \quad - \quad Cl^- \quad 125meq/l.$$
$$HCO_3^- \quad 45meq/l.$$
$$cations \quad 170meq/l.$$

These two theories are usually classed together as the Component Theory of Gastric Acid Secretion, which regards the gastric juice as a primary secretion of acid modified by

non-parietal cell secretion.

Teorell (1939 & 1940) however suggested the concept of
the gastric mucosa as an ion permeable membrane and used this
as the basis for a'diffusion theory', which could explain
the acidity-chloride variations in the gastric juice without
postulating neutralizing or diluting secretions. He observed
the passage of alkali and chloride from the blood to the
stomach. He demonstrated that the rate of acidity reduction
in the stomach was proportional to the hydrogen ion
concentration of the stomach contents; and explained the excess
neutral chloride over the acid chloride as being a product of
inward diffusion. He visualized a diffusion of sodium ions, in
fact a H^+/Na^+ exchange. Both component and diffusion theories
help to explain the phenomenon. The slight variation that
occurs between the osmolarity of plasma and gastric juice is
explained on the basis of the component theory as the
hydrochloric acid being neutralized by the bicarbonate of the
non-parietal secretion with formation of sodium chloride and
water, and a loss of carbon dioxide. Hirchowitz (1961) proposed
that a primary secretion of 160mM NaCl by the parietal cell
was modified to give a high 140mM Cl^- concentration in the
resting stomach and the inverse ratio between H^+ and Na^+ in
the secreting stomach. The potassium ion (K^+) concentration
was constant and the relation ($Na^+ + K^+ + H^+$) = Cl^- held. He
regarded chloride as the principle anion and the amount and

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concentration of the bicarbonate ion as negligible. These
views were held by both Heinz & Obrink (1954) and Rehm (1958).

That the formation of acid was an actual secretory process
was shown for frog by Gray et al. (1940). Using the technique
of an isolated frog gastric mucosa mounted between two chambers,
with known solutions on each side, they recorded any changes
in pH that occurred. The mucosal solution became acidic, and
though secretion ceased after a while, it was not due to a
build-up of acid concentration. Reversal of the tissue, so that
sodium chloride (NaCl) was bathing the serosal, instead of the
mucosal side, (Ringer solution on the other side), gave an
increase in pH of that solution; suggesting a release of alkali.
Isolated mucosa of other tissue did not produce the same result.
This acid secretion was peculiar to the gastric mucosa. Gray
(1941) also showed that the potassium ion, unlike the sodium
ion, is essential for acid production. Patterson et al. (1949)
got a similar release of acid from an **in-vitro** preparation of
rat gastric mucosa and this occurred even when the bathing
solution was 5% glucose, containing no salts.

Ussing and his colleagues made studies of the movement of
ions across the frog skin, using techniqes and concepts that
have been of great use to physiologists in studies of ion
movement across other tissues. The relationship between
electrical characteristics and movement of ions across cell
membranes, found by Nernst, has been shown by Ussing (1949) to

hold true only if the ions are moving passively. He deduced
the following equation for the flux ratios:

$$\frac{M_{1-2}}{M_{2-1}} = \frac{A_1}{A_2} \exp. \frac{EZF}{RT}$$

where:

M_{1-2} is the unidirectional flux from side 1 to 2.

M_{2-1} " " " " " " " " " 2 to 1.

A_1 and A_2 are the activities of the ion on sides 1 and

2 respectively.

E = electrical potential difference across the membrane.

Z = valency of the specific ion in question.

F = Faraday unit (96500 coulombs.)

R = gas constant (Ergs/mol/$^{\circ}$A.)

T = absolute temperature.

Any deviation from the above equation indicates that the ion
is being 'actively transported': that is that energy other than
the kinetic energy of the ion or that due to electrical or
chemical gradients is being used to move the ion. The
additional source of energy is called the 'active transport
potential', and can be written into the above equation so that
it can represent an actively transported ion.

$$\frac{M_{1-2}}{M_{2-1}} = \frac{A_1}{A_2} \frac{(\exp. EZF/RT)}{(\exp. E_{\measuredangle}ZF/RT)}$$

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where E_x is the active transport potential.

Levi & Ussing (1948) found that in the frog skin, sodium was the only ion transported from a lower to higher positive potential, when both sides of the skin were bathed with identical solutions. Under these experimental conditions any active transport of ions occurring will tend to be short-circuited by a passive movement of ions, (chloride ions being the most likely in frog skin). If the two sides of the skin are connected by reversible electrodes of very low resistance, the current produced, which will be equivalent to the rate of active transport, can be measured. Ussing overcame this difficulty by applying, across the tissue, an external electromotive force (E.M.F.) of appropriate sign and magnitude to reduce the natural potential difference across the membrane, to zero. The current then flowing in the external circuit is equal to the current set up by the short-circuit of passive ions. By the use of isotopic tracers Ussing & Zerahn (1951) showed that the rate of net sodium flux in the frog skin was exactly equal to the short-circuit current, thus proving that sodium was the only ion actively transported in this tissue. This approach made possible a description of the active transport process in a tissue, in terms of an electrochemical cell. The active transport potential was equal to the source of the electromotive force and had a resistance and a shunt resistance in series with it, across which passive ions would

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move to complete the circuit. The shunt resistance would cause the open-circuit potential difference to be less that the active transport potential. However, the open-circuit potential difference could be made equal to the active transport potential if the shunt resistance was very large. Ussing (1958) obtained this effect, practically, by replacing the chloride ion by the sulphate ion in the bathing solution, or by adding copper ions at 10^{-5}M concentration, to the mucosal side, which effectively reduced the chloride permeability to zero. The active transport potential of the frog skin was found to be 140mV and the series resistance about $1.5K\Omega/cm^2$.

$$\frac{Active\ transport\ potential}{short\text{-}circuit\ current} = internal\ resistance$$

The in-vitro Ussing technique was first applied to the problem of ion movement in gastric mucosa by Hogben (1955), using frog stomach. He stripped off the muscle coat and mounted the mucosal tissue between two chambers and found that the full potential difference still existed, confirming the view of Rehm (1946) that the potential difference originates in the mucosa. The tissue was originally flaccid, with low electrical resistance and did not secrete hydrogen ions or have a trans-mucosal potential difference. However after 30 to 60 minutes in an oxygenated saline medium it began to spontaneously secrete hydrochloric acid and to develop a full gastric potential difference of about 35mV; the serosal surface being

positive to the mucosal. Hogben found that the short-circuit
current was equivalent to the rate of net chloride transport,
measured isotopically, from the serosal to the mucosal surface,
minus the rate of hydrogen ion transport in the same direction.
The flux ratio of the sodium deviated slightly from that
expected in the case of passive transport of sodium, but could
be accounted for if 30% of the sodium movement from mucosa to
serosa was active.

Heinz & Durbin (1959) using Rana pipiens, replaced the
chloride ion with sulphate ion in the bathing solution and,
showed the open-circuit potential difference and the short-
circuit current to be of reversed sign; and that the latter
was approximately equal to the hydrogen ion secretion. There is
no net sulphate ion transport in this species.

An extensive study of the electrical properties and rates
of acid secretion, with varying composition of bathing solution,
has been carried out by Rehm and his co-workers, in the
amphibian and mammalian gastric mucosa: (1943, 1945, 1948, 1955,
1962, 1963, 1966.) The earlier work of Rehm dealt mainly with
the problem, could the stomach produce enough energy from its
electrical activities to maintain the secretion of hydrochloric
acid; but came to no definite conclusion. He proposed the
hypothesis that active transport of chloride from serosa to
mucosa gave rise to a potential difference which serves to
drive the hydrogen ions from serosa to mucosa. Thus artificially

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increasing the natural potential difference of the stomach
should give rise to an increase in acid secretion and vice-
versa. An experiment by Rehm et al. (1945) on dog stomach, with
intact blood supply, agreed with this theory and so did
experiments of Crane, Davies & Longmuir (1948). It has also been
shown that the maximum current that could be drawn from the
gastric mucosa was electro-chemically equivalent to the rate
of hydrogen ion secretion. Rehm (1945) demonstrated that
thiocyanate caused complete inhibition of acid secretion and an
increase in potential difference, but the acid secretion fell
before the potential difference rose, (in chloride solutions),
thus bearing out his hypothesis. Histamine also produces
results in agreement with this,(Rehm et al. 1963); a lowering
in potential difference and resistance runs parallel to an
increase in the rate of acid secretion. He suggested that a
carrier mechanism for the transport of the chloride ion could
be involved. The carrier R would combine with the chloride ion
and move across the membrane from the serosal to the mucosal
side; be reduced by an electron donor, setting the chloride
ion free into the mucosal solution. The reduced carrier then
moved back across the membrane, was oxidized in the membrane
or on the serosal side where it was free to combine with
another chloride ion. In the resting state the resistance to
movement of the uncombined R across the membrane was assumed to
be large, so that little net movement would occur: in the

secreting stomach however the resistance was assumed to be small. The main objection to Rehm's hypothesis was that the resistance across the stomach wall was too high (in the order of 200 ohms· cm^{-2} for dog.) to allow sufficient energy to be available for the concentration of the hydrogen ions against the electro-chemical gradient. Rehm (1950), however, analysed the resistance and showed that it was analogous to a capacitance in parallel with a resistance; these two components were considered to exist in the secreting cell. The serosal and muscular layers were considered to act as a higher resistance in series with these two components. The parallel resistance was shown to be extremely small in the resting stomach (3 ohms.cm^{-2}) and to fall to practically zero when secretion commenced; thus an adequate hydrogen current was able to be delivered by the electromotive force of the gastric mucosa. The zero value of the parallel resistance is explained on the basis of there being active chloride and active hydrogen ion secretion, (Rehm et al. 1956 & 1957). Hogben's work in 1955, with frog gastric mucosa, bears out this explanation.

The nature of the potential difference across the gastric mucosa still remains to be elucidated, but there are three possible theories for the existance of a potential difference across a living membrane. The membrane can act as a concentration cell, in respect to a particular ion in the system. This is demonstrated particularly well in nerve, muscle

and red-cell when a metabolic extrusion of ionic sodium gives
rise to an unequal distribution of potassium ion between the
external and internal cell contents: (in order to preserve
electrical neutrality). The potassium ion concentration on the
inside then gives rise to a diffusion potential, which then
becomes the equilibrium potential for potassium and is
described by the Nernst equation. Other passive ions which
are able to pass through the membrane are governed by the same
argument. The mucosal surface of the frog skin is relatively
impermeable to sodium but permeable to potassium ions, thus
acting like a potassium electrode; and the serosal surface
behaves like a sodium electrode, being permeable to sodium but
relatively impermeable to potassium ions. The total potential
difference across the skin is the sum of the sodium and
potassium equilibrium potentials. This theory of the origin of
the trans-membrane potential is termed non-electrogenic as no
current is involved. (Koeford-Johnsen & Ussing, 1958.)

Earlier it was thought that the potential difference was
across the frog skin was electrogenic in nature, (Ussing &
Zerahn, 1951); in this the active transport of sodium was
considered to be equivalent to a current passing through a
resistance.

The third possibility for the source of the potential
difference was described by Lund (1947). It was suggested it
may involve simply electron translocation (pH independent) or

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hydrogen transfer as well as electron translocation (pH dependent) and is termed the redox system.

Electronic conduction in non-metallic media has been confirmed, by Brillouin (1962), and has been demonstrated in a large number of biological macromolecules. Crane, Davies & Longmuir (1948) suggested that the ferrous/ferric (Fe^{++}/Fe^{+++}) system, redox type, could be involved in hydrochloric acid production. The mechanism would remove electrons from the hydrogen ion on the mucosal side of the oxyntic cells; and the potential difference would be at a maximum at rest, and fall during secretion, which does occur. They have also suggested that the resonance of the imidazole ring of histamine enables it to act as a hydrogen ion carrier in the electron transport system; which could possibily account for the stimulation of acid secretion caused by histamine.

There are also essentially three ways in which an ion can be transported against its electrochemical gradient. First, it can be accompanied by an ion of opposite sign, in combination with a single carrier, which is termed a unitary mechanism. A second possible mechanism is a forced carrier exchange between a given ion and one of the same sign moving in the opposite direction, as in the frog skin; both non-electrogenic mechanisms; and thirdly, one in which there is a net transport of charge,across the membrane, at the transport site. This type is referred to as an electrogenic mechanism, and, because

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of conditions of electro-neutrality there must be some electrical coupling between the electrogenic mechanism and ion transport at other sites in the membrane.

It is theoretically possible that an actual transport process may involve more than one type of mechanism. The hydrochloric acid secretory mechanism could be partially unitary, and partially electrogenic; the degree of electrogenicity of a mechanism, for a given ion, being defined as that fraction of the total transport of the given ion that results from the transport of charge of that ion. The degree of electrogenicity could also vary depending on experimental conditions or on species differences. Hogben's (1959) studies on dogfish indicate that the hydrochloric acid mechanism of this species is primarily non-electrogenic, whilst in sharp contrast are the studies of Rehm (1963) on the frog Rana pipiens and Rana esculenta, which show that, under certain conditions, the hydrogen ion secretory mechanism is 100% electrogenic. The electromotive force of the chloride 'pump' tends to make the serosal surface positive with respect to the mucosal, and the electromotive force of the hydrogen ion 'pump' tends to make the mucosa positive. The maximum E.M.F. is obtained when the stomach is in the resting state as only the chloride 'pump' is operative. Commencement of acid secretion tends to lower the potential difference: (experimentally demonstrated.) The observed fact that the rate of acid

secretion is increased by passing current from serosa to mucosa
is predicted by Rehm's separate site mechanism theory. The
relationship between acid secretion and resistance across the
mucosa, resistance decreasing as secretion increased, cannot be
predicted on the basis of a non-electrogenic mechanism: (Rehm,
1965.). In the separate site theory, because of the restriction
of electro-neutrality, in the absence of an applied current;
there must be electrical coupling between the two 'pumps', so
that an equal number of negative and positive charges are
transported across the lumen.

The 'pumps' must also be coupled in a biochemical sense,
since both require metabolic energy. Rehm has shown (1962,
1963) that the metabolic coupling is more than a simple
dependency on a common energy source. In sulphate Ringer, with
the potential difference clamped to the same level as in the
chloride Ringer solutions, the hydrogen ion secretion rate is
about one third of that in the chloride solutions. If the
hydrogen ion secretion rate depended only on the transmucosal
E.M.F. (produced by chloride transport) it should be identical
under the two conditions.

The present theories for gastric secretion of acid are
based on two main concepts. One school of thought favours the
idea of a 'redox process' involving the Flavine enzyme, F, and
a cytochrome chain. The reactions involved are seen as:

$$FH_2 + 2cyt.^{+++} = F + 2cyt.^{++} + 2H^+.$$
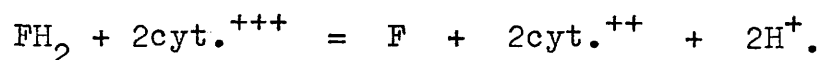
This type of mechanism has been suggested by Crane & Davies, (1948), Conway & Brady,(1950), and Rehm, (1950); and has received more recent support from Robertson, (1960) and Bannister (1965, 1966).

The other school suggests that adenosine triphosphate (A.T.P.) is the main energy source (Forte, 1963 & 1964) and the presence of an adenosine triphosphatase (A.T.P.-ase) stimulated by chloride has been described by Kasbekar & Durbin (1965).

Davies & Ogston (1950) suggested a redox mechanism coupled to a transfer process involving A.T.P., and more recently Kidder III et al. (1966) produced experiments which indicated that acid secretion was closely associated with a process which caused cytochrome 'c' to shift to a more reduced state, but as A.T.P. was also required, suggested that both an intact electron transport system and oxidative phosphorylation were required for the hydrogen ion transport process.

Hogben (1951) did suggest a theory involving carbonic anhydrase, of which a large supply is present in the stomach. He suggested that the hydrogen ion was actively secreted indirectly by the active transport of bicarbonate from mucosa to serosa with chloride exchanging for bicarbonate on a carrier system. The potential difference observed was said to be due to a passive diffusion of bicarbonate ion from serosa to mucosa. Rehm (personal communication to Heinz & Obrink, 1954) showed

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that the potential difference was insensitive to the changes in mucosal bicarbonate concentration. Good reviews of these theories have been given by Heinz & Obrink (1954) and Conway (1958).

A.T.P., which was discovered by Lohmann in 1929 and first synthesized by Lord Todd twenty years later; is thought to be the main storage source for immediate energy required by a living system. Hydrolysis of the two terminal phosphate bonds can release energy to the value of 11,000 cals/mol., for each bond; and were termed 'energy rich' bonds by Lipmann (1941); and are usually denoted by the symbol $\sim$: ie. R - 0 -(P)$\sim$(P)$\sim$(P). denotes the type of bonding found in A.T.P. When a cell needs free energy for any purpose it draws on its store of A.T.P.; and the adenosine diphosphate (A.D.P.) formed is ready to accept $\sim$(P) again. The reaction

$$A.D.P. + P_i \rightleftharpoons A.T.P.$$
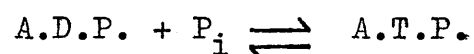
is readily reversible. The free energy available from A.T.P. is regarded as the driving force behind the chemical reactions in every living cell, and is under the immediate control of enzymes In the majority of living systems the ultimate controllers are the respiratory enzymes; the existance of which was first demonstrated by Harden & Young in 1904 and their importance not fully apparant until the work of Meyerhof, and Von Euler & Myrbäck in the 1920's. The enzyme, initially called Co-enzyme I,

then Diphosphopyridine nucleotide, and now nicotinamide adenine dinucleotide (N.A.D.), was shown to act as a 'universal aunt' to a family of dehydrogenases, and cause the oxidation of such substrates as lactate. Co-enzyme II, or nicotinamide adenine dinucleotide phosphate (N.A.D.P.), found later by Warburg & Christian (1932), behaved in a similar way for other substrates.

The active part of these compounds is the nicotinamide residue, which behaves as a weak quarternary base and is readily reduced and oxidized. One hydrogen ion enters the pyridine ring at position 4; and the other can be regarded as being split into a proton and an electron; the latter pairing off with the nitrogen ion (N^+) in the ring; the proton attaching itself to the oxygen ion (O^-) of the phosphoric acid residue. The overall reaction is usually written as

$$N.A.D. + H_2 \rightleftharpoons N.A.D.H_2.$$
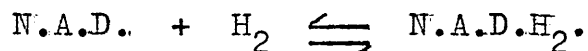
Pioneer work on another group of enzymes, the flavoproteins, was done by Warburg, but a more detailed knowledge of the chemical behaviour dates from 1933-35 from Kuhn & Kauer, The yellow colour of these compounds, flavine adenine dinucleotide (F.A.D.) and flavine mononucleotide (F.M.N.), is due to 6:7 dimethyl-alloxazine, which is the active part and is easily oxidized and reduced. Some flavoproteins react directly with oxygen; $FP.H_2 + O_2 \rightleftharpoons FP + H_2O_2$, ie. aerobic dehydrogenases; and others, ie. F.A.D. remove the hydrogen ion

(H^+) from N.A.D.H_2. and N.A.D.P.H_2. and are anaerobic
dehydrogenases which cannot act directly with molecular oxygen,
but must go via the cytochrome chain.

The cytochrome chain consists of a series of proteins which
contain iron and have the property of being readily oxidized
and reduced. Keilin during the period from 1925 to 1959 enabled
identification of the various cytochromes to be made by their
absorption spectra; and distinguished them by the letters 'a',
'b' and 'c'. The fact that cytochromes have been identified in
all living cells except those of anaerobic bacteria, which will
not grow in the presence of even a trace of oxygen, suggests
that these pigments are concerned with oxidation reactions; but
they do act only as electron carriers and not as H^+ ion
carriers, like N.A.D. or N.A.D.P. Considered as a whole series
of oxidation reactions, the system is as follows: Initially,
with N.A.D. and N.A.D.P. and the flavoproteins, the hydrogen is
actually transferred and enters the pyridine and iso-alloxazine
rings; then for the next step the hydrogen can be regarded as
splitting into protons and electrons, and the electrons reduce
the cytochrome 'c' from the ferric to the ferrous state; and as
cytochrome 'c' can only accept one electron at a time it is
reduced and oxidized twice as each pair of hydrogen atoms pass
along the chain. Cytochrome oxidase (a_3) finally causes the
electrons to unite with oxygen, one at a time to form water.

metabolite
oxidized

$$\text{oxidized}\bigg) \Bigg(\begin{array}{c} _nN.A.D.H_2 \\ N.A.D. \end{array}\Bigg) \Bigg(\begin{array}{c} _nF.P.H_2 \\ F.P. \end{array}\Bigg) \Bigg(\begin{array}{c} _ncyt.'c'\ Fe^{++} \\ cyt.'c'\ Fe^{+++} \end{array}\Bigg)\Bigg(\begin{array}{c} _nH_2O \\ \tfrac{1}{2}O_2 \end{array}\Bigg)$$

metabolite
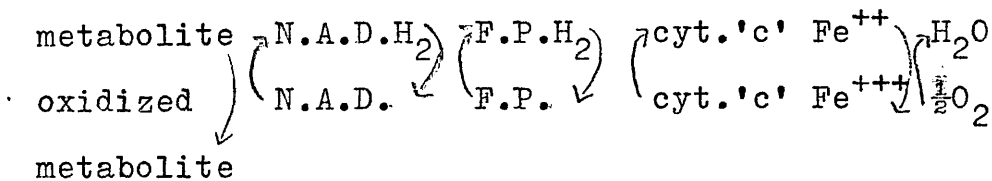
When electron (or hydrogen ion) transport occurs in normal living cells there is a simultaneous phosphorylation of A.D.P. to A.T.P.. This process called oxidative phosphorylation has been demonstrated by carefully controlled experiments by H.M. Kalckar, S. Ochoa, V. A. Balitoser, Lipmann, Hardy and Lehninger, among others. Their experiments show that when a metabolite,like lactic acid, is oxidized, three energy rich phosphate bonds; ie. three molecules of A.T.P. from A.D.P. and inorganic phosphate; are formed for each pair of hydrogen ions passed up the chain to oxygen. This finding is commonly expressed as the phosphorous/oxygen ratio; ie. P/O = 3. Some metabolites such as succinate yield a P/O of 2.

The sites of formation of A.T.P. occur when there is a large change in energy between the reduced and oxidized products. These sites have been calculated and experimentally demonstrated to be:

1. $N.A.D.H_2$ + FP $\longrightarrow$ N.A.D. + FPH_2. with antimycin A as inhibitor, by Copenhaver & Lardy, (1952).

2. FPH_2 + cyt.'c'Fe^{+++} $\longrightarrow$ FP + cyt.'c'Fe^{++}. by Slater, (1955) with cytochrome 'c' as the hydrogen acceptor.

3. cyt.'c'Fe^{++} + $\tfrac{1}{2}O_2$ $\longrightarrow$ cyt.'c'Fe^{+++} + H_2O. Lehninger, (1954).

The energy change in each case is more than enough to generate a pyrophosphate bond of about 8,000 cal./mol.. The system is usually called the respiratory chain phosphorylation. The mechanism is still obscure but can be symbolically displayed as:

(a) $X-OH + P_i \rightleftharpoons X-O-\textcircled{P} + H_2O$

(b) $X-O-P - 2e^- \rightleftharpoons X^{++}-O \sim \textcircled{P}$

(c) $X^{++}-O \sim \textcircled{P} + A.D.P. \rightleftharpoons A.T.P. + X^{++}OH$

(d) $X^{++}OH + 2e^- \rightleftharpoons X - OH$
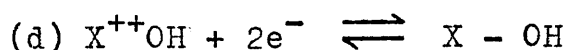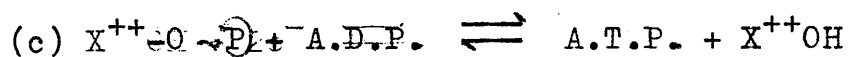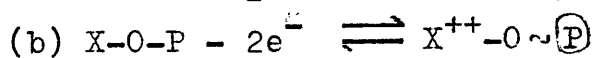
The majority of the present knowledge on sources of energy and mode of utilization for the work processes carried out in living cells, has come from a careful study of the effects of metabolic inhibitors: (amytal and rotonone, (Chance & Hollander, 1963); oligomycin, (Lardy, Johnson & McMurray, 1958, and Ernster & Lee, 1964); dinitrophenol, (Lardy, 1945); iodoacetate, (Racker, 1965); fluoride, (Warburg & Christian, 1941)): isotopic tracers, especially ^{14}C; and analysis of build-up products, (Krebs, Knoop & Szent Györgyi). A good general review of this topic has been presented by Racker, (1965).

The present uncertainty about the actual mechanism of oxidative phosphorylation, despite rapid progress in the elucidation of the steps involved by Chance, Lehninger, Slater and others; makes the interpretation of some of the results obtained by the use of metabolic inhibitors, difficult. In some tissues, where ions such as sodium and potassium are

actively transported and isolation of a Na^+/K^+ stimulated
A.T.P.-ase has been made (ie. Skou, (1957) in nerve; and Glynn,
(1962) in red-cell membranes); dependence of the active
transport process on A.T.P. seems to be more or less
demonstrated. One main point in favour of this view is the
inhibitory effect, on both active sodium transport and on the
isolated A.T.P.-ase, of cardiac glycosides. A good review on
this subject is one by Glynn (1965).

However in the case of active transport of other ions, in
particular of the hydrogen ion, analysis of the results is not
so straight forward. / The basis of an oxidation system is
removal of hydrogen atoms from the substrate; and the initial
respiratory enzymes (ie. N.A.D. & F.A.D.) actually combine with
the hydrogen atoms, but the cytochromes react with only the
electron so that separation of the electron from the hydrogen
ion is assumed to occur, over a small distance. The likelihood
of the hydrogen ion being able to diffuse through the lipoid
structure of the membrane as quickly as the electron will move
down the cytochrome chain, is slight. This could result in
hydrogen ions on one side of the membrane and hydroxyl ions
being produced, when the electron is passed to oxygen, in the
presence of water, on the other. The membrane thickness required
for this process is small, about $75^{\circ}A$ (that in gastric secretory
cells is about $100^{\circ}A$). Such a separation in the right membrane

structure, would result in a separation of hydrogen from hydroxyl ions without the direct intervention of an energy rich phosphate. Coupling between oxidation and phosphorylation can be broken by 2.4.dinitrophenol (D.N.P.) and also by azide. Phosphorylation ceases but oxidation is unaffected.

As both these compounds inhibit gastric acid secretion, this is one of the main points upon which the requirement, for acid secretion, of a high energy phosphate bond material (ie.A.T.P.) is based. However the proponents of the redox theory argue that inhibition by D.N.P. does not necessarily mean a requirement for A.T.P.; and propose a hypothesis for the mode of action of D.N.P. which could cause inhibition of acid secretion, although it is formed by a redox process. As outlined by Robertson (1960), D.N.P. could leave oxygen uptake unaffected, but prevent the separation of positive and negative charges. Both acid secretion and the formation of A.T.P. would be inhibited on the basis that separation of positive and negative charges leads either to acid secretion and A.T.P. formation; or acid secretion, or A.T.P. formation.

In the gastric mucosa the hydrochloric acid secretion occurs against a large electrochemical gradient, and in the mammalian forms, the secretory product can achieve an acidity as high as 160mN from a nutrient solution of pH 7.4. The energy required for acid secretion can be estimated from the

concentration gradients and the transmucosal potential

difference. The minimum free energy required for secretion of an

ion is given by

$$\triangle G = nRT \ln. \frac{a_1}{a_2} + nzFE.$$

n = number of gram ions per mole.

a_1= the activities of the secretory product.

a_2= the activities of the nutrient product.

z = net charge of the ion.

E = the potential difference between the solutions.

R = the gas constant $(ergs/mol/^\circ A)$.

F = Faraday unit (96500 coulombs).

T = the absolute temperature.

The estimated requirement is approximately 10,000 cals/g.mole.

or 1,600 cals/litre of gastric juice; this is the minimum free

energy needed if the system was 100% efficient, which is

unlikely. The gastric mucosa of the frog needs 8,800 cals/mol.

for secretion of 0.12N hydrochlofic acid; simultaneous

measurements of the rate of acid secretion and oxygen

consumption give approximately 2 moles of hydrochloric acid

per mole of oxygen consumed by the tissue. (Crane & Davies,

1951; Davenport, 1952; Forte & Davies, 1964; Teorell, 1949.)

Since the complete oxidation of glucose yields about 114,000

cals. per mole of oxygen utilized; there is an adequate amount

of energy in the system to account for the observed rate of

acid secretion.

If an oxidation – reduction scheme, as proposed by Conway (1948), Crane & Davies (1951) and Bannister (1965) provides the basis for the operation of the hydrogen ion mechanism, the number of univalent ions transported per molecule of oxygen utilized, is limited to 4.0; the electrochemical equivalent of molecular oxygen. Various workers have made attempts to measure the uptake of oxygen due to acid secretion alone; and not to the whole mucosa as the values quoted above. Crane & Davies (1951) made bags of the amphibian stomach and measured the change·in oxygen uptake before and after histamine was added. The species used did not secrete acid before addition of histamine. They found, in the majority of cases, that the ratio of the entire rate of acid secretion after histamine, to the change in oxygen consumption, was above 4.0. Forte & Davies (1963, 1964) using both total oxygen consumption and change in oxygen consumption, associated with reversible inhibition of acid secretion by thiocyanate, also obtained values for $\dfrac{Q_{HCl}}{Q_{O_2}}$ of above 4.0.

However Davenport (1952) observed a linear correlation between acid secretion and oxygen uptake in a large number of experiments using sacs of frog gastric mucosa. The coefficient of regression of acid secretion on oxygen uptake, which was considered to give the mean value of the ratio between acid

secretion and the associated oxygen uptake, has a value below 2.15 and 99% confidence limits are 0.95 and 3.71. Experiments on whole mouse stomachs (Davenport & Chavre, 1953) gave ratios varying from 1.2 to 4.8. Detailed reviews of this field are given by Heinz & Öbrink (1954) and Conway (1958).

Recent work in support of the redox theory has been produced by Bannister (1965a & b, 1966); who used single everted sacs of frog gastric mucosa and measured oxygen uptake by a Warburg flask technique. In two-thirds of his experiments he obtained a good linear relationship between oxygen uptake and acid secretion addition of substrate did not alter the relationship; though pyruvate and glucose caused increased activity. His mean ratio was 2.3 $\pm$ 1.0 but some values were greater than 4.0. The effects of various inhibitors, and complete lack of acid secretion during anaerobic conditions supported his views on the redox mechanism.

The argument used by the proponents of the redox theory to explain the results of ratios in excess of 4.0, is that oxygen consumption in the resting state is not identical with the non-acid producing oxygen consumption; a fact assumed by these workers: (Crane & Davies, 1951). Bannister (1965a) presents evidence for a change in the resting rate of oxygen consumption in non-acid secreting and acid secreting mucosa.

Forte, Adams & Davies (1965) studied the relationship

between phosphate metabolism and acid secretion. Bullfrog gastric mucosae were divided in half and one part kept as a control while the other half was subjected to various procedures during which the rate of acid secretion was measured. When the nucleotide concentrations were measured in both control and experimental tissue, a linear correlation ($r = 0.78$) existed between the rate of acid secretion and the level of A.T.P. in the mucosal tissue. Anoxia decreased the rate of acid secretion, but still a significant rate was measurable after 60 minutes in oxygen-free solution, and anaerobically produced A.T.P. seemed able to maintain some acid secretion until levels fell below $0.4 - 0.5\mu g$/gram of tissue. Inhibition of anaerobic glycolysis by iodoacetate caused acid secretion to fall to zero and tissue A.T.P. to fall to very low levels; thus it was concluded that A.T.P. from anaerobic sources could maintain acid secretion, though at much reduced rates. On re-oxygenation of anaerobic mucosae, acid secretion only returned when the A.T.P. concentration was restored. Thiocyanate (SCN^-) caused 90% inhibition of acid secretion, even though the A.T.P. levels were still high, thus they postulated that SCN^- affected the acid secreting mechanism at some point distal to the production of energy metabolites. Forte estimated that at least 1.5Eq. of hydrogen ions could be produced from one mole of A.T.P..

Kasbekar & Durbin (1965) in support of the direct

involvement of A.T.P. in acid secretion, isolated a membrane-
bound A.T.P.-ase by differential centrifugation from frog gastric
mucosa. The enzyme was neither stimulated by sodium and
potassium, nor inhibited by cardiac glycosides, but possessed
properties which seemed to link it with acid secretion. The
halide ions, at concentrations of up to 40mM, caused slight
stimulation of activity, but higher concentrations caused
inhibition. Bromide caused the greatest degree of stimulation,
chloride the least. The bicarbonate ion caused marked
stimulation, again in concentrations up to 40mM. The thiocyanate
ion, which reversibly inhibits acid secretion in-vivo and
in-vitro, exerted similar reversible inhibition proportional to
concentration, on the gastric A.T.P.-ase preparation. These
results were confirmed by Sachs, Mitch & Hirschowitz (1965) and
Forte, Forte & Bils (1965). However the thiocyanate ion has
been shown to have similar inhibitory effects on A.T.P.-ase
preparations from other tissues such as liver. There is thus
evidence in favour of both the two main theories for the
mechanism of acid secretion.

A small number of studies on electrolyte transfer across
foetal membranes have been made. Garby (1957) was unable to
show a spontaneous potential difference or electrolyte transfer
across human amniotic membrane. Wright (1959, unpublished)
obtained similar results with sheep, rabbit and human amniotic

-33-

membranes. Active transfer of calcium ions from maternal to foetal tissue was suggested by Comar (1956) on the basis of 'against the gradient' transfer; but nothing was experimentally demonstrated. However in 1960 Crawford & McCance showed that a spontaneous P.D. did exist across the chorio-allantoic membrane of pig, in-vitro, and that the S.C.C. was equivalent to the net sodium transport in the foetal to maternal direction. This active transport was inhibited by the presence of a high carbon dioxide tension and fall in pH on the foetal side; but was not affected by an extract from the neurohypophysial gland, unlike the frog skin which is stimulated; (Koeford-Johnson & Ussing, 1958). The activity was only present in the chorio-allantoic membrane, the function was abolished if the chorion was stripped. A metabolic dependence between the two membranes was suggested.

The early work of Van Puteren, relating presence of acid in its stomach to the length of the foetal rabbit, was verified and expanded by work of Wright (1962, 1962). He showed that acid was present in the foetal rabbit stomach only on and after the 23rd day of the gestation period; and a large increase occurred about the 27/28th day. Stomachs of this age would secrete acid in-vitro. These results were related to the appearance, on the 23rd day, in the gastric mucosa of oxyntic cells which suddenly became more numerous on the 27th day; (Menzies, 1958). Prior to

this only undifferentiated cells were present. Wright demonstrated, using a modified Ussing technique, that before the 23rd day the P.D. across the stomach wall, serosa positive with respect to mucosa, was wholly dependent on the presence of sodium ions in the mucosal bathing fluid. After the 23rd day, until birth, removal of the mucosal sodium ions and replacing with choline chloride or a potassium solution caused a decrease in the P.D. and S.C.C. but left a residual fraction of about 20-30%. From chemical analysis of the mucosal solution in the whole stomach, used in-vitro, a net transport of sodium from mucosa to serosa, chloride from serosa to mucosa, and hydrogen ions from serosa to mucosa was demonstrated. No net transport of potassium or bicarbonate ions was observed. The result could be explained on the basis of active transport of sodium from mucosa to serosa accounting for the major part of the current and the sodium independent fraction on the basis of an active transport of chloride ions from serosa to mucosa; which would agree with the chemical analysis. From the little work that has been done on the metabolism of foetal tissues, it appears that the foetus can survive in an anoxic condition for a longer period of time than the adult. Whittam (1960) and Capék (1961) have shown that foetal kidney can utilize energy derived from anaerobic metabolism to maintain the mineral integrity of the cells.

The presence of glycogen in foetal tissues was first demonstrated by Claud Bernard (1859) and he stated that it was probably of importance in the development of the organs. However, after more accurate methods for analysis were developed, Needham (1931) stated; 'This belief died out when it was found that glycogen is not present in embryonic tissues to a greater extent than in adult ones'. Shelley (1961) reviewed this subject of the presence and importance of glycogen in foetal tissues. Under anoxic conditions it was found that cardiac and brain glycogen fell to 20%, liver also fell but skeletal and lung glycogen were unchanged. There was a close correlation between cardiac glycogen and the ability of the foetal animal to survive anoxia.

The present work was carried out as an extension of the previous work by Wright; and in order to obtain quantitative data relating to Wright's hypothesis that in the foetal stomach there is an active sodium transfer from mucosa to serosa and an independent chloride transfer in the opposite direction. It was also desired to study the metabolic factors on which the ionic movements and potential difference depended and to see whether these were typical of adult stomachs or were in any way specialised to foetal conditions.

CHAPTER II.

IDENTIFICATION OF THE SHORT-CIRCUIT CURRENT

BY THE USE OF ISOTOPIC TRACERS.

CHAPTER II

<u>OPERATIVE PROCEDURE</u> .

Adult rabbits were mated overnight, and conception known to within 12 hours. The foetus used were aged from 24 to 31 days gestation. The foetus were taken from the mother by Caesarian section, decapitated, and the whole stomach rapidly removed and washed with ice-cold Krebs' Ringer; and they were stored in Krebs' bicarbonate Ringer solution, at $2^{\circ}C$. Under these conditions the stomachs were usable for experiment for up to five days after removal from the mother. The same order of potential difference and short-circuit current was obtainable from the stomachs whether they were used immediately or stored for five days; but in the case of the stored stomachs, a greater period of time was required to obtain the maximum potential. Fresh tissues reached maximum potential difference after 5 to 15 minutes, whereas one stored for five days could take up to six hours.

<u>MOUNTING PROCEDURE.</u>

The stomachs were cut through the cephalic surface and opened out to form a flat membrane, the <u>in-vivo</u> contents being washed away with a Krebs' Ringer solution. The piece of washed stomach wall was sandwiched between two perspex chambers, based on the design used by Ussing & Zerahn (1951), so that a membrane with a useful area of $0.6cm^{2}$ was obtained. The volume
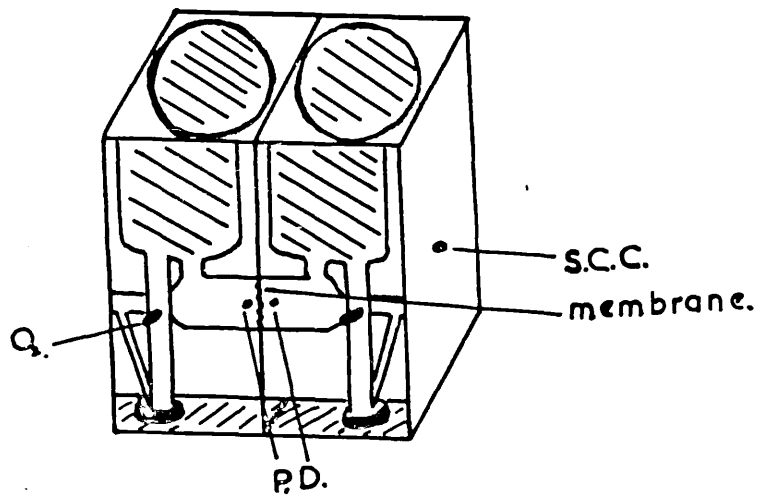
Fig.1.   Showing the perspex chambers between which the
stomach membrane was clamped. See text for details.

of each chamber was 5mls.(Fig.1). The chambers were provided

with an oxygen lift which gassed and stirred the solutions.

Holes were drilled into the chambers in order that salt-bridges;

thin polythene tubes filled with 3M KCl in 2% agar, could be

inserted with their tips as close as possible to the membrane.

The other ends of these bridges were dipped into a pair of

calomel electrodes, connected to a Vibron 33B electrometer,

which was used to measure the potential difference (P.D.) across

the membrane. Both the salt-bridges and the electrodes were

checked so that the residual P.D., if any, could be recorded.

A second pair of holes was drilled in the chambers so that two

other salt-bridges could be inserted with their tips lying

along the normal to the plane of the membrane. These bridges

were connected with a pair of silver/silver chloride electrodes

which connected with a circuit for passing current through the

system. These two pairs of electrodes enabled the short-circuit

current, (S.C.C.), direct current (D.C.), resistance and the

open-circuit P.D. to be measured. The chambers were placed in

a water-bath containing an aquarium heater, thermostat and a

stirrer. The temperature of the bath was adjusted so that the

solutions in the chamber maintained a constant temperature of

$35^{\circ}C$.

SOLUTIONS

The Krebs' bicarbonate Ringer had the following

composition (mM): Na^+ 135.7, Cl^- 132.2, K^+ 14.9, Ca^{++} 3.5,

HCO_3^- 25.3, glucose 24.0. Sodium-free solution was 154mM

choline chloride with 24mM glucose.

Measurement of the Sodium Chloride Transport.

The net transport was obtained as the difference between

the two absolute fluxes across the membrane. In each experiment

the absolute sodium flux from mucosal to serosal surface was

measured first. Known volumes of Krebs' bicarbonate Ringer were

dispensed to the mucosal and serosal chambers and allowed to

equilibrate. Duplicate samples of the serosal solution were

taken and used as a background and blank. 20µC of ^{24}Na, as

isotonic sodium chloride were added to the mucosal chamber,

and after a few minutes for complete mixing, a sample of the

mucosal fluid was taken and diluted 1:10. After about 90-120

minutes a second pair of samples were taken from the serosal

chamber; and a second sample from the mucosal, again diluted

1:10. This enabled any evaporation loss to be accounted for.

20µC of ^{22}Na were then added to the serosal chamber, and the

serosal to mucosal flux was determined using analogous

procedures. The timing between samples was exactly the same in

each half of an experiment. The concentration of sodium in the

mucosal solution was determined by flame photometry. The samples

of radioactive solution were dried and counted, at infinite

thinness, on recessed planchettes for 1000 seconds, using a
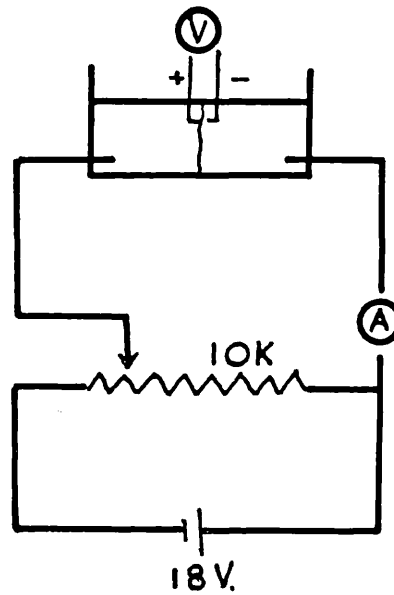
Fig.2.   Showing the circuit used for passing current and

recording the potential difference.

V   —   the potential difference across the membrane.

A   —   the recorded short-circuit current.

Geiger-Müller counter. The count rates in the weakest samples were at least five times background.

The amount of sodium transferred from mucosal to serosal solution was calculated from the equation:

$$\text{absolute flux} = [\text{Na}]_m \frac{C_s/t_s}{C_m/t_m} \cdot V_s$$

where $[\text{Na}]_m$ is the sodium concentration in the mucosal solution.

C_s/t_s is the count rate in the serosal solution,

(corrected for background and decay.)

C_m/t_m is the count rate in the mucosal solution.

V_s is the volume of the serosal solution.

The serosal to mucosal absolute flux was calculated in the same manner, but whereas the ^{24}Na samples were counted immediately, the ^{22}Na samples were counted after about three weeks when the activity due to the ^{24}Na had decayed to insignificant levels. Attempts to measure the absolute fluxes simultaneously were unsuccessful as the fluxes were too small.

The net flux of chloride across the stomach was also measured as the difference between two absolute fluxes. Ideally it should have been measured using the two chloride isotopes, ^{36}Cl and ^{38}Cl in the same stomach, as with the sodium. The use of ^{38}Cl, with its short half life (38 minutes), was impracticable as no close source of the isotope was available. Instead the experiment was carried out on a pair of stomachs

from the same litter, stored for the same length of time and having a very similar P.D. and S.C.C. agreeing to within 4%. One preparation was used for the mucosal to serosal flux, the other for the serosal to mucosal flux, using ^{36}Cl (added as 0.1M KCl) as the isotope in each case. The methods of sampling and counting were similar to those used for radioactive sodium. The serosal side was bathed in Krebs' bicarbonate Ringer, and when the mucosal solution was sodium-free, choline chloride was used.

Electrical Measurements.

Figure 2 shows the circuit used for passing the current and recording the P.D.. The integrated S.C.C. was obtained by a method described by Wright (1965). The sodium-dependent component was measured by washing the mucosal surface with choline chloride solution, and deducting the residual S.C.C. from that in the presence of sodium; (Wright, 1962). The sodium-independent component of the S.C.C. was similar prior to and immediately after measurement with sodium isotope present on the mucosal side, the values usually being identical.

When the preparation was dead, as judged by the absence of a potential difference, no diffusion potential greater than 1.0mV was observed with sodium-free solution on the mucosal side.

Choline
Chloride

160

120

$\mu A/cm^2$

80

40

0   40   80   120 mins.
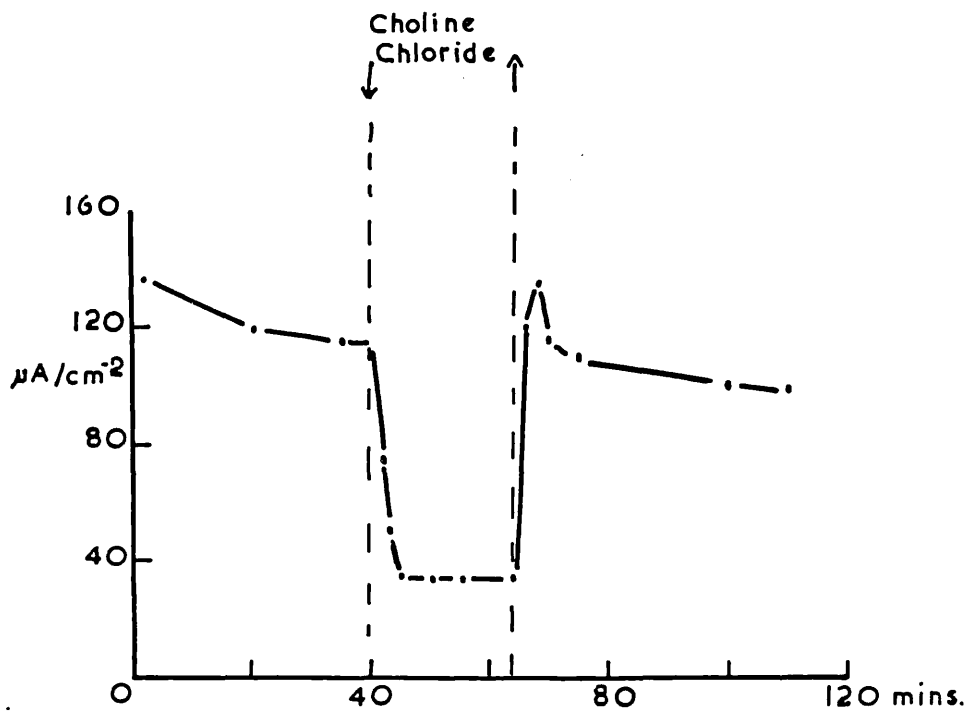
Fig.3.   The short-circuit current of a stomach from a

28 day foetus showing the dependence of the S.C.C. on the

presence of mucosal sodium.   Choline replaced the Na^+ ion

in the mucosal solution.

Table I   Na$^+$ fluxes and short-circuit current. Influx is from serosa to mucosa, efflux from mucosa to serosa. The units of flux are μEquiv.cm^{-2}hr.$^{-1}$ The short-circuit current is expressed in the same units as the fluxes, and is that part of the short-circuit current dependent on sodium being present.

Experiment	Influx	Efflux	Net Flux	S.C.C.
10.2.65	0.46	4.48	4.02	4.47
12.2.65	1.17	5.15	3.98	3.98
16.2.65	1.93	3.68	1.75	1.36
17.2.65	0.44	2.58	2.13	1.49
18.2.65	0.54	1.54	1.00	0.91
19.2.65	0.47	3.01	2.54	2.48
23.2.65	0.57	2.17	1.60	1.50
25.2.65	0.40	3.44	3.04	2.28
26.2.65	1.73	5.56	3.88	3.87
2.3.65	1.08	4.47	3.39	3.07
3.3.65	0.92	5.63	4.70	3.10
16.3.65	0.46	1.20	0.74	0.99
19.3.65	0.10	1.78	1.68	2.00

Section II

Results.

The way in which the S.C.C. is dependent on the presence
of sodium ions in the mucosal bathing solution is shown in
Fig.3. It was found that about 70% of the total S.C.C. is
reversibly dependent upon the presence of the sodium ion in the
mucosal solution; the remaining fraction appearing to be
independent. The result shown was obtained from a solution
containing chloride ions, but a chloride-free solution gave the
same effect. The overshoot was always seen but not yet explained.

The results of the 13 experiments, in which the net flux of
sodium was measured using the isotopes, are shown, along with
the sodium-dependent current, in table I. The same results are
expressed graphically in Fig.4. The regression line for this
figure is the equation

$$y = 0.98x - 0.01 \qquad P < 0.001$$

There is no significant intercept of the regression line on
either axis and the slope of the line is 45°, indicating that
the net flux of sodium from mucosa to serosa, is equal to the
sodium dependent component of the S.C.C., I_{Na}.

The net flux of chloride, determined in the absence of
sodium in the mucosal chamber, by radioactive chloride, gave
the results expressed in table II.

In this case due to the use of two separate membranes, for
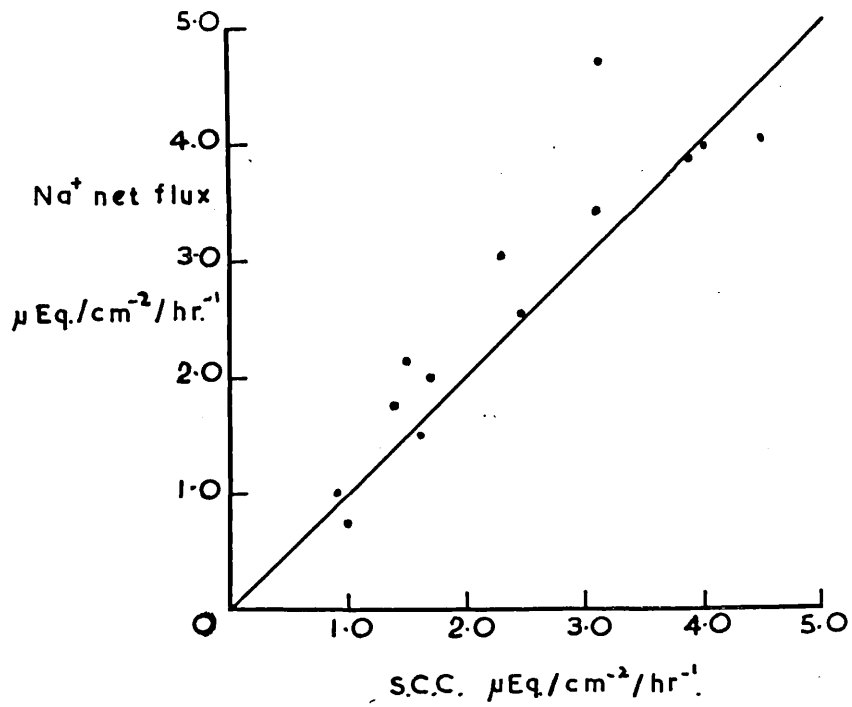
Fig.4. The relation between the net flux of sodium from mucosa to serosa and the sodium dependent short-circuit current. The equation of the regression line is
$y = 0.98x - 0.01$, $P < 0.001$.

Table II   Cl$^-$ fluxes and Na$^+$ independent short-circuit current. Fluxes and units are defined as in Table I. The net flux is defined as positive when in the direction serosa to mucosa.

Experiment	Influx	Efflux	Net Flux	S.C.C.
15.3.66	4.37	6.56	2.19	1.37
4.4.66	3.81	5.86	2.05	1.27
4.4.66	7.31	4.00	-3.31	0.64
5.4.66	5.62	7.58	1.95	1.15
6.4.66	2.08	4.91	2.83	0.69
25.4.66	5.39	8.20	2.81	0.78
26.4.66	5.94	6.95	1.01	2.14
27.4.66	6.44	7.72	1.28	2.58
28.4.66	5.95	7.81	1.86	1.89
2.5.66	9.14	6.12	-3.02	1.46
3.5.66	5.30	9.06	3.76	1.64
3.5.66	4.53	7.00	2.47	1.32
9.5.66	4.41	6.14	1.73	0.79
10.5.66	3.97	8.28	5.31	1.25
10.5.66	4.56	6.06	1.50	1.19
16.5.66	4.27	6.57	2.30	1.72
16.5.66	3.37	5.31	1.94	1.24
17.5.66	4.78	6.33	1.55	1.01
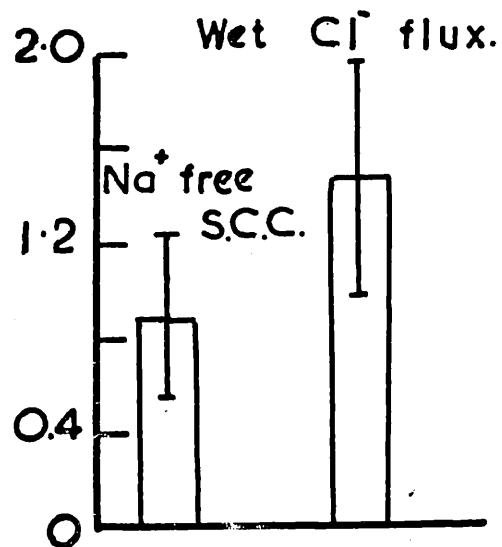
Fig.5. The left-hand column shows the mean Na^+ independent short-circuit current and S.D. for sixteen experiments. The right-hand column shows the mean net flux of Cl^- from serosa to mucosa and σ for the same sixteen experiments. The t test for related means gives t_{n-1} = 2.24, $0.01 < P < 0.025$ which shows that the difference of the means is significant.

each absolute flux determination, the results show more variation.
The overall result from these experiments indicated that the
mean net flux of chloride from serosa to mucosa was 166% of the
mean sodium independent component. The statistical significance
of this result was investigated using the t test for related
means, which gave

$$t_{n-1} = 2.24 \qquad 0.01 < P < 0.025;$$

the difference of the means was considered to be significant:
Fig.5.

Since the net flux of chloride was greater than the sodium
independent S.C.C., there must be a net movement of some other
ion. The most likely ion was hydrogen, and attempts were made
to determine to what extent the active transport of the hydrogen
ion into the lumen contributed to this component of the S.C.C..

Attempts to measure directly the rate of hydrogen ion
appearance in the mucosal chamber, using a glass electrode and
0.01N sodium hydroxide (NaOH) were unsuccessful due to the mucus
production and high degree of frothing which occurred when the
mucosal volume was reduced to give a higher surface area to
volume ratio; at which pH changes would be detectable.
Oxygenation of the serosal solution alone was not adequate.

A technique was tried to determine indirectly the
contribution of the hydrogen ion secretion to the S.C.C. by
replacing the chloride ion by an anion which would not be

Table III Effect on the Na^+ independent S.C.C. of replacing Cl^- by SO_4^{--} or glucuronate. Serosa positive to mucosa.

Experiment	Anion	P.D. mV.		S.C.C. $\mu A/cm^2$.	
		before	after	before	after
17.5.66	SO_4^{--}	5.5	17.5	30	140
18.5.66	SO_4^{--}	4.2	34.0	22	90
23.5.66	glucuronate	12.5	21.0	56	100
24.5.66	glucuronate	10.0	26.0	54	100
25.5.66	glucuronate	12.0	30.0	52	100

Mucosal solutions were Na^+ free throughout. The values of P.D. and S.C.C. are those existing immediately before replacement of Cl^- in both mucosal and serosal bathing fluids, and 2 hours after replacement.
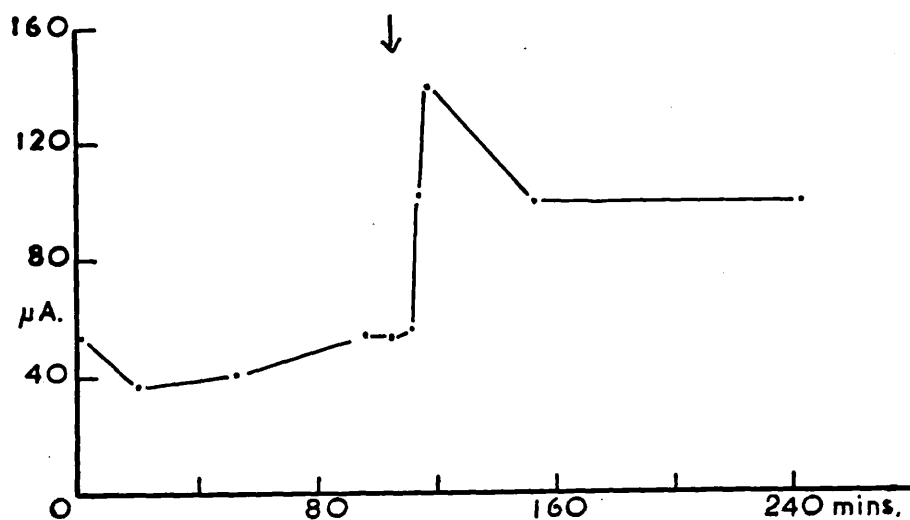
Fig.6.   The effect on the short-circuit current of a 28-day
stomach of replacing Cl^- by glucuronate in mucosal and serosal
solutions. The mucosal solution was Na^+ free at all times.
Area of membrane = $1.15cm^2$.
Replacement of Ringer solution by glucuronate solution is
shown by the arrow.

expected to be subject to active transport. Under this condition, and with sodium-free solution on the mucosal side, any active transport of hydrogen ion would result in a potential difference and S.C.C. in the reverse direction to that obtained under normal conditions; if no other ion was actively transported. Results of this nature have been obtained with frog gastric mucosa, with the chloride replaced by sulphate ions. (Rehm et al., 1963; Heinz & Durbin, 1959.) Neither of the ions used in this case, glucuronate or sulphate, produced a reversal of the gastric P.D. and S.C.C.; instead a large increase in P.D. and S.C.C. was obtained: (Kendall & Wright, 1967.) see table III and Fig.6.

CHAPTER III.

THE EFFECT OF METABOLIC INHIBITORS ON THE

SHORT-CIRCUIT CURRENT OF THE FOETAL GASTRIC MUCOSA.

CHAPTER III

Section I

The effect of all classes of metabolic inhibitors on the
S.C.C. and P.D. of the foetal stomachs was observed in an
attempt to elucidate the source of the energy required for the
active transport of the sodium ion and acid secretion. Using the
previously established fact that the total short-circuit current
is composed of a sodium dependent , and a sodium independent
fraction; the present experiments were carried out in such a
manner that the effect of the drug on the total and sodium
independent fractions was observed and the effect on the sodium
dependent fraction estimated. A change in the observed S.C.C.
and P.D. was taken to be due to the influence of the given drug;
as the tissue was always allowed to maintain a constant P.D.
and S.C.C. for at least 30 minutes before the drug was added.
Control experiments indicated that the tissue could maintain a
steady P.D. and S.C.C. over a period of 3 to 6 hours.

Method.

The tissue was mounted between two chambers as before in
Krebs' bicarbonate Ringer solution containing glucose, and well
oxygenated. The P.D. and S.C.C. were recorded at intervals
until a steady value was obtained over about 30 minutes. The
mucosal surface was washed with 154mM choline chloride plus
glucose at 24mM several times until a constant value for the

P.D. and S.C.C. were obtained, when it was desired to record
the sodium independent component. The Krebs' solution was
returned to the mucosal chamber and stable conditions once
again obtained. The drug was then added to the bathing solution,
on the serosal side, unless otherwise stated, to give a known
final concentration. Both chambers contained the same volume of
fluid, to prevent osmotic differences; and were partially covered
to prevent splashing and evaporation. The bathing solutions
were kept at $35^{o}C$ and all the drugs were brought to the same
temperature before addition. All the stomachs used in this set
of experiments were from foetuses of from 27 to 30 days
gestation period. It was not possible to use the stomachs from
younger foetuses to study sodium movement, (before the acid
producing cells were present) because their size was too small
for the present technique.

For study of the sodium independent component, after a
stable P.D. and S.C.C. had been attained in Krebs' solution, the
mucosal surface was washed several times in choline chloride,
154mM, with glucose, until a stable P.D. and S.C.C. were
observed; then a volume of choline chloride, equal to that of
the serosal Krebs' solution, was placed in the mucosal chamber,
and a short time allowed for stablization. The drug was then
added to give a known final concentration. Throughout, the
S.C.C. was recorded continuously; the P.D. intermittently.
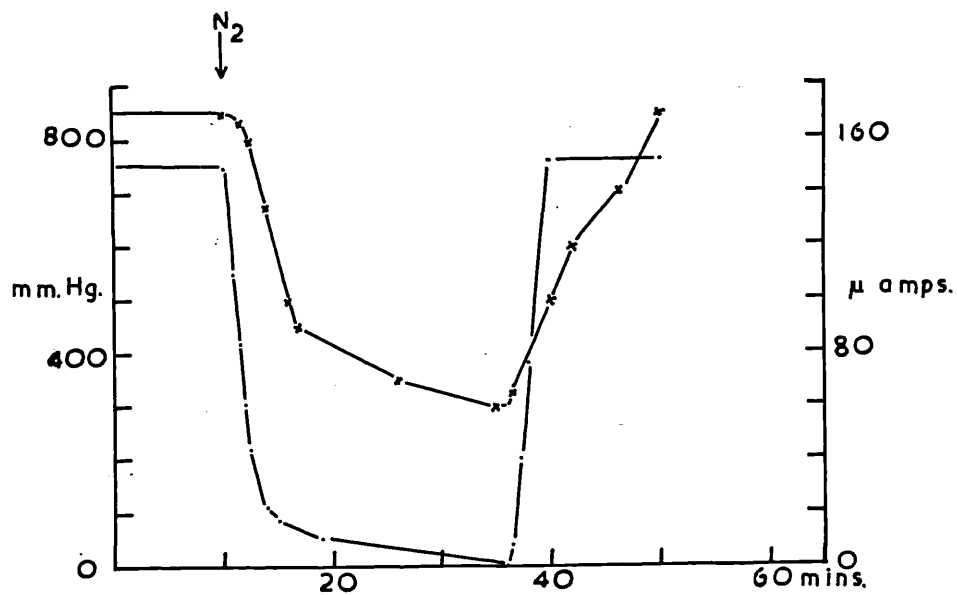
Fig.7. Showing the rate of fall of oxygen pressure in the solution and the total S.C.C. when nitrogen is bubbled through the system. Area of membrane = $0.6 cm^2$. Age, 28 days.

x —— x shows S.C.C.

o ——— o shows oxygen pressure.
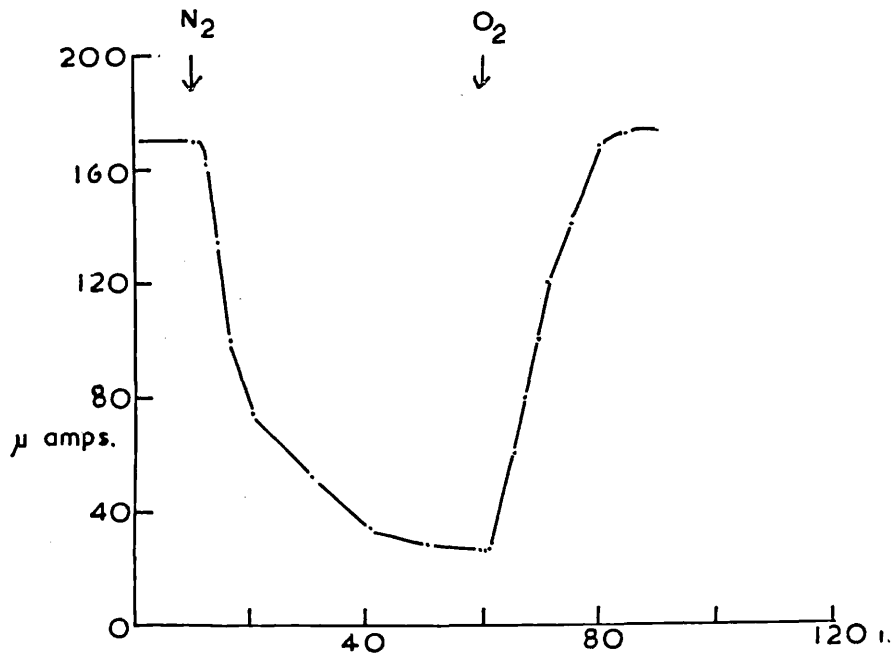
Fig.8.   The effect of prolonged anoxia on the total S.C.C. of a 28-day stomach. Area of membrane = $0.6cm^2$.

Section II

The Effect of Anoxia

(1) on the total current.

Anoxic conditions were brought about by replacing the oxygen/carbon dioxide supply with a 95% Nitrogen/5% Carbon dioxide gassing mixture. Fig.7 shows, by use of an oxygen electrode, that the oxygen pressure in the bathing solutions began to fall immediately when nitrogen was administered; and that after 5 minutes was virtually zero. The short-circuit current however began to decline only after a time lag of about 2 minutes and then it also fell rapidly; but was much slower than the fall in the oxygen pressure. Re-administration of oxygen caused an immediate rise in the oxygen pressure and the maximum value was obtained within 3-4 minutes. The short-circuit current however took a much longer time to reach its initial value. Fig.7 shows the result of an experiment designed to illustrate how quickly the gas pressures of the bathing solutions change with change of the gas mixtures bubbled through them. Fig.8 shows the effect of prolonged anoxia on the total S.C.C.. After a short time lag of 2 to 3 minutes, during which there is no change, inhibition starts to develop and 50% inhibition is obtained within 8 to 10 minutes. The total inhibition obtained was 85-90%. A return of the oxygen mixture, after a 2 to 3 minute lag, reversed the inhibition and in some

-60-
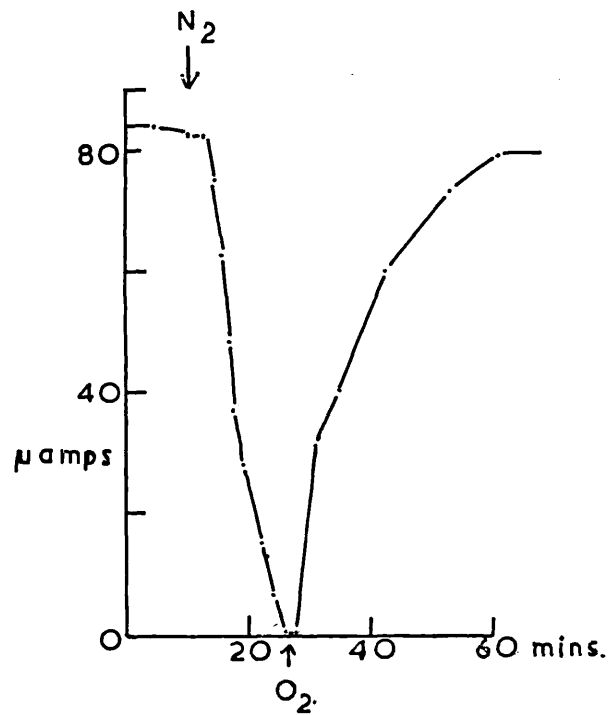
Fig.9.   The effect of anoxia on the sodium independent S.C.C.
of a stomach from a 29-day foetus. Area of membrane = 1.3cm^2.

cases a 100% recovery was recorded; and usually well over 90%.
The degree of recovery depended on the length of time the
tissue was subject to anaerobic conditions but after an hour a
good recovery was still obtainable.

(2) on the sodium independent current.

Fig.9 gives a typical result. There was an initial time lag
of 2 to 3 minutes after administration of the nitrogen/carbon
dioxide mixture, and then inhibition began to develop; 50%
inhibition was obtained within 5 minutes; and unlike the effect
on the total current, complete inhibition seemed to be obtained
but as the largest sodium-free current recorded was usually in
the order of 60 to 80μA, an error of about 5% was possible with
the recording technique used. Return of oxygen gave 100%
recovery of the sodium independent component.

As about 10-15% of the total current remains in anoxic
conditions and virtually none of the sodium independent current;
this suggests that approximately 10-15% of the sodium current
can be maintained by anoxic mechanisms.

The Effect of Amytal

(1) on the total current.

Sodium amytal was dissolved in distilled water, and added,in
volumes of 0.1ml., to give a known concentration in the bathing
solution of either 2mM or 4mM. In the majority of cases amytal
had to be added to the mucosal chamber before inhibition
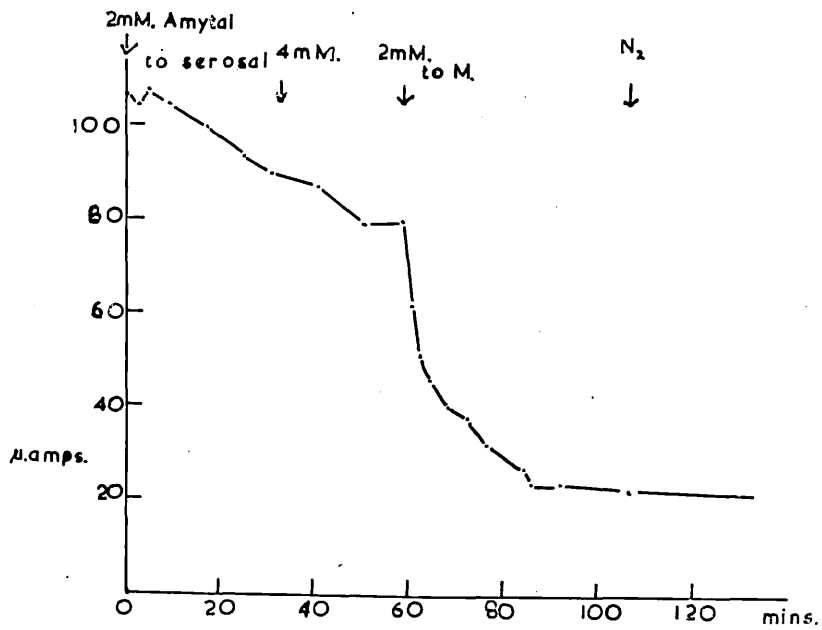
Fig.10.   The effect of Amytal on the total S.C.C. of a
stomach from a 28-day foetus. The drug was added initially
to the serosal and finally to the mucosal chamber.
Area of membrane = 0.6cm^2.

4mM.

to Mucosal
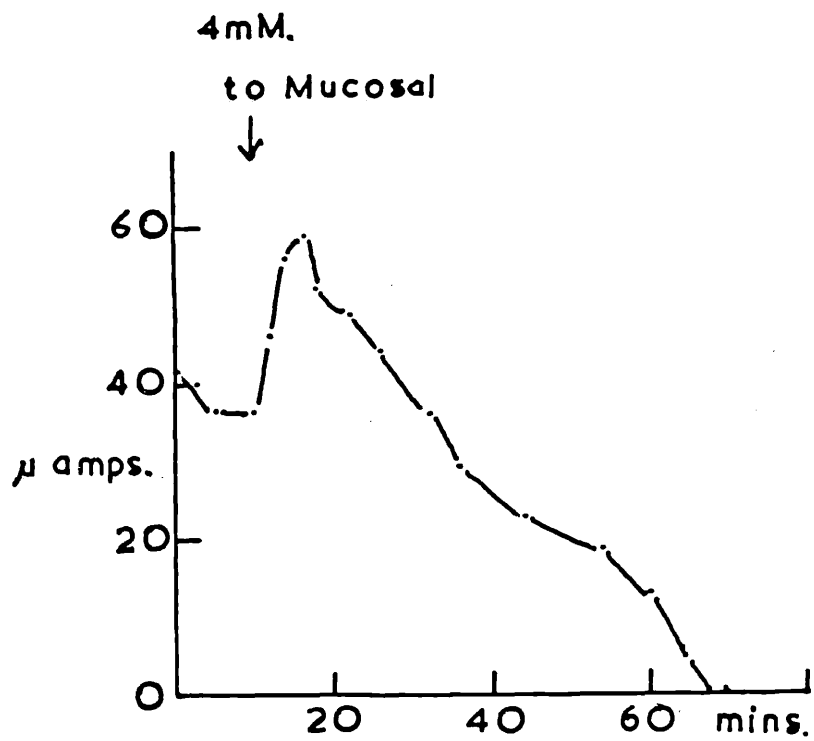
Fig.11.   Showing the effect of Amytal on the sodium
independent S.C.C.. Foetal age, 30 days. Area of membrane
= 1.3cm^2.

occurred; but in a few experiments the same degree of inhibition was obtained with the drug on the serosal side. The time lag between giving the drug and the initial fall in S.C.C. was no more than 1 or 2 minutes. No relationship between the length of storage of the tissue and this effect could be found. In the event of no effect of the drug, when in the serosal solution, the drug was left for 20 to 30 minutes.

Fig.10 shows a maximum inhibition of 80%, the value obtained in all experiments varied from 70 to 80%, the average value being 75%. 50% inhibition was achieved in 30-40 minutes; and gassing with N_2/CO_2 mixture after maximum inhibition produced very slight, if any, further decrease in the S.C.C..

(2) on the sodium independent fraction.

The drug was again more effective in the mucosal chamber. With the same concentration complete inhibition was obtained in about 50-60 minutes; and 50% inhibition in approximately 20 minutes. In most cases, as in Fig.11, a slight initial rise in S.C.C. was observed immediately on giving the drug, and it lasted for about 5 minutes.

Amytal acts on the cytochrome chain by blocking the first site of phosphorylation and inhibits respiration only when linked to phosphorylation; and has been stated (Chance & Hollunger, 1963) to exert an inhibitory action on 'energy transfer as well as electron transport'.
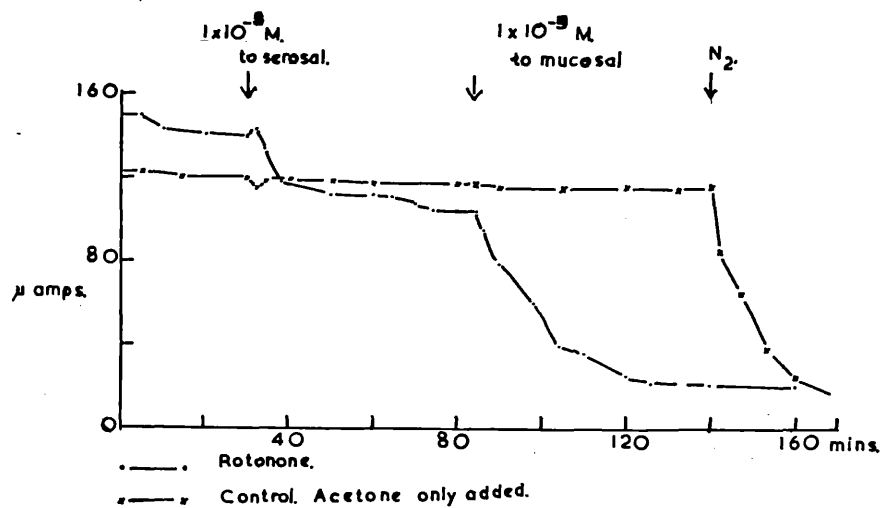
Fig.12.   The effect of Rotonone (in acetone solution) on the total S.C.C. of a stomach from a foetus aged 27/28 days. Also the effect of an equivalent concentration of acetone on the S.C.C.. Membrane area = $0.6cm^2$.

. ———— .   shows the effect of Rotonone.

x ———— x   shows the acetone control.
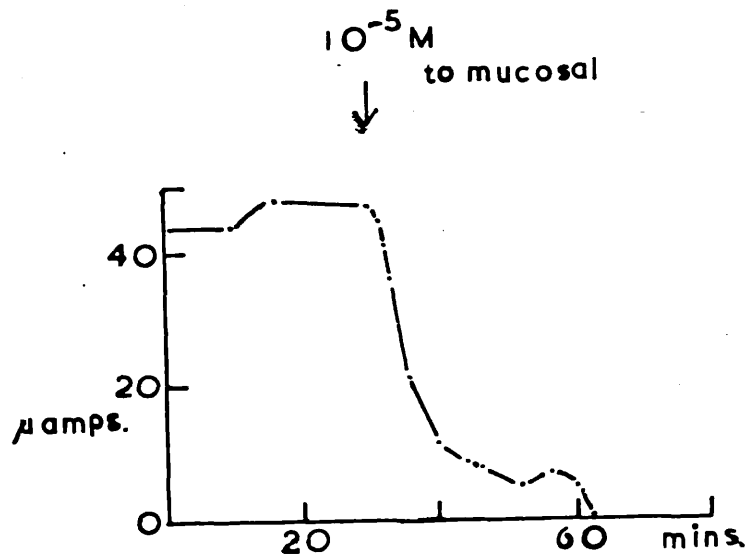
Fig.13. The effect of Rotonone on the sodium independent S.C.C.. Foetal age - 29/30 days. Membrane area = $1.3cm^2$.

The Effect of Rotonone

(1) on the total current.

Rotonone was dissolved in an acetone solution and given in 0.1ml. aliquots to give a final concentration of 1×10^{-5}M in the bathing solutions. Control experiments, adding the acetone solution only, produced a very slight effect on the S.C.C. and P.D.,and it occurred immediately. With the drug added to the serosal chamber a degree of inhibition was produced but the rate was very slow; addition to the mucosal chamber however produced rapid inhibition, 50% occurring in about 20 minutes: (Fig.12). A maximum inhibition of 85-95% was obtained which was affected only slightly by anoxic conditions.

(2) on the sodium independent current.

With the drug placed on the mucosal side, as in Fig.13, a rapid inhibition was obtained, 50% occurring within 5 minutes; and almost complete inhibition within 30 minutes.

Rotonone blocks the phosphorylation at site I in the cytochrome oxidation chain.

The Effect of Antimycin A

(1) on the total current.

Antimycin A was used in an alcoholic solution and added as 0.1ml. aliquots to give concentrations of 20 or 40μg per chamber; ie. 4 or 8μg per ml. of bathing solution. Control experiments showed that alcoholic solutions added in equivalent quantities

O·8 cc.

N₂·

150

120

80

μ.amps.

40

O   40   80   120   160 mins.

——— effect of drug. 40 μg.

•——+ control.
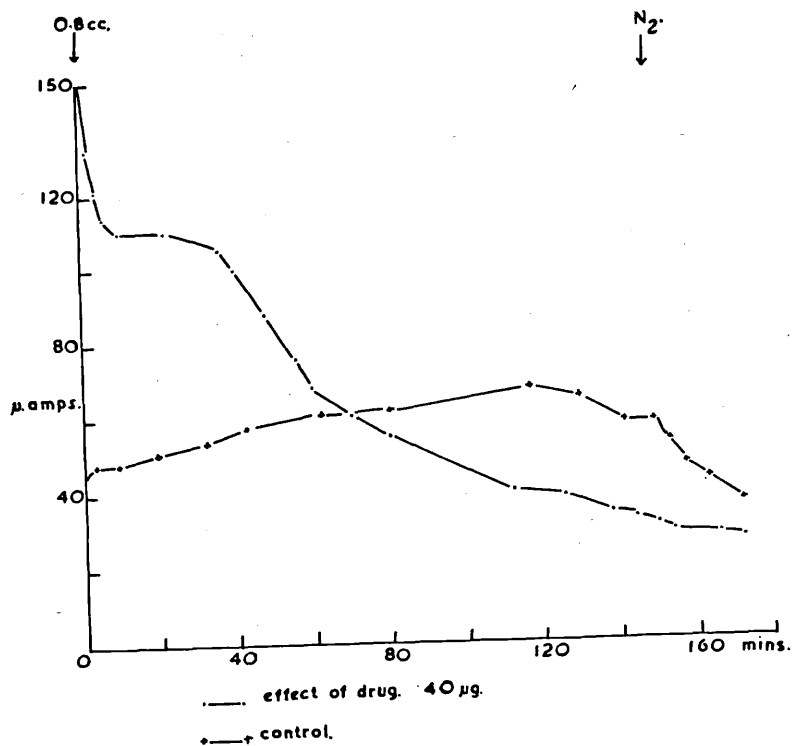
Fig.14.   Showing the effects of alcoholic solution and

Antimycin A, in alcoholic solution, on two tissues from the

same litter of 30 day foetuses. Area of membrane $= 0.6\text{cm}^2$.

o ———— o shows the effect of Antimycin A. on the total S.C.C.

x ———— x shows the effect of alcoholic solution alone.

40μg to S.   20μg to M.
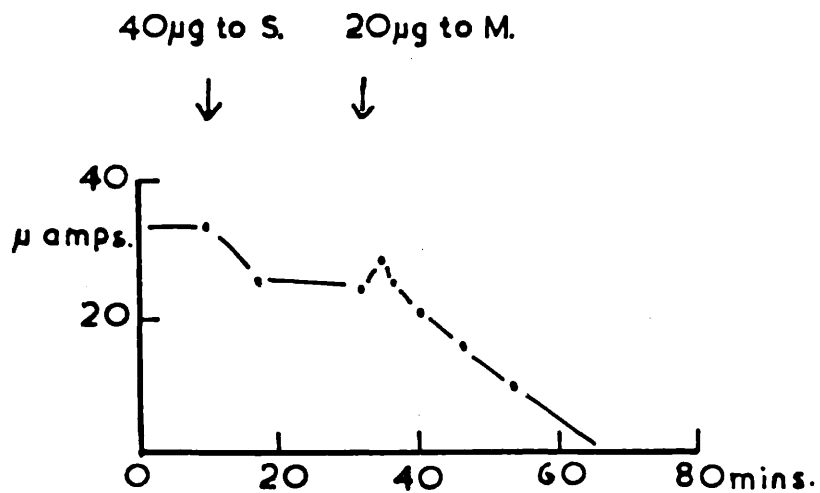
Fig.15.   The effect of Antimycin A on the sodium independent

S.C.C.. The drug was added initially to the serosal and then

to the mucosal chamber. Foetal age = 28 days.

Area of membrane = $0.6cm^2$.

caused a stimulation of the S.C.C. and P.D. over a period of

one or two hours. However addition of 35µg of antimycin A to

the serosal chamber (Fig.14) caused a rapid inhibition with 50%

inhibition occurring over a period of 35 to 45 minutes and a

maximum inhibition of 80% was obtained. Inhibition did occur

with the drug in the serosal solution, but in some cases a more

rapid decline occurred when antimycin A was placed on the

mucosal side. This could be due to the rate of diffusion of the

drug into the tissue. Stomachs from older foetuses of 30/31

days gestation period, where the muscle coat is thicker than in

stomachs from foetuses of 27/28 days, tended to need the drug

in the mucosal solution for inhibition. Anaerobic conditions

had no further effect on the degree of maximum inhibition

obtained with the drug; thus respiration had probably been

completely inhibited by the antimycin A.

(2) on the sodium independent component.

Addition of 40 or 60µg of antimycin A to the serosal chamber

had very little effect on the S.C.C.. However addition of this

amount of the drug to the mucosal side produced 50% inhibition

within 15 minutes of giving the drug; complete inhibition was

obtained: (Fig.15).

The Effect of 2.4.Dinitrophenol (D.N.P.)

(1) on the total current.

D.N.P. is a true uncoupler of oxidative phosphorylation,
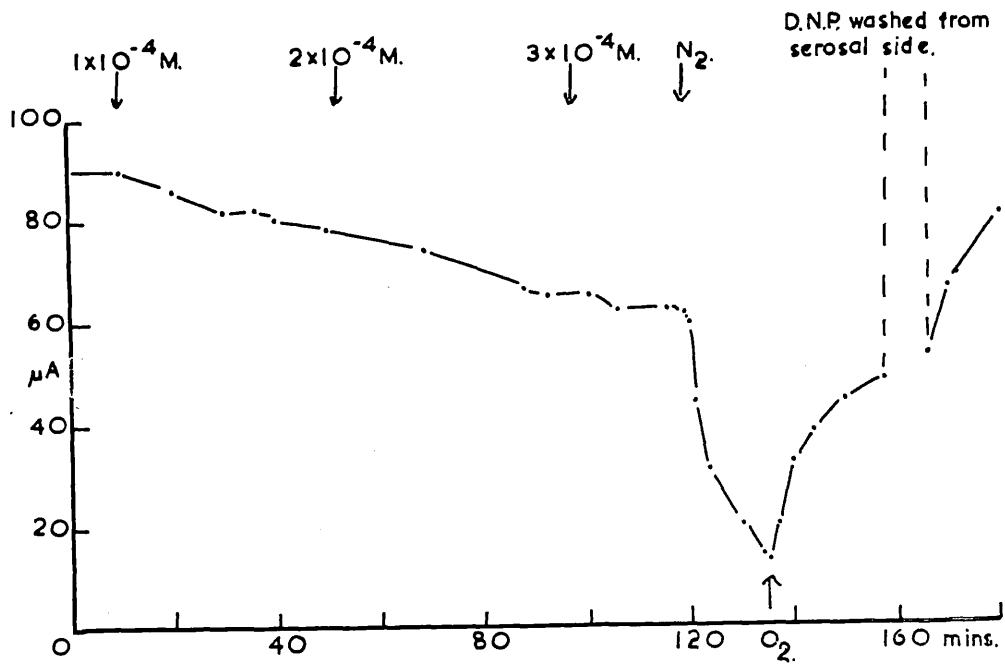
Fig.16.   Showing the effect of an aqueous solution of

Di-nitrophenol (D.N.P.) on the total S.C.C. of a stomach from

a 30-day foetus; and the recovery of the current after D.N.P.

was washed out.   Area of membrane $= 0.6 \text{cm}^2$.
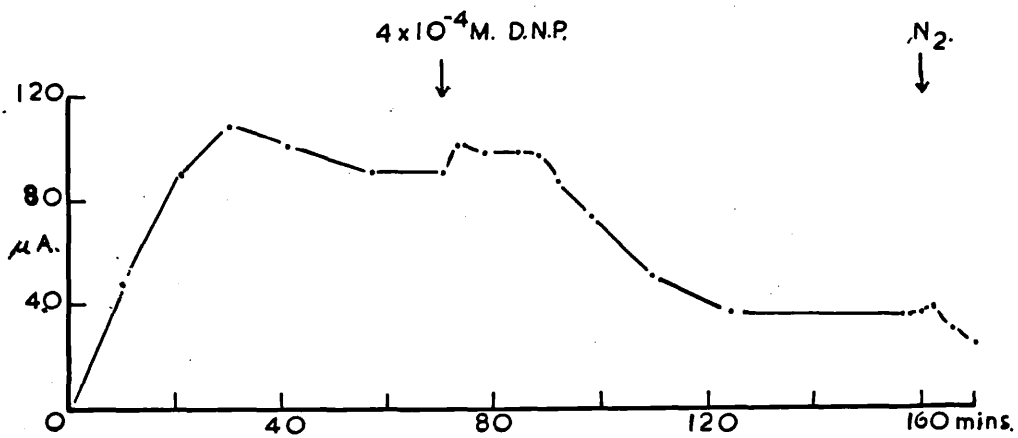
Fig.17.   The effect of Di-nitrophenol (D.N.P.) in acetone

solution on the total S.C.C.. Foetal age, 29/30 days.

Area of membrane = $1.3cm^2$.

and leaves oxidation uninhibited, usually stimulated.

The drug was initially used in aqueous solution, but did not dissolve readily and the concentration of the drug added was not accurately known. An aqueous solution of D.N.P. (Fig.16) caused a very slow rate of inhibition, less than 15% in one hour, despite increasing the concentration of the drug. Subjecting the tissue to anoxia immediately reduced the S.C.C., leaving about 15% of the initial current: and return of the oxygen returned the current to its previous value. If D.N.P. was washed out from the serosal chamber, as seen in Fig.16, the S.C.C. returns towards its initial value and the inhibition is released.

Acetone was tried as a solvent, and it was found that if the D.N.P. was initially dissolved in a little acetone, then the volume made up with water (1 part acetone to 9 parts distilled water) the drug was completely dissolved. 0.1ml aliquots of this solution were added to the serosal chamber to give a final concentration of 1×10^{-4}M. Acetone in these quantities was shown to have little effect on the S.C.C. and P.D. (Fig.12). Fig.17 shows the effect of 4×10^{-4}M D.N.P. (in acetone solution) on a 28 day stomach. Initially there was a slight increase in the current which was maintained for a period of 10-15 minutes, and then the current fell fairly rapidly giving a 50% inhibition in less than one hour.
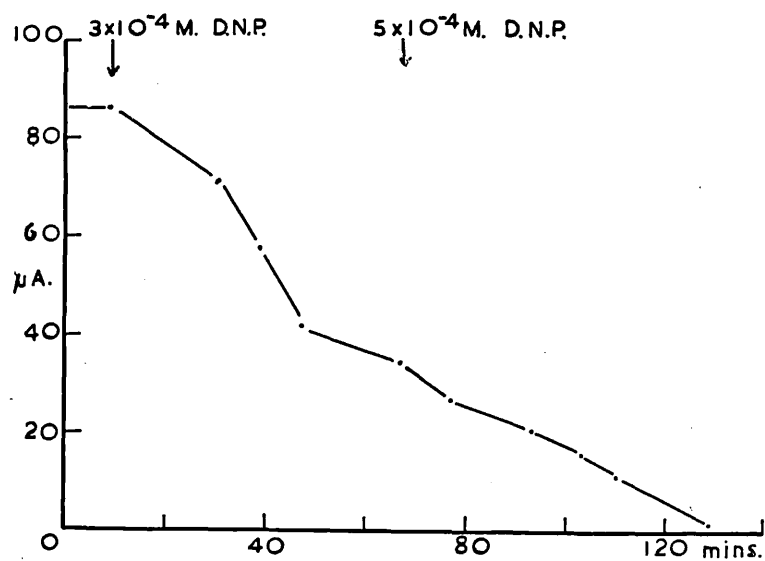
Fig.18.   The effect of D.N.P. in aqueous solution, on the

sodium independent S.C.C. of the stomach from a 30-day foetus.

Area of membrane = $1.3 \mathrm{cm}^2$.
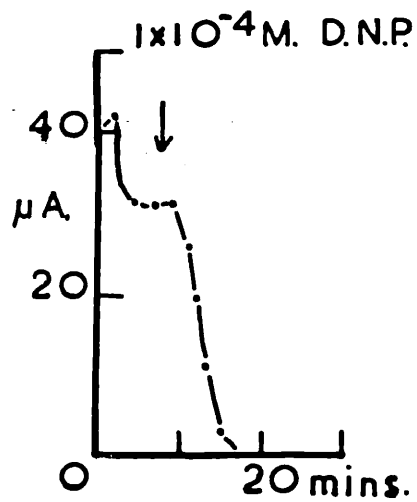
Fig. 19.   The effect of D.N.P. (in acetone solution) on the

sodium independent S.C.C. of a stomach from a 28 day foetus.

Area of membrane $= 1.3\text{cm}^2$.
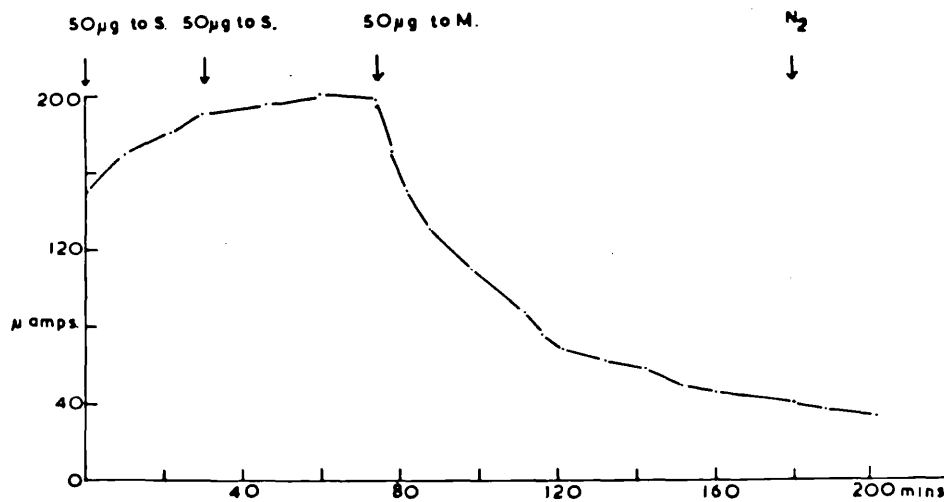
Fig.20.   The effect of Oligomycin, added to both serosal

and mucosal bathing solutions, on the total S.C.C. of a

stomach from a 28 day foetus. Area of membrane = $0.6cm^2$.

Approximately 20 to 30% of the initial current remained after maximum inhibition had occurred. Passing nitrogen through the bathing solutions caused a further reduction in the S.C.C., leaving about 15 to 20% of the initial current. The resistance of the tissue was increased after addition of the drug.

(2) on the sodium independent component.

With the D.N.P. in aqueous solution a 0.1 ml aliquot caused 50% inhibition in about 40 minutes, (Fig.18), total inhibition in about two hours. However with the acetone solution (Fig.19) the effect was more rapid, probably due to the greater concentration of the drug present. Complete inhibition was obtained within 30 minutes.

The Effect of Oligomycin

(1) on the total current.

Oligomycin was used in an alcoholic solution and 50μg were added to the mucosal chamber. Addition to the serosal chamber caused hardly any inhibition over a 40 minute period: (Fig.20). However 50μg added to the mucosal chamber (ie. 10μg/ml.) caused 50% inhibition in 25 to 30 minutes. The maximum inhibition was approximately 70-75%, which passage of N_2/CO_2 reduced slightly.

(2) on the sodium independent current.

(Fig.21) shows the effect of 50μg of oligomycin added to the mucosal chamber: 50% inhibition occurred in 15-20 minutes; and usually complete inhibition was obtained within one hour.

50μg to mucosal.

80

40

μ amps.

0   40   80 mins
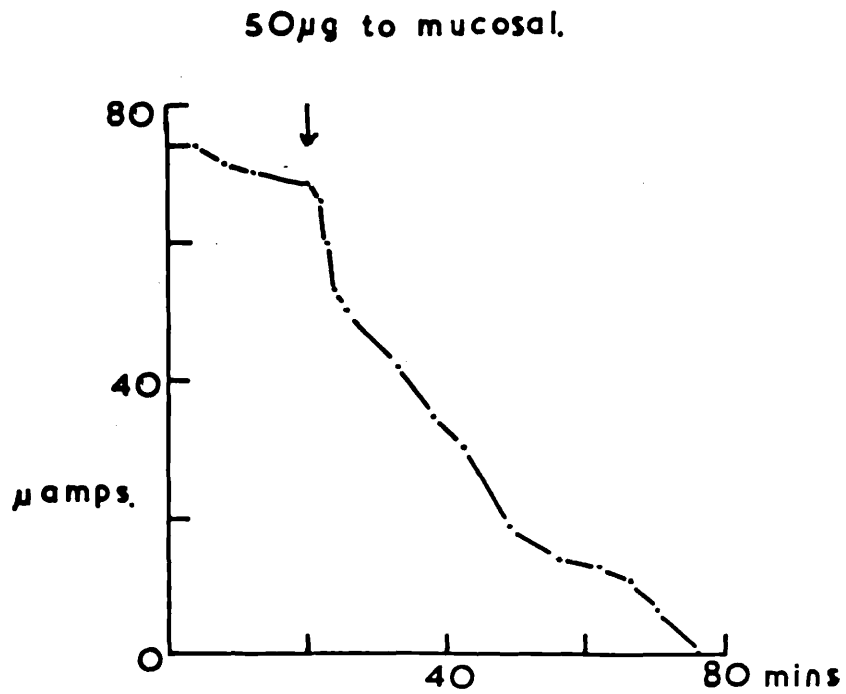
Fig.21.   The effect of Oligomycin on the sodium

independent S.C.C. of a stomach from a 28 day foetus.

Area of membrane = 1.3cm^2.

-79-

50 μg to S.   50 μg to M.   1 x 10^{-4} M. D.N.P.   N$_2$.

480

320

μ amps.

160

0

40   80   120   160 mins.
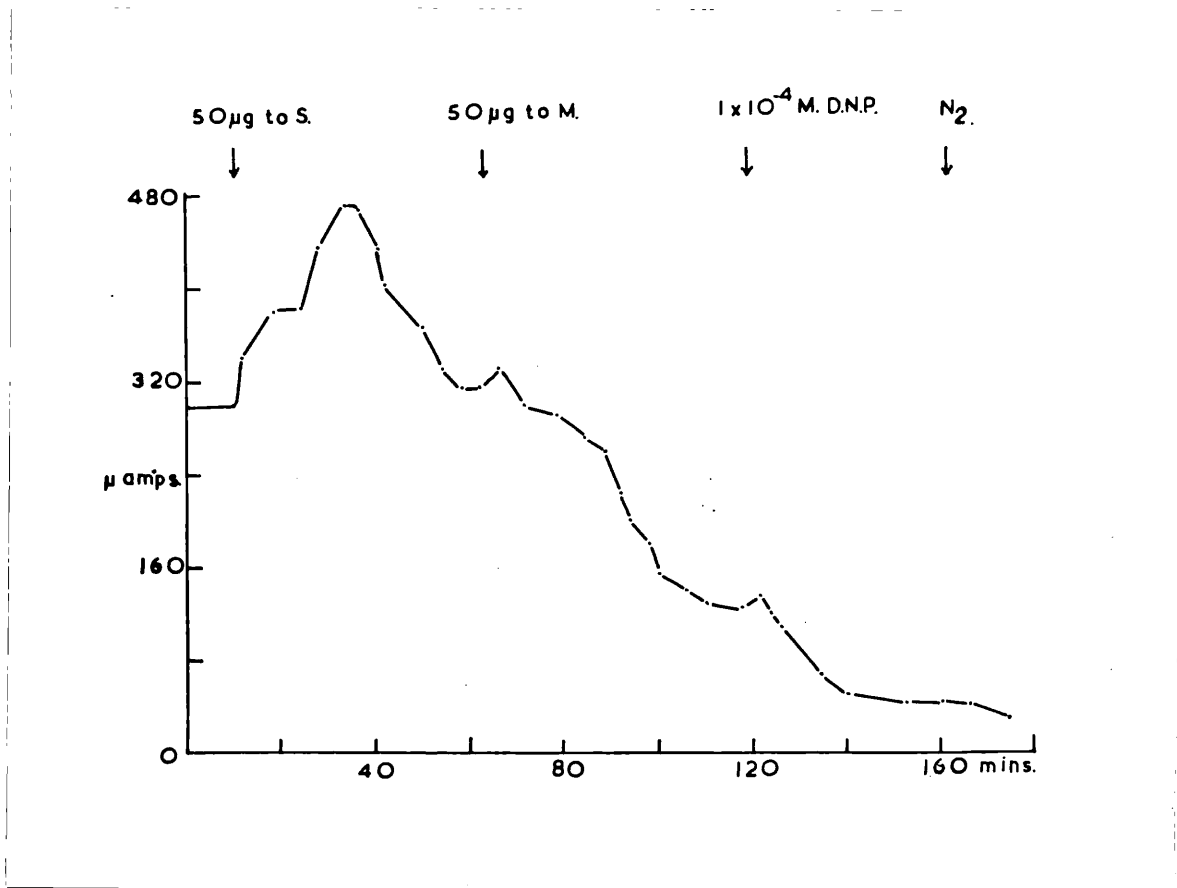
Fig.22.   The effect of D.N.P. on the inhibition of the total S.C.C. caused by Oligomycin. Age of foetus = 29 days. Area of membrane = 1.3cm^2.
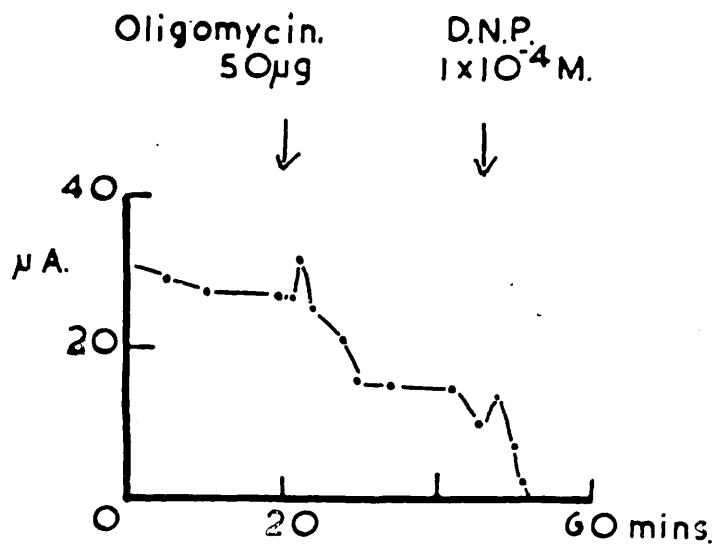
Fig.23.   The effect of D.N.P. on the inhibition of the

sodium independent S.C.C. of a stomach from a 30 day foetus

caused by 50ug. of Oligomycin in the mucosal chamber.

Area of membrane = 1.3cm^2.

Oligomycin is an inhibitor of oxidative phosphorylation
which depresses oxidation steps only when they are coupled to
phosphorylation; not in an uncoupled system; (Lardy et al.,1958).
This drug acts between cytochromes 'b' and 'c' in the cytochrome
chain; site II of phosphorylation.

In an attempt to see if oxidation alone was adequate to
sustain either the sodium dependent or sodium independent
current, or both; a partial inhibition with oligomycin was
obtained and then 1×10^{-4}M. D.N.P. was added to the system to
see if any reversal of the inhibitory effect occurred. D.N.P.
should uncouple the oxidative phosphorylation mechanism, and
thus release the respiration from the inhibitory effect of the
oligomycin. Both total current and sodium independent current
were studied and in neither did release of inhibition occur.
With the total current, as in Fig.22, about 15% of the initial
current remained after both drugs, an average value was 15-20%,
and with the sodium independent component (Fig.23) complete
inhibition was obtained.

The Effect of Azide

(1) on the total current.

Azide was used in aqueous solution and added in 0.1 ml.
aliquots to give a final serosal concentration of 2mM. As in
Fig.24, addition of azide was usually followed by a slight
stimulation in the S.C.C. which lasted for only 2 to 3 minutes;

Fig.24.
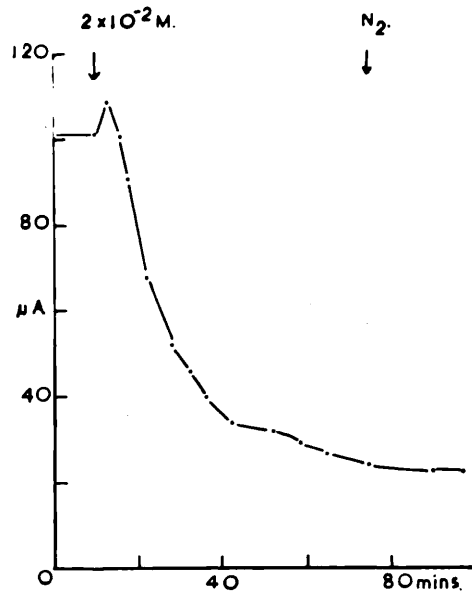
Fig.25.
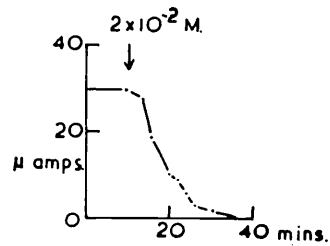
Showing the effect of Azide:

Fig.24.   on the total S.C.C.. Area of membrane = $0.6cm^2$.

Fig.25.   on the sodium independent component of the

S.C.C.. Area of membrane = $0.6cm^2$.

Age of foetuses = 27/28 days.
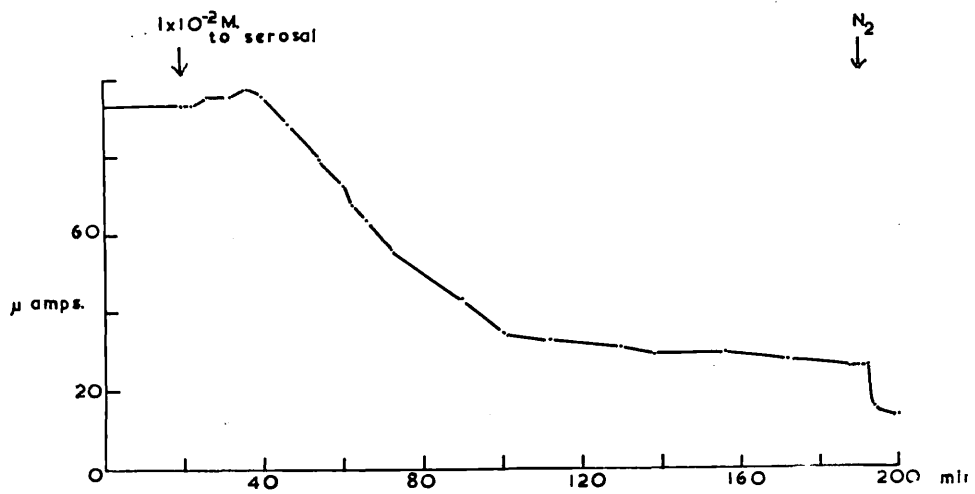
Fig.26.   The effect of Fluoride on the total S.C.C..
Foetal age: 28days.   Area of membrane = $0.6cm^2$.

then the current began to fall; 50% inhibition occurring after about 45 minutes, and a maximum inhibition of 70-80% was obtained. This was not affected by bubbling a N_2/CO_2 mixture through the bathing solutions. A small decrease in the D.C. resistance of the tissue was usually observed.

(2) on the sodium independent current.

As shown in Fig.25, 50% inhibition was obtained within 8 minutes after azide was added. The time varied from 8-15 minutes; complete inhibition occurred in each case.

Sodium azide acts as an uncoupling agent for oxidative phosphorylation; and has been reported (Myers & Slater, 1957) to stimulate A.T.P.-ase in mitochondria, and then inhibit it.

The Effect of Fluoride

(1) on the total current.

Fluoride was added to give a final concentration of $2 \times 10^{-2} M$ in the serosal chamber. In all experiments on the total current there was a time lag of 20-30 minutes after the addition of the fluoride, before any inhibition of the S.C.C. or P.D. occurred. The time for 50% inhibition averaged about 90-100 minutes after the addition of the drug, and a total inhibition of 65-70% was recorded. Fig.26 gives a typical experiment. The remaining fraction of the current was subjected to a 95% $N_2/5\%$ CO_2 mixture and a further inhibition occurred leaving only about 5% which could possibly have been some degree of recording

-85-

1×10^{-2} M.

40

μ amps.

20

0

20   40   mins.
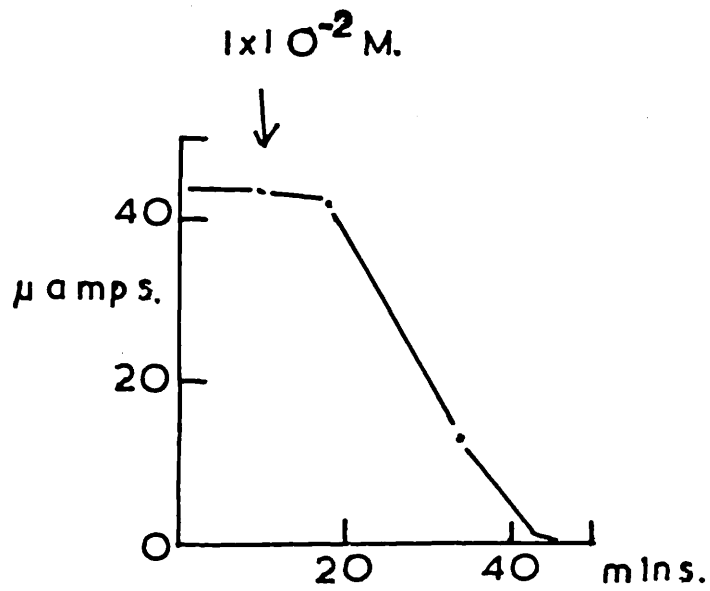
Fig.27.   The effect on the sodium independent component of the S.C.C. of Fluoride.   Age of foetus = 28/29 days. Area of membrane = 1.3cm^2.
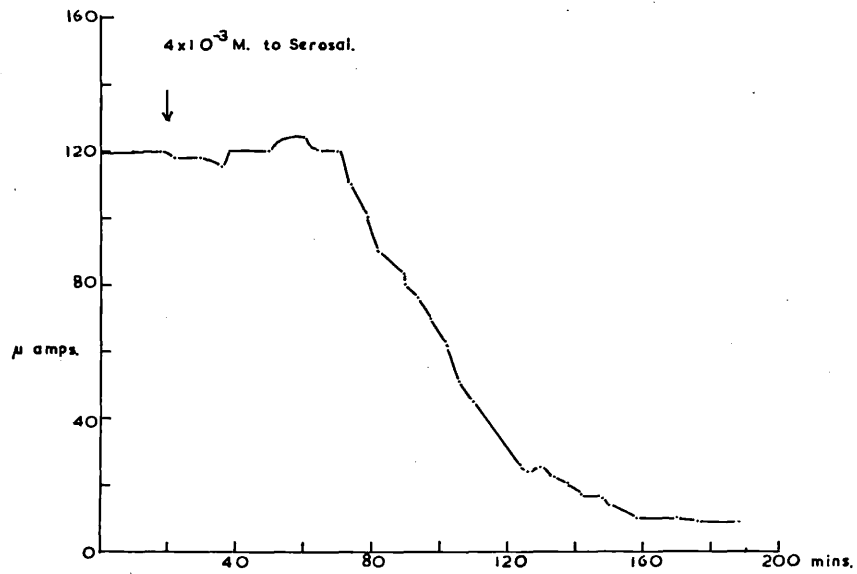
Fig.28.   The inhibition produced by Iodoacetate on the
total S.C.C. of a stomach from a 28/29 day foetus.
Area of membrane = $0.6cm^2$.

error. Slight fluctuations in resistance of the tissue occurred, but there was no overall large change.

(2) on the sodium independent component.

After addition of the sodium fluoride the S.C.C. fell only very slightly over about the first 10 minutes then a fairly rapid decline occurred until complete inhibition was obtained in about 30 to 40 minutes: (Fig.27). The current fell less rapidly than under anoxic conditions.

Fluoride has been shown (Warburg & Christian, 1941) to inhibit the enzyme enolase, and believed to form a complex with the enzyme, phosphate and magnesium on the enzyme surface. Fluorocitrate is also formed in the glycolytic cycle, preventing complete oxidation of glucose.

The Effect of Iodoacetate

(1) on the total current.

Sodium iodoacetate was added to give a final concentration of 1×10^{-3}M in the serosal solution. A time lag of 30 to 40 minutes (Fig.28) between addition of the drug and decline in S.C.C. was always noted. 50% inhibition occurred after 70 to 80 minutes and a maximum inhibition of about 90% was obtained.

(2) on the sodium independent component.

After addition of iodoacetate a small increase in current was observed and then after about 10 to 12 minutes the current began to fall; (Fig.29). Complete inhibition was obtained and took
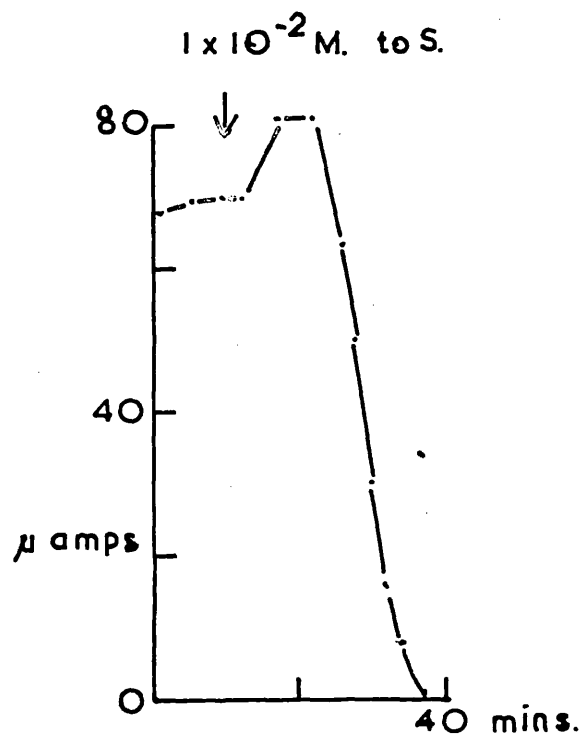
Fig.29.   The effect of 1 x 10^{-2}M Iodoacetate on the sodium

independent component of the S.C.C. of a stomach from a

28/29 day foetus.   Area of membrane = 1.3cm^2.
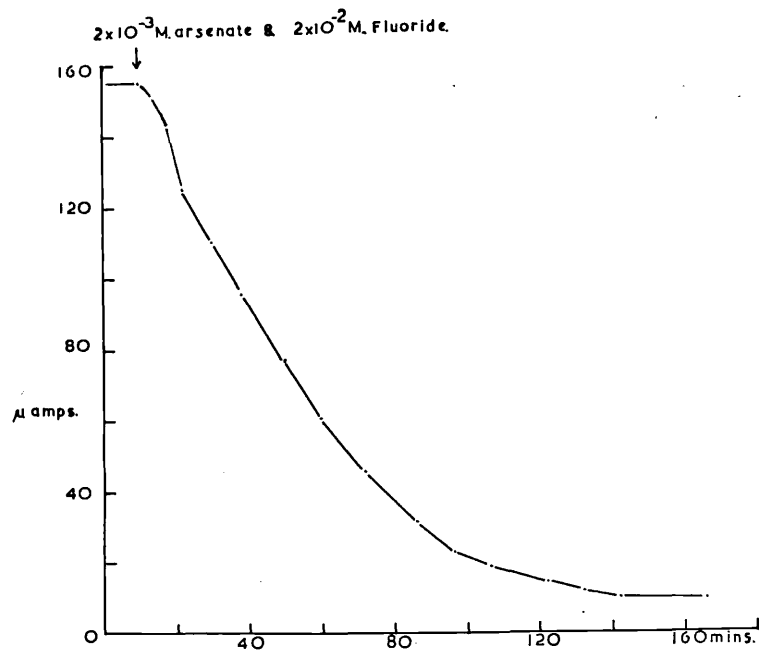
Fig.30.   The combined effect of Arsenate and Fluoride on the total S.C.C. of the stomach from a 28-day foetus. Area of membrane = 0.6cm^2.
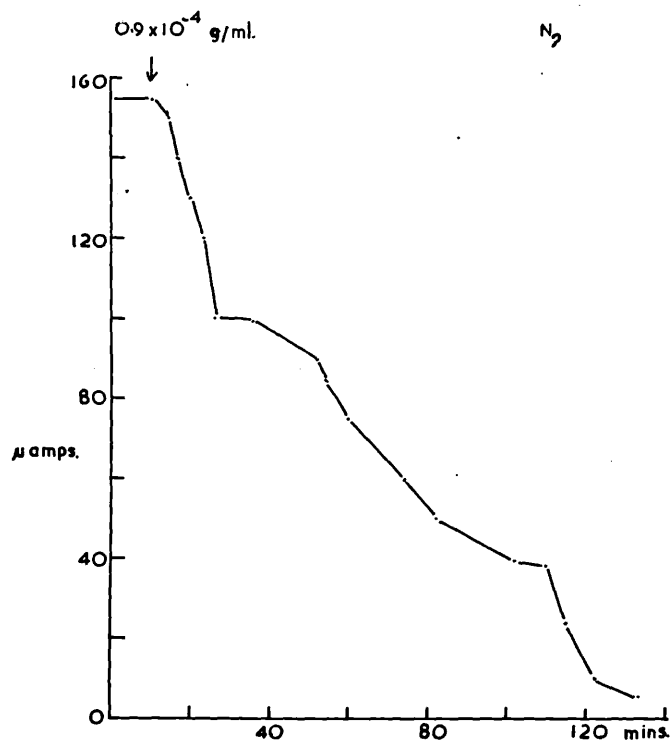
Fig.31.   The effect of the cardiac glycoside, Ouabain,

on the total S.C.C. and the effect of anoxia when maximal

inhibition had occurred.   Foetal age = 28 days.

Area of membrane = $0.6cm^2$.

about 20 to 25 minutes. The general effect of the fall was
rather like that under anoxia conditions; however it was slightly
slower. Iodoacetate is a potent inhibitor of the SH groups: and
thus through this medium causes inhibition of glyceraldehyde-3-
-phosphate dehydrogenase and the N.A.D. bonding with hydrogen;
and also forms a complex with Co-enzyme A of the Krebs'
tricarboxlyic acid cycle.

The Combined Effect of Arsenate and Fluoride.

Fig.30 shows the very reproducible result of the combined
effect of these two drugs, used at a concentration of arsenate,
2×10^{-3}M, and fluoride, 2×10^{-2}M, on the serosal side. With
the combined drugs the delay in inhibition obtained with fluoride
was eliminated and a 50% inhibition was obtained within about
40 to 50 minutes. A maximum inhibition of 95 to 98% was
recorded; the small remaining fraction could possibly be
recording error.

The Effect of Cardiac Glycosides.

(1) on the total current.

Cardiac glycosides are known to cause inhibition of active
sodium transport in, among other tissues, red-cells and frog
skin. Ouabain was used to give a final concentration of between
0.5×10^{-4} and 4×10^{-4}gm/ml; in the order of 10^{-5}M. There was
no time lag before a fall in the S.C.C. commenced, and the fall
was initially rapid; and then became more gradual: (Fig.31).
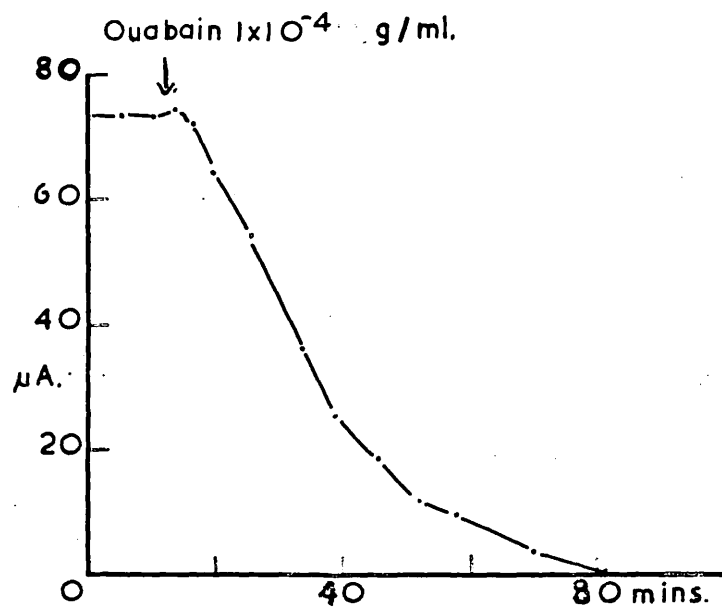
Fig.32. Showing the effect of the cardiac glycoside, Ouabain, on the sodium undependent component of the S.C.C.. Foetal age = 29/30 days. Area of membrane = $1.3cm^2$.
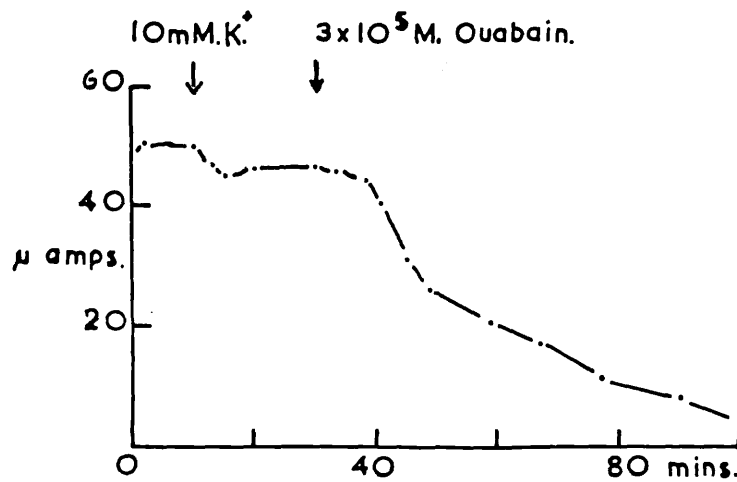
Fig.33.   The effect of increasing the potassium ion (K$^+$)

concentration by 10mM in both chambers, on the inhibition of

the sodium independent S.C.C. by Ouabain.   Foetal age = 28 days.

Area of membrane = 1.3cm^2.

50% inhibition occurred within 40 to 50 minutes and a maximum
inhibition of 75 to 80% occurred. The passage of nitrogen
reduced the remaining current to practically zero.

(2) on the sodium independent current.

Inhibition began immediately the drug was added and was
rapid. With ouabain concentration at 1×10^{-5}M in the serosal
chamber, 50% inhibition was obtained within 10 to 20 minutes,
as shown in Fig.32 and complete inhibition occurred within one
hour. Assuming that the changes in this fraction of the current
can be taken to indicate changes in acid secretion, a similar
inhibitory effect with ouabain has been obtained in frog gastric
mucosa (Davenport, 1962). In that case the effect was blocked
by increasing the potassium ion concentration. The potassium
ion concentration was increased in this tissue by 10mM in each
chamber, and then 10^{-5}M ouabain was added. It caused some
decrease in the rate of inhibition: Fig.33 shows the effect
obtained.

The Effect of Substrate-free Solution.

The time lag between administration of the drug and
commencement of inhibition of the S.C.C., which occurred with
the compounds which interfere with metabolism through either
the Krebs' cycle or A.T.P. synthesis rather than directly
with electron transport along the cytochrome chain; suggested
that the tissue might have some energy store. To try to verify

Fig.34.   The effect on the total S.C.C. of a stomach

from a 28 day foetus when bathed in a substrate-free

solution.   Area of membrane = $0.6 cm^2$.
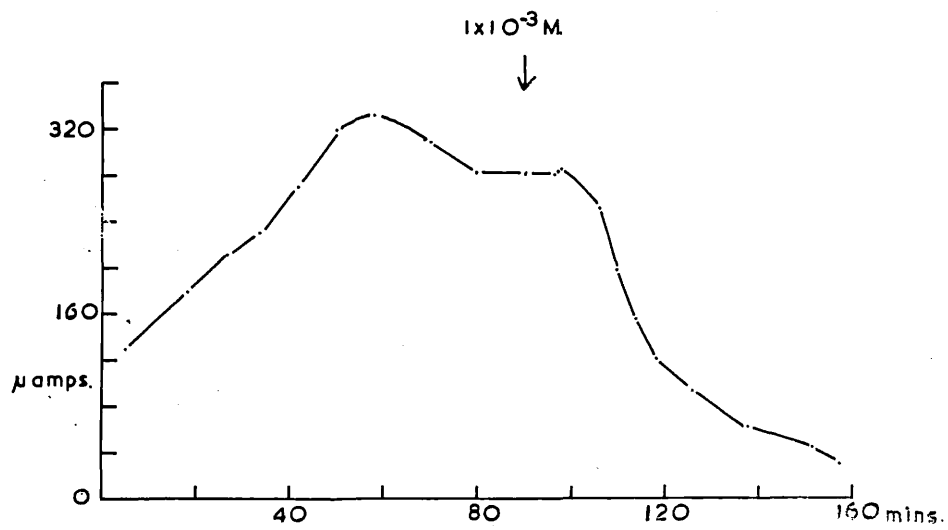
Fig.35.   The effect of Iodoacetate on the total S.C.C. of

a stomach from a 28/29 day foetus, when the Krebs' Ringer

was substrate-free.   Area of membrane = $1.3cm^2$.

this point, the tissues were mounted in substrate-free Krebs'
Ringer solution. The P.D. and S.C.C. were recorded from the
time of mounting. Fig.34 gives a typical example. The tissue
used in this particular experiment had been stored in ice-cold
Krebs' Ringer and hence its mounting P.D. was low. However the
tissue began to recover and to produce a fairly normal P.D.
and S.C.C. (slightly below that of a fresh preparation). About
two hours after mounting a slow decline in S.C.C. and P.D.
began; and about six hours after mounting was less than 50% of
the maximum value obtained. Addition of glucose to the serosal
bathing solution, to give a final concentration of 24mM, caused
a reversal of the decline after only a short time lag. The S.C.C.
returned to its optimum value well within one hour.

The Effect of Iodoacetate when the Ringer is Substrate-free.

As shown in Fig.35 there is a small time lag of about 10
minutes between addition of the iodoacetate and start of fall
of the S.C.C.. In all experiments, where the tissue was bathed
in a substrate-free solution, the time lag of about 10 minutes
was obtained, before the maximum rate of inhibition occurred,
whether the iodoacetate was given when a steady maximum S.C.C.
was reached or when the current began naturally to slowly
decline. Inhibition once started, was 3 or 4 times more rapid
than in experiments using glucose Krebs' solution.
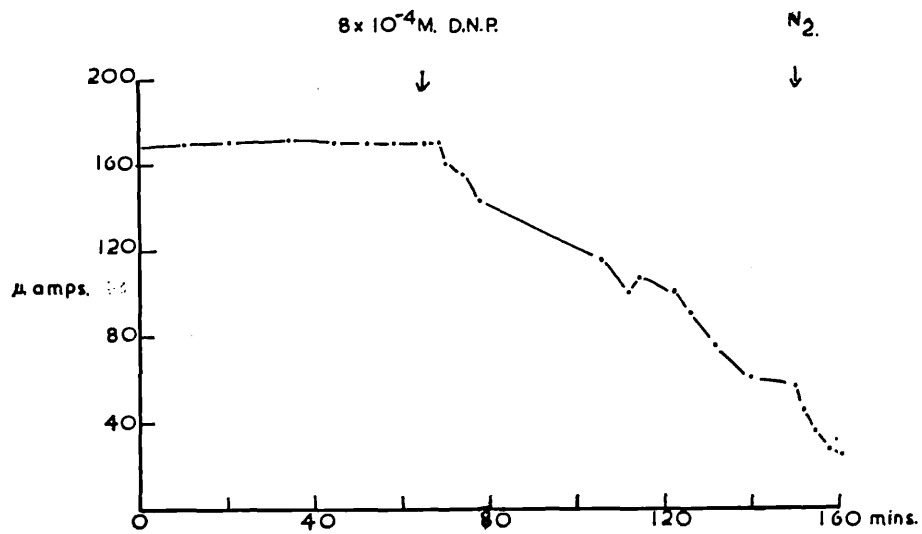
Fig.36.   The effect of Di-nitrophenol (D.N.P.) on the total

S.C.C. of the foetal stomach when bathed in a substrate-free

Ringer solution.   Foetal age = 28/29 days.

Area of membrane = $1.3cm^2$.

The Effect of D.N.P. in Substrate-free Solutions.

The tissue was mounted in a substrate-free solution and the
S.C.C. and P.D. recorded. When the tissue had been mounted for
about an hour, but the S.C.C. had not yet begun to fall,
8×10^{-5}M. D.N.P. was added to the serosal solution and the
effect on the S.C.C. was recorded: (Fig.36). The current began
to fall immediately.

Experiments Using β-hydroxybutyrate as Substrate.

The previous experiments using a substrate-free Ringer
indicated that the tissue needed an exogenous substrate in
order to keep up its maximum P.D. and S.C.C. over a period of
several hours; and that glucose fullfilled the requirements. In
some experiments lactate or pyruvate were added, in similar
concentrations to glucose, but the fall in current was not
reversed. There was the possibility that the decline in S.C.C.
observed when iodoacetate or fluoride were added,was due to an
overall exhaustion of A.T.P. and not of A.T.P. specific to the
maintainance of the S.C.C.. A.T.P. is required in some steps of
oxidation of glucose; and if A.T.P. is not available there
would be no oxidation of glucose to produce the three-carbon
compounds to enter the Krebs' cycle, and the reduced N.A.D. for
the cytochrome chain. For this reason a substrate not requiring
A.T.P. for its breakdown was used. One of these compounds is a
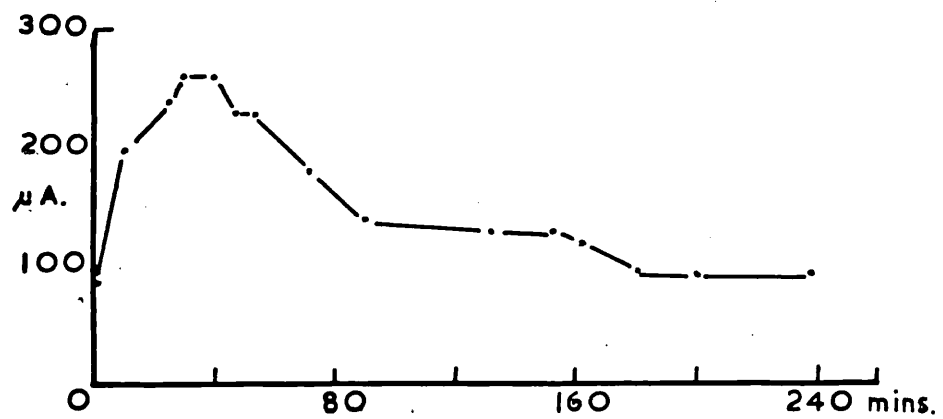
Fig.37.   The effect on the total S.C.C. of a stomach

from a 28/29 day foetus, of using 30mM β-hydroxybutyrate

as substrate.   Area of membrane = 1.3cm^2.
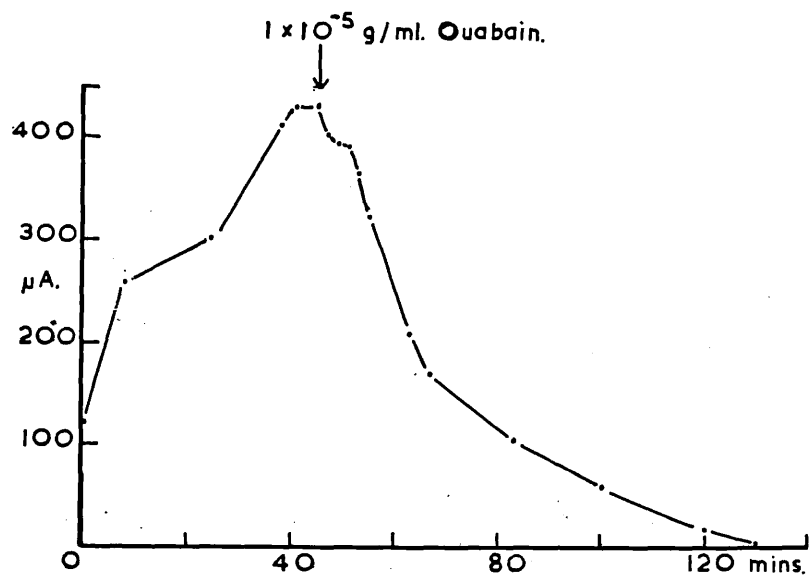
Fig.38. Using /B-hydroxybutyrate as substrate, the effect of Ouabain on the total S.C.C. of a stomach from a 29/30 day foetus is shown. Area of membrane = $1.3cm^2$.

fatty acid derivative, /B-hydroxybutyrate, which produces two-
carbon fragments which are incorporated with Co-enzyme A into
the Krebs' cycle; the first stage of oxidation being
transference of two hydrogen atoms to N.A.D..

Tissues were mounted in Krebs' Ringer containing about 30
to 40mM /B-hydroxybutyrate as substrate. The P.D. and S.C.C.
were recorded from mounting the tissue. Fig.37 shows the result
of one of these experiments. The tissue rapidly attained a high
and steady S.C.C., but in less than an hour, in this case, the
current began to fall. In no experiment did /B-hydroxybutyrate
maintain the tissue at its maximum S.C.C. for longer than a
substrate-free solution.

The effects of such drugs as iodoacetate and ouabain were
very similar to those obtained in substrate-free solutions. A
time lag of about 10 minutes was obtained with iodoacetate
before the inhibition commenced. Fig.38 shows the effect of
1×10^{-5}g/ml. of ouabain in the serosal chamber, on a stomach
from a 28 day foetus, using /B-hydroxybutyrate as substrate.
The S.C.C. began to fall immediately after the addition of the
drug, 50% inhibition occurring in 15 minutes and only about 5%
of the initial current was left, compared to 20 to 30%
remaining when the tissue was bathed in Ringer containing
glucose.

CHAPTER IV.

STUDY OF THE A.T.P.-ase ISOLATED FROM THE FOETAL GASTRIC MUCOSA.

CHAPTER IV

Section I

METHOD.

In experiments aimed at studying the presence of an

A.T.P.-ase in foetal gastric mucosa, the stomach was dissected

out from the foetus and preparation of the enzyme was usually

carried out on the same day as the dissection. When the stomachs

of younger foetal age were used, sometimes one litter was

stored overnight so that a second litter could be added to give

a more workable amount of material. The stomachs were used from

foetuses of 28-30 days gestation age and also 24-26 days; the

method of preparation being the same for both. Stomachs from

foetal rabbits of 24-26 days gestation age were used, as at this

age only a few oxyntic cells were present; and if the A.T.P.-ase

was directly linked to acid secretion, it might be expected

that there would be a small amount of activity only in these

extracts, compared to those from foetuses nearer term, which

have larger numbers of oxyntic cells. A few enzyme preparations

were made from adult gastric mucosa for comparison.

Preparation using an acetate - tris buffer.

The stomachs were cut open, freed of contents, and blotted

to remove excess water. The mucosal tissue was scraped from the

muscle coat, weighed, then chilled and maintained near $0^{\circ}C$ in an

homogenizing medium containing:

-105-

sucrose	250mM.
K^+ (as sulphate)	25mM.
Mg^{++} (as acetate)	5mM.

and tris-acetate buffer, 50mM at 7.6.pH.

The volume of homogenizing medium was such as to give a 10%
suspension, weight of tissue to volume of medium. The tissue
was macerated finely with scissors and then homogenized in
a ground-glass homogenizer. The suspension was centrifuged
(according to Durbin, 1965) at 600 x'g' for 10 minutes, the
supernatent fluid at 10,000 x'g' for 10 minutes; the supernatent
from this at 105,000 x'g' for one hour, in each case at a
temperature of about 1-2°C. The pellet from the last
centrifugation was washed in a suspension medium containing:

sucrose	250mM.

Tris-acetate buffer, 50mM at pH 7.6.,
re-homogenized, then centrifuged at 105,000 x'g' for one hour.
The final pellet was homogenized in this solution to yield
approximately 1mg protein equivalent per ml. This suspension
could be stored, frozen, for about two weeks without any
substantial deterioration.

Protein Determination.

The protein equivalent of the enzyme was estimated according
to the method of Lowry et al. (1951).

Reagents.

<u>A</u>   2% Na_2CO_3 in 0.1N NaOH.

<u>B</u>   0.5% $CuSO_45H_2O$ in 1% sodium potassium tartarate.

<u>C</u>   Alkaline copper solution, 50ml. of A with 1ml. of B, which was made up immediately before use.

<u>D</u>   Dilute Folin Reagent.   (the concentrated reagent diluted

1.: 2.3.)

Method.

The standard solution was bovine serum albumin, made up to a concentration of 125µg protein/ml. The standard quantities used were:

100 µg protein using 0.8ml standard soln. & 0.0ml distilled H_2O.

75 µg   "   "   0.6ml   "   "   " 0.2ml   "   "

50 µg   "   "   0.4ml   "   "   " 0.4ml   "   "

25 µg   "   "   0.2ml   "   "   " 0.6ml   "   "

0 µg   "   "   0.0ml   "   "   " 0.8ml   "   "

To each was added 4mls. of reagent C and allowed to stand for at least 10 minutes; then 0.4ml. of Folin reagent (D) and the colour allowed to develop over a 30 minute period. The colour densities were read on an OPTICA U.K. spectrophotometer at 750mµ wavelength, against a reagent blank.

The unknown enzyme was used concentrated, diluted 1:2 and 1:5. In each case 0.2ml. of the preparation was made up to 0.8ml. with distilled water. 4mls. of reagent C were added, left to

stand for 10 minutes, then 0.4ml. of reagent D, and then left

for 30 minutes. The samples were compared with the reagent

blank.

Analysis of A.T.P.-ase activity.

The incubations were carried out in a small stoppered flask

at $37^{\circ}C$. The incubating medium was a basic tris-acetate buffer,

20mM, magnesium acetate 1mM, A.T.P. 2mM (disodium salt), with

a final pH of 8.0 and a total volume of 2.5mls. To study the

effects of various anions and cations on the A.T.P.-ase activity,

the above medium was modified by addition or removal of these

ions. The enzyme was added in volumes of 0.1ml. giving a

protein equivalent of about 100 μg per incubation. In each case

the exact amount of protein added was known.

The activity of the A.T.P.-ase enzyme was measured by the

amount of inorganic phosphate released. This was estimated

using a modified Allen method.

Reagents.

15% perchloric acid.

2.5% ammonium molybdate.

Amidol   – made by dissolving 20gms. of sodium meta-

        bisulphate and 1gm. 'amidol' in 100mls. of

        distilled water and filtering. This was made up

        just before use.

After a specific time two 0.5ml. samples were taken from each

flask and pipetted into tubes containing 0.5ml. of ice-cold

perchloric acid, and 2.2mls. of ice-cold distilled water were

added. To each tube was added 0.5ml. of amidol and then 0.3ml.

of ammonium molybdate: shaken, and then left for 15 minutes

for colour development. These were read at $750m\mu$ on a

spectrophotometer against a reagent blank and the amount of

inorganic phosphate present determined from a standard phosphate

curve. The tubes were kept cold to minimise spontaneous

hydrolysis of any A.T.P. still present in the samples, during

the time lapse between reading the first and last samples in

the spectrophotometer. Control samples showed that very little

if any spontaneous hydrolysis of A.T.P. took place; and any

correction required was made.

Each set of incubations included a control flask containing

only the incubating medium and any ions added, to determine the

amount of normal hydrolysis of the A.T.P.; and one containing

the enzyme suspension in the pure incubating medium, to

determine the rate of enzyme activity before the various ions

were added. The results are expressed in two forms:

(1) As the total amount of inorganic phosphate, (P_i), in

μMoles, liberated per mg. of protein per minute.

(2) As a percentage of the control tube containing enzyme

in the unmodified incubating medium: (both corrected

for spontaneous A.T.P. hydrolysis.)

-109-
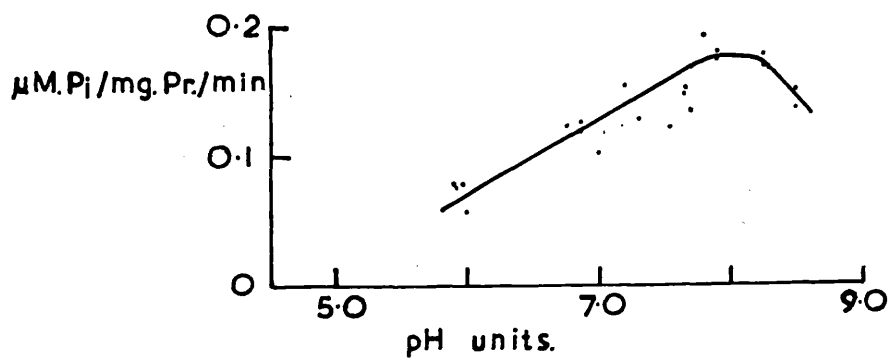
Fig.39.   Showing the variation of the A.T.P.-ase activity

of the enzyme, with varying pH values. A Tris-acetate

buffer was used with 1mM Mg^{++} ions as the incubating medium.

The peak of activity was the same for both foetal ages of

24/26, and 28/30 days, and for adult gastric mucosal enzyme.

Section II

RESULTS.

Effect of pH variation on the A.T.P.-ase activity.

Incubating medium.

Tris-acetate buffer	20mM.
Mg^{++}	1mM.
A.T.P.	2mM.
enzyme.	

The pH of the incubating medium was varied over the range 5.5 to 9.5. A peak of activity was obtained around pH 8.0 to 8.3 and this range was used for the rest of the experiments. This peak was obtained with both foetal ages and with the adult preparations: (Fig.39).

When the experiments on the stomachs from 24-26 day foetuses were performed it was decided to see if the muscle coat of the stomach contained much of the A.T.P.-ase enzyme, which reacted in a similar way to that from the mucosal tissue alone. If not, the whole stomach could have been used, instead of scraping off the very thin mucosal layer. However the muscle fraction caused the release of as much inorganic phosphate as the mucosal fraction; and it responded in a very similar way to many of the variations in ionic concentration. In many of the experiments a graph indicating the results for a muscle extract will be included.

Fig.40.
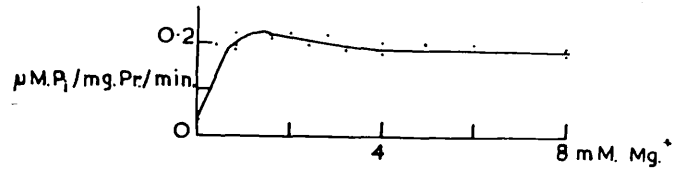
Fig.41.
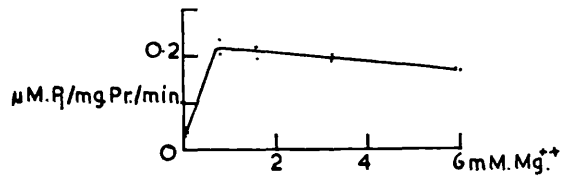
Fig.42.
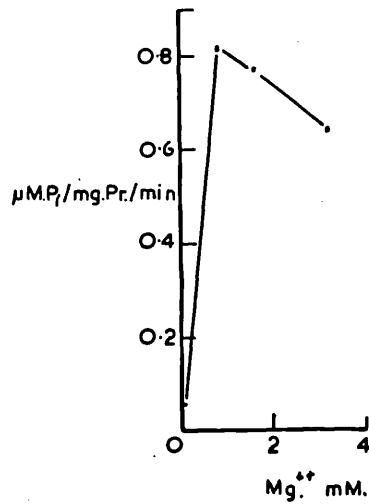
Showing the stimulation caused by the presence of Mg^{++} ions on the mucosal A.T.P.-ase enzyme activity from:

Fig.40.   stomachs from foetuses of age 28 to 30 days.

Fig.41.   stomachs from foetuses of age 24 to 26 days.

Fig. 42.   stomachs from adult animals.
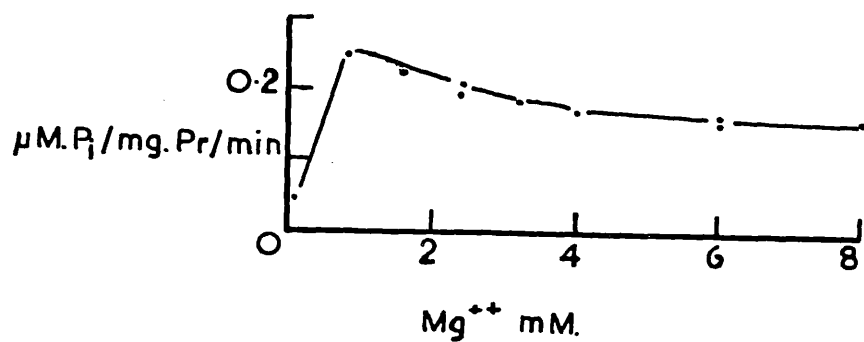
Fig.43. Showing the effect of increasing concentrations of Mg^{++} ions on an A.T.P.-ase enzyme extract from the muscle of stomachs of 28 to 30 day foetuses.

Effect of Mg^{++} ion on A.T.P.-ase activity.

Incubating medium.

Tris-acetate buffer   20mM,   pH 8.0.

A.T.P. (disodium salt)   2mM.

enzyme (about 100/ug protein)

Without the presence of magnesium (Mg^{++}) ions in the incubating medium release of inorganic phosphate from A.T.P. was small. However, as shown in Figs. 40, 41,& 42, in all preparations used the activity of the enzyme greatly increased in the presence of Mg^{++} ions in the incubating medium; as little as 0.4mM Mg^{++} produced a 20-fold increase in the enzyme activity. The maximum activity was obtained with a Mg^{++} ion concentration of 0.8mM. The level of activity remained fairly constant for concentrations up to 3-4mM Mg^{++} in both of the foetal preparations; and further addition of Mg^{++} ions caused a slight inhibition. The adult preparations had a definite peak of activity at 0.8mM Mg^{++}. The muscle extract (Fig.43) was also stimulated by Mg^{++} ions and showed a definite peak of activity; the extract shown was from a 28 day foetus. The Mg^{++} ion was used at a concentration of about 1mM throughout the rest of the experiments.

The Effect of Calcium ions.

Incubating medium.

Tris-acetate buffer   20mM, pH 8.0.

-114-
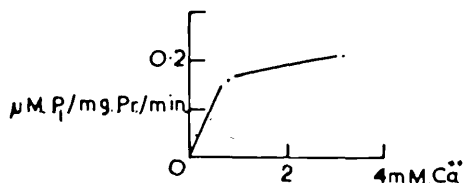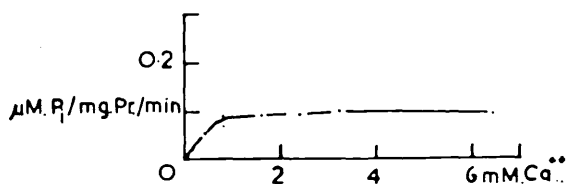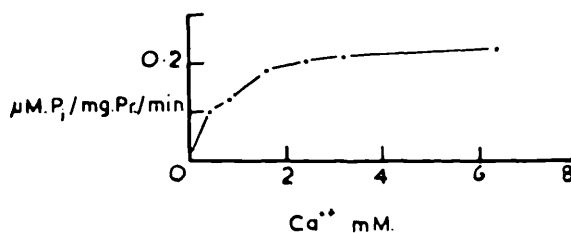
Fig.44.

Fig.45.

Fig.46.

Showing the effect of Ca^{++} ions (no Mg^{++} ions present) on the _mucosal_ A.T.P.-ase enzyme extract from:

Fig.44.   stomachs from foetuses aged from 28 to 30 days.

Fig.45.   stomachs from foetuses aged from 24 to 26 days.

Fig.46.   The effect of Ca^{++} ions on the _muscle_ A.T.P.-ase enzyme from stomachs from foetuses aged from 24 to 26 days.
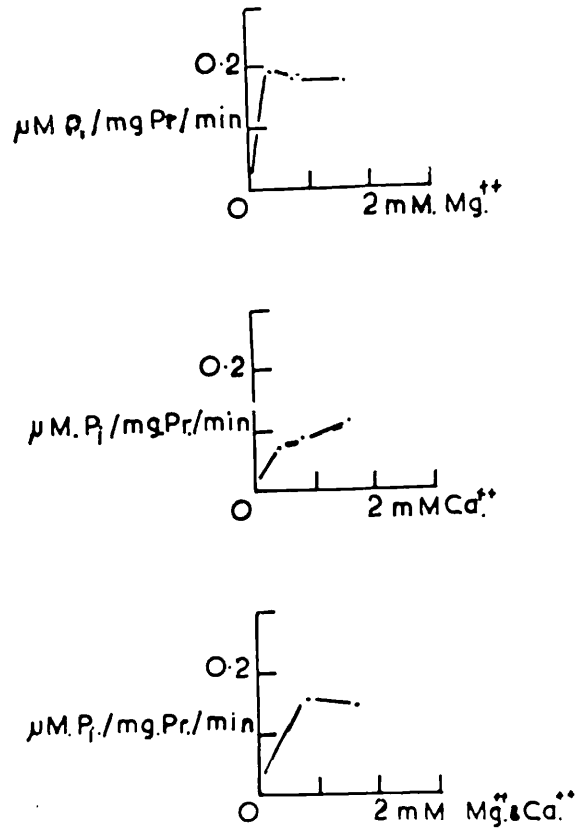
Fig.47.   Showing the antagonistic effect of Ca^{++} ions on
Mg^{++} ions. Upper figure – the effect of Mg^{++} ions alone;
central figure – the effect of Ca^{++} ions alone; and the lower
figure – the amount of activity when both Mg^{++} and Ca^{++} ions
are present in equal amounts, giving the same final ionic
concentration together, as was present with Mg^{++} and Ca^{++}
ions alone.   Foetal age – 28 to 30 days.
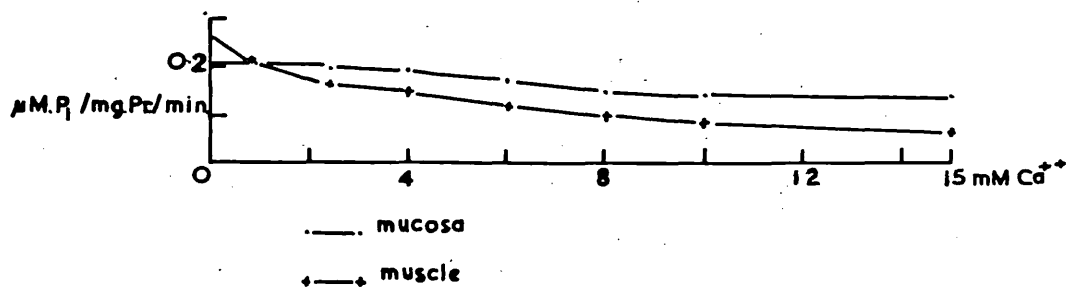
Fig.48.   Showing the greater degree of inhibition

obtained in the muscle A.T.P.-ase enzyme extract to that of

the mucosal A.T.P.-ase enzyme extract when Ca^{++} is added in

the presence of 0.8mM Mg^{++}, at the optimum pH of 8.0.

Both enzyme extracts were from the stomachs of 28 to 30

day foetuses.

A.T.P. (disodium salt)   2mM.

enzyme.

In Mg^{++}-free incubating medium the calcium (Ca^{++}) ion
caused a stimulation in the activity of the A.T.P.-ase enzyme;
in the stomach extracts from foetuses aged 24-26, and 28-30 days;
but a greater concentration of Ca^{++} ion was required to produce
the maximum degree of activity: (Figs. 44 & 45). There was a
variation between the mucosal and muscle extract in respect to
this ion. The muscle extract was stimulated to a much greater
extent, (Fig.46). Figs.45 & 46 show the mucosal and muscle
extracts taken from stomachs of foetuses in the same litter,
aged 24-26 days.

Effect of Calcium ions when Magnesium ions were present.

The addition of Ca^{++} ions to the medium containing 1mM Mg^{++},
caused an inhibition of activity. Fig.47 shows the effect of
Mg^{++} ions alone, Ca^{++} ions alone and Mg^{++} ions with Ca^{++} ions,
on the same mucosal extract from a 28 day foetus. A divergence
between mucosal and muscle extracts again occurred; (Fig.48),
a greater degree of inhibition occurred with the muscle extract.
Similar effects were obtained with 24-26 day foetal and with
adult extracts.

Effect of adding Sodium and Potassium ions.

In all cases the addition of either sodium or potassium, or
both ions caused a degree of inhibition of the activity

-118-
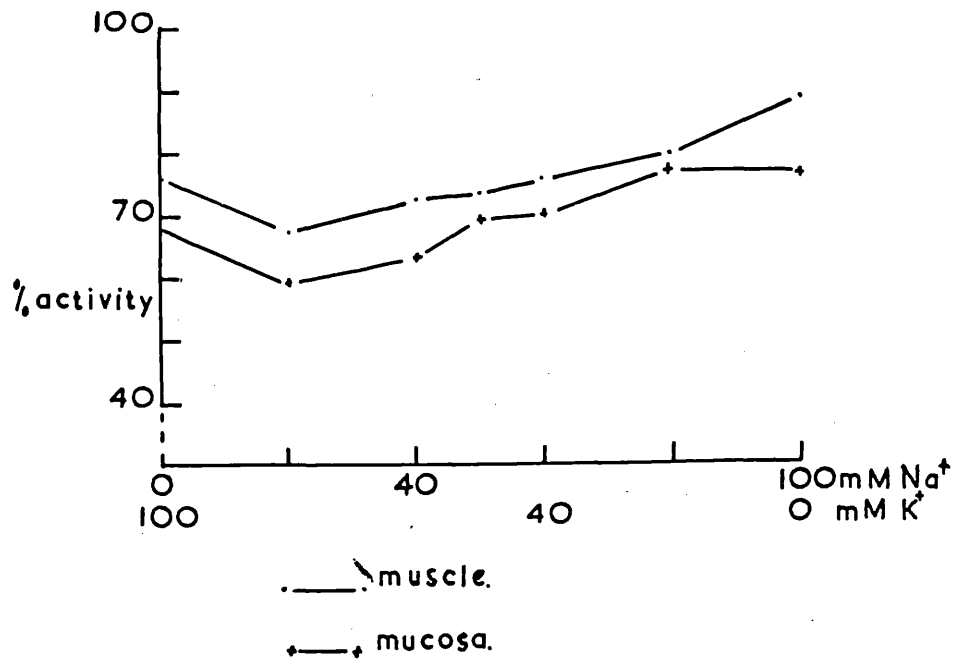
Fig.49. Showing the effect of adding $(Na^+ + K^+)$ to the incubating medium. The combined concentration of the two ions was 100mM. The figure shows both mucosal and muscle extracts from the stomachs of foetuses aged from 28 to 30 days.
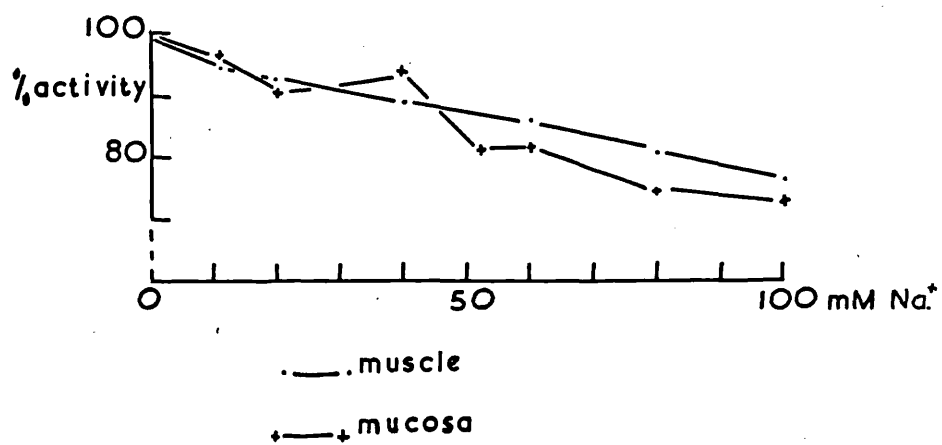
Fig.50.   The effect of the addition of increasing

concentrations of Na^+ ions to the mucosal and muscle

A.T.P.-ase extracts from stomachs of foetuses aged from

28 to 30 days.
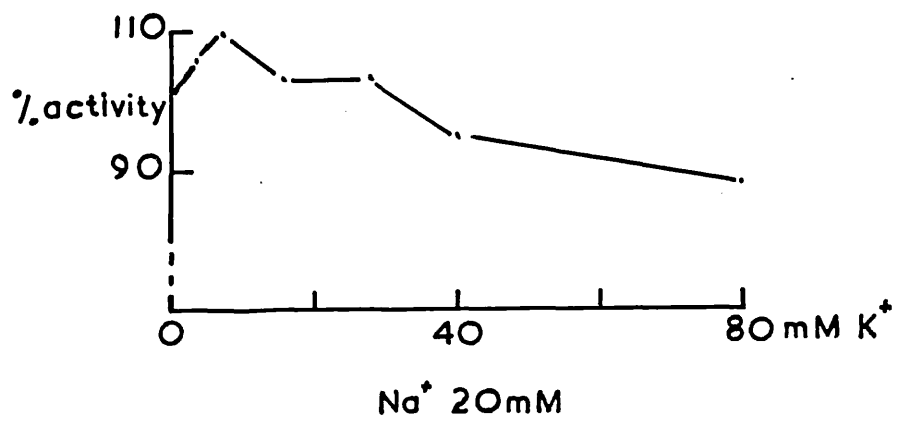
Fig.51. The effect of varying the K^+ ion concentration with a constant Na^+ ion concentration of 20mM, on a mucosal A.T.P.-ase enzyme extracted from the stomachs of 24 to 26 day foetuses.

compared to that obtained in the unmodified medium. In these
experiments the unmodified medium was:

Tris-acetate buffer   20mM, pH 8.0.

A.T.P. (disodium salt) 2mM.

Mg^{++} 1mM.

Regarding the latter as 100% activity, the inhibition was about
25%. Fig.49 shows a typical result obtained from extracts of
both mucosal and muscle tissue from a 28 day foetus. The
combined total concentration of both Na^+ and K^+ ions was 100mM
in each case. 24-26 day foetal and adult extracts produced a similar
similar pattern.

The effect produced when Na^+ ion was added alone is seen in
Fig.50, (the mucosal and muscle extracts were from stomachs
from foetuses aged from 28-30 days). Once again inhibition
occurred, but to a lesser extent.

Fig.51 shows the effect of keeping the Na^+ ion concentration
constant at 20mM, and varying the K^+ ion concentration. In this
case 100% activity is the activity in a medium containing:

Tris-acetate buffer   20mM, pH 8.0.

A.T.P.(disodium salt)   2mM,

Mg^{++} ion   1mM.

Na^+ ion(total conc.)   20mM. and enzyme.

A slight stimulation, up to 10%, occurred with low concentrations
of K^+ ion, up to 30mM; then inhibition occurred. Similar results
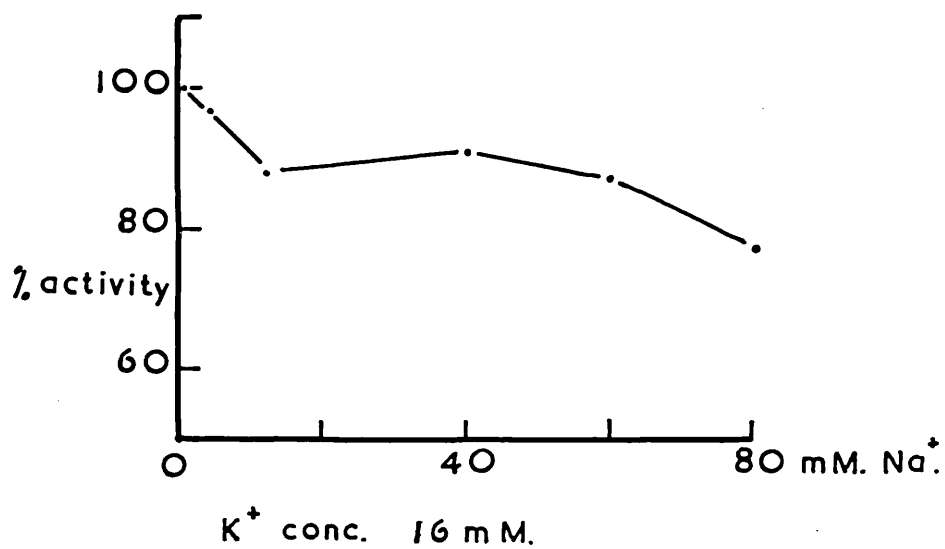
Fig. 52.   Showing the effect produced when a constant
K$^+$ ion concentration of 16mM is used and the Na$^+$ ion
concentration is varied. An A.T.P.-ase enzyme from the
gastric mucosa of 28 to 30 day foetuses was used.

were obtained with both extracts from 28 day foetuses and from adult tissue, with Na^+ ion concentrations constant at 40 and 60mM.

With the K^+ ion concentration constant, increase in Na^+ ion concentration produced a decrease in activity: Fig.52.

As increasing the Na^+ or K^+ ion concentrations produced a decrease in activity compared to a medium containing neither ion, experiments similar to those with the calcium ion were carried out to see if any degree of competition could be occurring between the Mg^{++} ion, and the Na^+ and K^+ ions. The effect of Na^+ ions, K^+ ions, and both Na^+ and K^+ ions in the absence of Mg^{++} ions was noted.

Addition of Na^+ ions alone caused no activation of the enzyme if Mg^{++} ions were not present; and K^+ ions alone were also without effect. (see table IV). Muscle preparations again gave similar results.

Effect of Ouabain.

Experiments of Skou (1960) and also by Glynn et al. (1965) have shown that if an A.T.P.-ase is stimulated by (Na^+ & K^+) it will be inhibited by the cardiac glycosides. An inhibitory effect of cardiac glycosides (eg. ouabain) has been demonstrated on the isolated foetal rabbit stomach and it was thought possible that a similar inhibitory effect might be shown to occur with the isolated gastric A.T.P.-ase.

Table IV   The effect of the cations Mg^{++}, Na^+ and K^+ on the A.T.P.-ase activity of a mucosal stomach extract from a 28 day foetus.

Cations present.	Enzyme activity in μMP_i/mg.Pr./min.
None	0.011
With 1mM Mg^{++}	0.169
With increasing K^+ concentration	0.011 to 0.022
With increasing Na^+ concentration	0.012 to 0.016
With Na^+ and K^+ ions.	0.008 to 0.019.

Table V   The effect of the presence of ouabain on the A.T.P.-ase activity; expressed as μMP_i/mg.Protein/min. Ouabain concentration 2.5g/litre.

Experiment	Activity without ouabain.	Activity with ouabain.
1	0.217	0.164
2	0.205	0.152
3	0.172	0.125

The action of ouabain on the activity of the A.T.P.-ase
enzymes, prepared in a tris-acetate buffer, was studied. No
inhibitory action could be found on the enzymes fromstomachs
of foetuses aged either from 24-26 days, or from 28-30 days.
Since there was a possibility that the activity was masked; the
enzyme was left in a urea solution of 1.0M for periods of 12 to
48 hours; and then incubated with Na^+, K^+ and ouabain, to see
if any ouabain-sensitive fraction had been unmasked. Under these
conditions there was a slight indication of an ouabain-sensitive
fraction.

Isolation of the enzyme was then tried in a tris-chloride
buffer containing E.D.T.A. (ethylene-diamine-tetra-acetate):

        sucrose   250mM.

        E.D.T.A.   2mM.

        tris-chloride buffer 30mM, pH 7.8. (according to

                                        Glynn, 1962)

and urea was added to the enzyme suspension to give a final
concentration of 1.0mM and left for 48 hours at $4^{\circ}C$.

With an incubation medium containing:

    NaCl - 100mM, KCl - 20mM, $MgCl_2$ - 2mM, sucrose-16mM,

    A.T.P. - 3mM, Tris-chloride buffer - 30mM, pH 7.8,

    and ouabain added to some flasks at a concentration of

    2.5g/litre;

a 25% inhibition with ouabain was obtained. Urea used in these
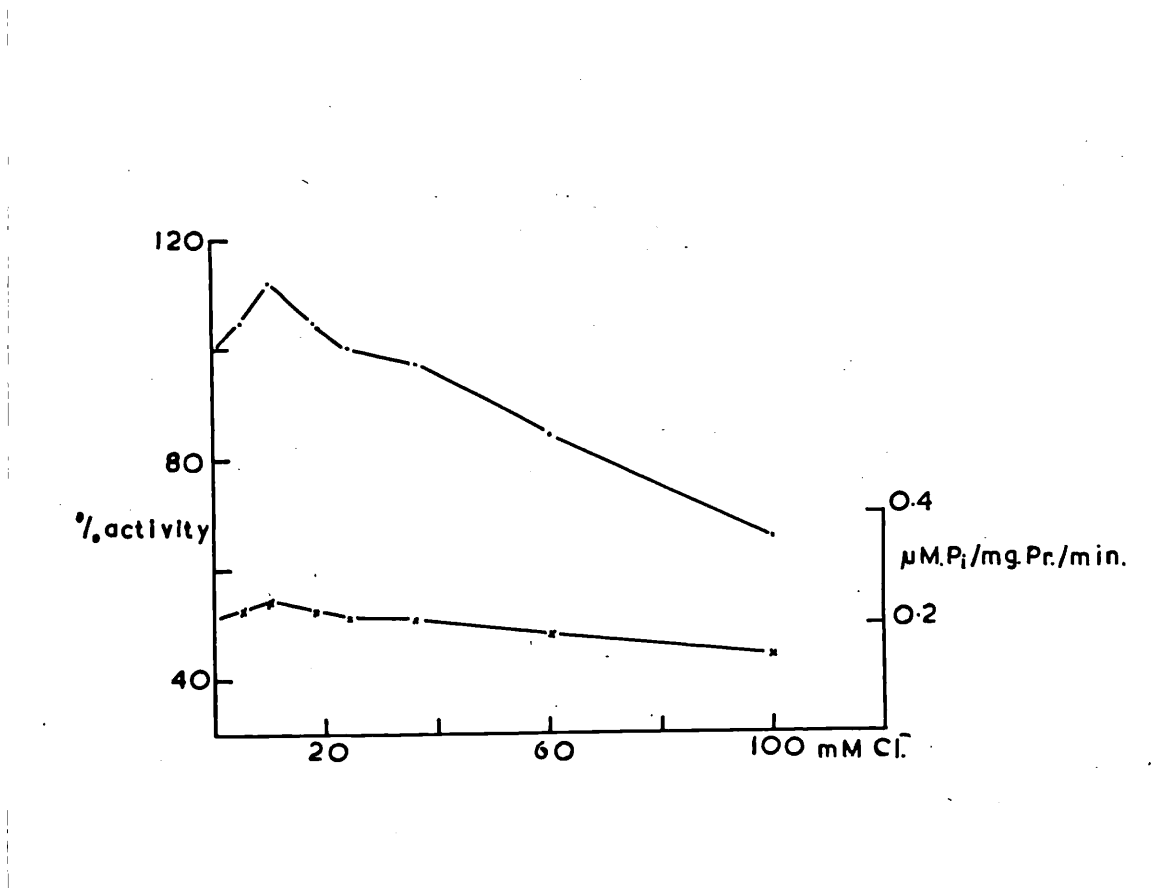
Fig. 53.   Showing the effect of adding Cl⁻ ions to the

mucosal A.T.P.-ase enzyme from stomachs of foetuses aged

from 24 to 26 days.

x ———— x shows μMP_i/mg.Pr./min.

.———— . shows % activity: 100% activity being when there

is no Cl$^{\pm}$ in the incubating medium.

-127-
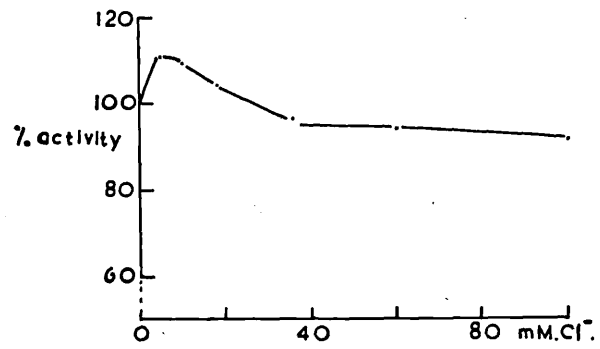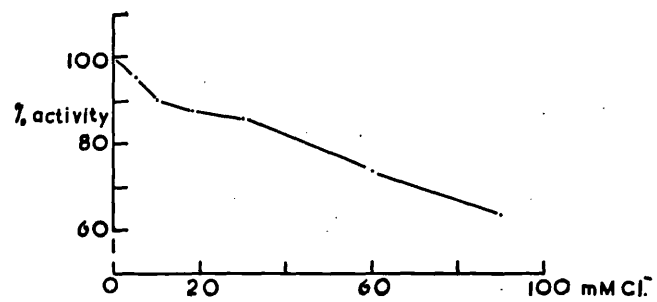
Fig.54.

Fig.55.

The effect of adding Cl⁻ ions to the A.T.P.-ase enzyme
from gastric mucosa:

Fig.54.   from foetuses aged 28 to 30 days.

Fig.55.   from female adult rabbits.

concentrations did not cause inhibition of the enzyme activity.
See table $\underline{V}$ for results.

Effect of the Halides.

If the mechanism of acid secretion is directly linked to
A.T.P. breakdown; it is possible that a chloride-stimulated
A.T.P.-ase is present in the stomach. A tris-acetate solution
was used as a chloride-free buffer.

Effect of Chloride Ions.

Incubating medium: Tris-acetate buffer   20mM,   pH 8.0.

Mg^{++} —   1mM.   A.T.P.   —   2mM.

enzyme.

The chloride was added as choline chloride. Concentrations of
up to 20mM Cl^- stimulated the enzyme, maximum stimulation was
obtained at approximately 10mM chloride. Higher concentrations
than 20mM caused inhibition of the activity, which was much
more marked in the enzyme prepared from stomachs of foetuses
aged 24-26 days; 100mM Cl^- gave only 75% activity, Fig.53. With
the 28 day foetal stomachs, 100mM Cl^- gave 90% activity, Fig.54.
Enzyme extracts from adult stomachs, Fig.55, showed no
stimulation with addition of chloride; the degree of inhibition
being more like that observed in extracts from the stomachs
of the younger foetuses than the 28-30 day ones.

Bromide Ions.

Addition of low concentrations of bromide ions caused slight
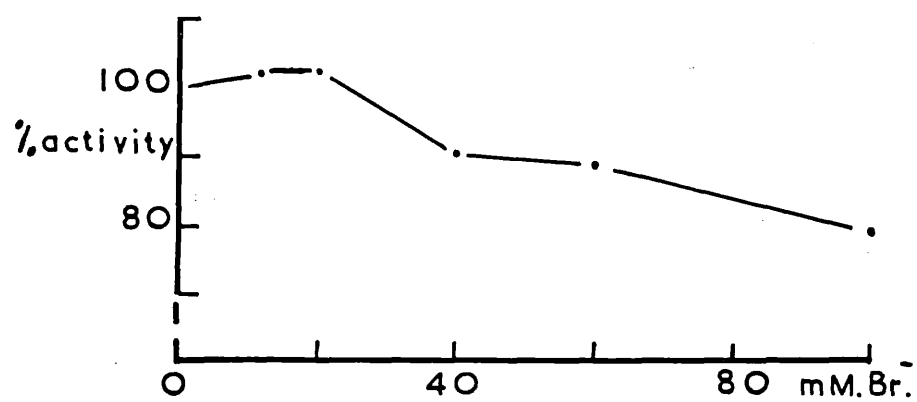
Fig.56. The effect of Bromide ions on the gastric mucosal
A.T.P.-ase enzyme preparation from foetuses aged 28 to 30
days.

Fig.57.
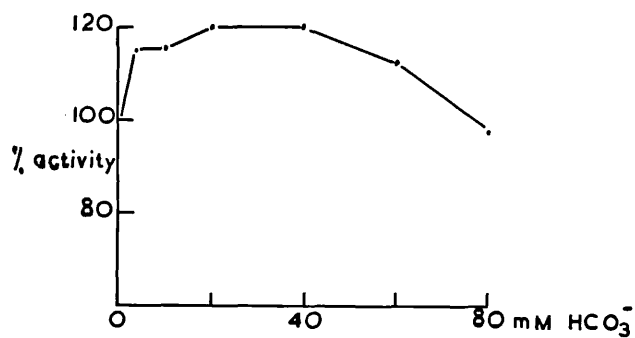
Fig.58.
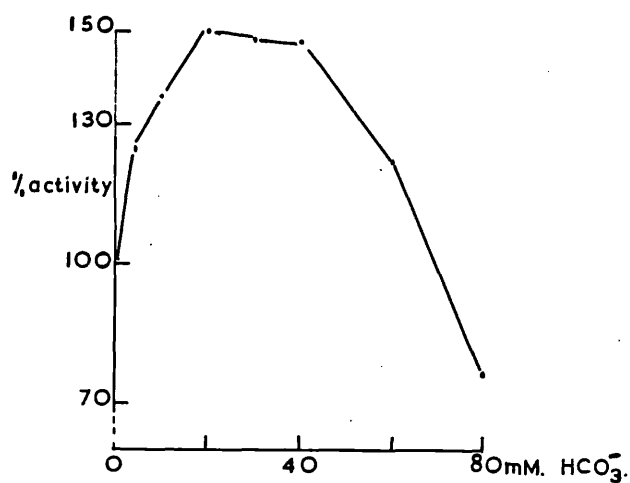
The stimulation produced, by the addition of the HCO_3^- ion, in the activity of the gastric mucosal A.T.P.-ase enzyme:

Fig.57.   from foetuses aged 28 to 30 days.

Fig.58.   from female adult rabbits.

stimulation of activity , but an inhibition occurred after 12 to 15mM bromide. The effect was very similar to that obtained with chloride. Fig.56 shows an experiment using a mucosal extract from the stomach of a 28 day foetus.

Effect of the Bicarbonate Ion.

This ion was used as it has been involved in one of the theories for acid secretion. Care was taken to ensure that the final pH of the incubation medium was not affected by the addition of the bicarbonate ion; and the pH value used was that used before. A definite stimulation was obtained with this anion with both foetal (Fig.57) and adult (Fig.58) mucosal preparations; of approximately 120-130% activity in the presence of the HCO_3^- ion, up to a concentration of 40mM; then the activity declined again. Muscle preparations gave similar results,

The Effect of the Thiocyanate Ion.

The thiocyanate ion is known to inhibit acid secretion both in-vivo and in-vitro; so its effect on the gastric A.T.P.-ase was determined. The degree of inhibition obtained was directly proportional (Fig.59) to the log. concentration of the thiocyanate ion (SCN^-); and at high SCN^- ion concentrations, $10^{-1}M$, the enzyme was about 70% inhibited. The addition of chloride ion gave release of the inhibition caused by $10^{-3}M$ SCN^- ion. If the activity of the enzyme when neither SCN^- nor Cl^- ions were present in the incubating medium, is regarded as
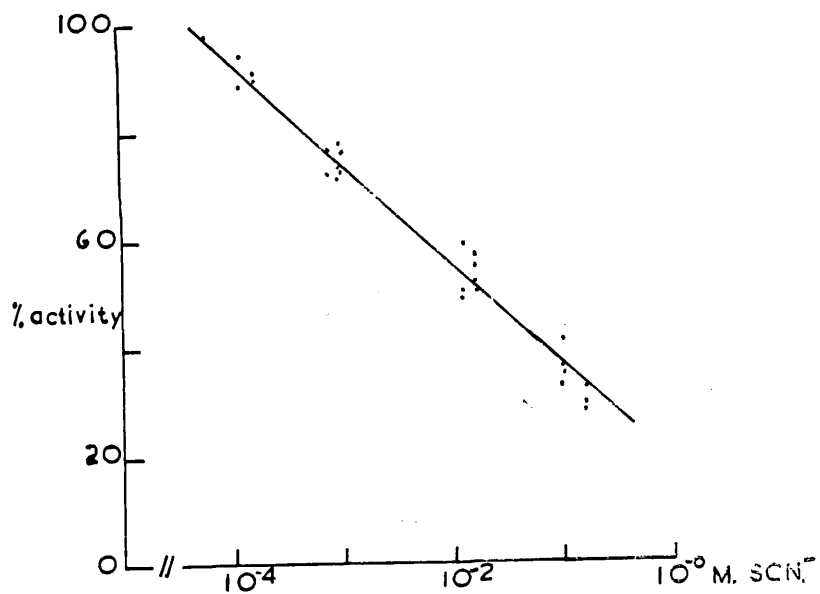
Fig.59.   The effect of the addition of the thiocyanate ion

(SCN⁻), on the activity of the A.T.P.-ase enzyme from

stomachs of foetuses aged from 28 to 30 days.

Table VI   The effect of the SCN^- ion on mucosal and
muscle enzyme activity; expressed as a percentage of the
activity when SCN^- not present.

SCN^- concentration.	Mucosa % activity.	Muscle % activity.
zero	100	100
2.4×10^{-4}M.	92	91
1×10^{-3}M.	78	81
$1. \times 10^{-1}$M.	41	42

Table VII   The release of SCN^- ion inhibition of A.T.P.—ase
activity by the HCO_3^- ion in both mucosal and muscle extracts.

SCN^- conc.	HCO_3^- conc.	Mucosal activity.	Muscle activity.
zero	zero	100%	100%
1×10^{-2}M.	zero	79%	82%
1×10^{-2}M.	20mM	101%	105%
$1. \times 10^{-2}$M.	40mM	103%	106%

100%; then:

$$\text{Activity with } 10^{-3}\text{M SCN}^- \quad - \quad 75\%$$

$$\text{Activity after the addition of } 100\text{mM Cl}^- \quad - \quad 94\%$$

However chloride did not release the inhibition caused by 10^{-2}M SCN$^-$; about 50%.

The bicarbonate ion, however, caused a much greater release of inhibition; 40mM HCO_3^- gave an activity of 117%, when activity with SCN$^-$ alone was 76%.

When the thiocyanate ion was added to the muscle extract a more or less identical picture was produced: table VI.

The bicarbonate ion also produced a release from thiocyanate induced inhibition in the muscle extract which was comparable with that for mucosal extract: table VII.

CHAPTER V.

A DISCUSSION OF THE RESULTS OBTAINED.

CHAPTER V.

Discussion.

Section I.

The stomach of the foetal rabbit was used as the experimental tissue in the study of the phenomenon of ion transport and metabolism in the mammalian stomach, because it is one of the few mammalian stomachs which can survive under the techniques used; and it was desired to know whether the metabolism of the foetal stomach was very similar to that of the adult animal of if it had any properties specific to foetal conditions. Rat stomachs have been clamped as a membrane between two chambers (Pattensen & Stetton, 1949), but the blood supply had to be kept intact if the tissue was to retain its spontaneous P.D. over any period of time. The mouse stomach was the only mammalian stomach capable of surviving under in-vitro conditions (Crane & Davies, 1948), until Wright (1962) used the stomach of the foetal rabbit. Maintainance of the spontaneous P.D. of the foetal rabbit stomach depends on, primarily, an adequate oxygenation of the bathing solutions, any blockage in the supply of oxygen causes a rapid reduction in the spontaneous P.D.. The thickness of the muscle coat of the stomach probably acts as a barrier to the diffusion of oxygen, and in the foetal stomach of 27/28 days the muscle coat is very thin, but gets gradually thicker towards full term; and it is just possible to

get a stomach from a one day post natal animal to survive under in-vitro conditions, but constant stirring and adequate oxygenation are essential.

This tissue was also chosen because of the earlier work on the cytology of the foetal rabbit stomach, by Menzies (1958); and that of Wright (1962 & 1963) on the electrolyte transfer and relationship between the P.D. and S.C.C. and the presence of specific ions. Wright initially showed the dependence of 70% of the S.C.C. of the stomach on the presence of mucosal sodium.

The measurements of the net flux of sodium and the S.C.C. have shown that I_{Na} can be used as a direct measure of the net flux of sodium from mucosa to serosa. This result could have been expected from Wright's work, but there remained the possibility that the sodium independent component could have been altered by the presence of sodium on the mucosal side. The results presented show that this does not in fact occur.

Previous work of Wright (1963) had shown, and the author confirmed, that the spontaneous P.D. in the stomachs of foetuses of 21/22 days gestation, was totally dependent on the presence of sodium ions on the mucosal side; and as Menzies (1958) had shown that only non-differentiated cells were present; the active transport of sodium from mucosa to serosa is a function performed by the non-differentiated cells. In the same paper Wright also showed that the onset of hydrochloric acid

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secretion into the mucosal solution co-incided with the appearance of oxyntic cells in the gastric mucosa. After this time (the 23rd day) the P.D. was no longer totally dependent on the presence of mucosal sodium; and thus the sodium independent P.D. and S.C.C. were assumed to arise from the oxyntic cell activity associated with acid secretion.

The results presented in Chapter II have shown that the sodium independent S.C.C. and the net chloride flux are in opposite directions and that the latter is 166% of the former. Attempts to measure directly the rate of acid secretion were made; but it was not possible to get a large enough Area (of membrane) to Volume (of solution) ratio with the various sizes of chamber used. If a small volume was used, oxygenation and mucosal mucus caused frothing and hence loss of volume; and serosal oxygenation alone was not adequate to sustain the P.D. The area of the membrane was naturally limited by the size of the foetal stomachs; the largest area obtainable from nearly full term foetuses was about 1.3 to 1.4sq.cm.. Acid secretion could be measured if a whole stomach was mounted on a canula, as a bag, but in this case, although the P.D. could be recorded, the S.C.C. could not, due to the inability to find an electrode of low enough resistance. Results for the replacement of chloride with an ion not expected to be subject to active transport have been given.

If the hydrogen ion and chloride ion were secreted at the same rate, no S.C.C. would be recorded with acid secretion. The following explanation may be considered:

(1) The chloride is transported electrogenically; the hydrogen transport is non-electrogenic; and no other electrogenic process is occurring. Hydrogen and chloride ions may or may not be secreted in equivalent quantities.

(2) Both chloride and hydrogen transport is electrogenic, more chloride being secreted than hydrogen; and no other electrogenic process occurring.

(3) Both chloride and hydrogen being secreted electrogenically in equivalent amounts; some other anions being subjected to electrogenic transport from serosa to mucosa.

(4) Electrogenic transport of both chloride and hydrogen ions in equivalent amounts; and some other cations being subjected to electrogenic transport from mucosa to serosa.

(5) Neither chloride nor hydrogen ions being subjected to electrogenic transport; but some other anion being electrogenically transported from serosa to mucosa.

(6) Neither chloride nor hydrogen ions being subjected to electrogenic transport; but some other cation being electrogenically transported from mucosa to serosa.

Chloride has been demonstrated (Wright, 1963, 1964) to be secreted always in excess of hydrogen ion secretion, hence

ruling out possibilities (3) and (4). The potassium ion has
been shown to move down its electro-chemical gradient, in
either direction; and substitution of the choline ion for
potassium in sodium-free solutions caused no alteration in P.D.
or S.C.C. (Wright, 1964). Thus the sodium independent S.C.C.
would receive no contribution from choline or potassium, the
only cations present in the mucosal solution, so ruling out
alternatives (4) and (6). Alternative (1) would only apply if
the chloride net flux was exactly equal to the sodium independ-
-ent S.C.C.; and it has been shown to be 166% of it. Since the
chloride flux appears to bear this direct relationship to the
sodium independent S.C.C., it would appear to be electrogenic;
thus ruling out (5) and (6). This leaves alternative (2) as the
possible explanation. However if this was so, replacing the
chloride by some other non-actively transported anion would be
expected to reverse the P.D. and S.C.C.; however this has been
shown not to be the case; and it is suggested that the S.C.C.
which persists is due to non-specific anion transport: this
point needs verification by labelled anions.

It could be suggested that chloride is being transported
out of the cells and giving rise to the current observed.
However calculation of the maximum amount of chloride that could
exist in these intracellular pools at the beginning of the
experiment indicated that it could not provide the amount of
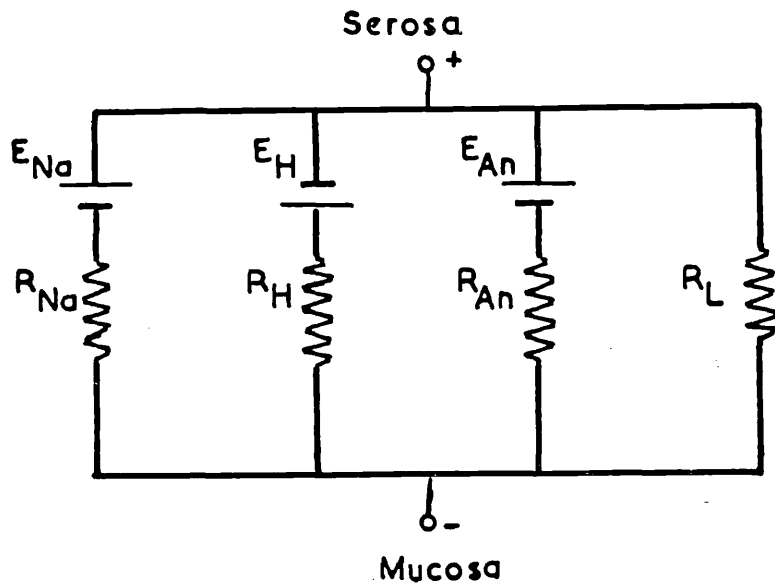
Fig.60.   Showing a possible equivalent circuit for the

stomach of the foetal rabbit. E_{Na}, E_H and E_{An} are the E.M.F.'s

of the sodium, hydrogen and non-specific anion transport

systems. R_{Na}, R_H and R_{An} are the internal resistances of these

systems, and R_L is the leakage pathway through the mucosa.

charge which was transported by the S.C.C. over the duration of
the experiment. Using Fig.6, where the mean S.C.C., after
addition of glucuronate in place of chloride, on both sides and
with sodium-free solution on the mucosal side, was $100\,\mu A/1.15cm^2$
over a period of 2 hours; calculation showed this to be
equivalent to the charge carried by $7.4\,\mu eq$ of chloride. A
typical value for the wet weight of a preparation is 0.14gm.
and 20% of this is assumed to be intracellular water in which
chloride is assumed to be present at a concentration of 150meq/
litre, then the amount of chloride initially contained in the
cells is $4.2\,\mu eq$.

The results presented in Chapter II, considered together
with those of Wright (1963, 1964) lead to the conclusion that
the majority of the S.C.C. of the stomach of the foetal rabbit
(aged from 27 to 30 days) is due to a specific transport of
sodium from mucosa to serosa; and the remaining fraction is
associated with secretion of hydrochloric acid from serosa to
mucosa; the chloride perhaps being transported by a non-specific
anion transport system. These results could be represented by
the equivalent circuit (Fig.60) (Kendall & Wright, 1967) where
E_{Na}, E_{An}, E_H are the E.M.F.'s of the sodium, anion and hydrogen
transport systems. R_{Na}, R_{An} and R_H are the internal resistances
of these systems and R_L is the leakage pathway through the
mucosa. A similar circuit was proposed by Bornstein, Denis and

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Rehm (1959) to describe the ionic movements in the resting stomach of the dog where sodium was found to be actively transported.

Section II.

The metabolic dependence of the ionic movements in the foetal stomach is of interest both from the possible light it might throw on the source of energy for acid secretion, and as a study of the metabolic activity in a foetal tissue. Anoxia is about the only condition which the foetus could have to face in its natural environment; and several papers indicate that the foetus could have a large supply of glycogen, which it could metabolize anaerobically if it so required (Shelley, 1961); and could survive for up to 20 minutes after the death of the mother.

When the foetal stomach was subjected to anoxic conditions, it was 2 or 3 minutes after nitrogen was given before there was any fall in the P.D. or S.C.C.. Oxygen electrodes, in the bathing solution, showed that the oxygen tension had dropped to about 200 or 250mm Hg., before any decline in the S.C.C. occurred, and was zero after 5 minutes, when S.C.C. readings were still high. If the sodium transport mechanism was directly dependent on oxidation, the rate of fall would have been more rapid; and no fraction of the current would have remained after long periods of anoxia. The results obtained indicate that

approximately 10 to 15% of the total sodium current can be maintained under complete anoxic conditions; which suggests an ability to utilize the glycolytic mechanism. The sodium independent current, and hence acid secretion, is completely dependent on adequate oxygenation and the rapid decline in current suggests a possible direct dependence on oxygen. The sodium current declines in such a way that it suggests another source will support the cation movement, but that it is not as efficient as the oxidative mechanism, hence the decline in S.C.C. If a similar effect occurs in the foetus in situ, this could be described as a type of protective mechanism. The acid secretion which requires a large amount of energy, and is not necessary for immediate survival, is rapidly inhibited; the reabsorption of sodium is considerably reduced, but a fraction is retained: salt retension being a primary consideration of animals. The foetal kidney also has the ability to utilize energy from anaerobic mechanisms (Whittam, 1960) in order to retain sodium.

Amytal and rotonone both inhibit oxidative phosphorylation by blocking the reaction at the first site of phosphorylation, their rates of inhibition being about the same. The lack of 두 further effect of anaerobic conditions, after a maximal inhibition has been reached, in both sets of experiments, indicates that respiration was also inhibited by these drugs. As amytal will inhibit respiration only when linked to

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phosphorylation, this shows that oxidative phosphorylation is occurring in the isolated gastric mucosa. The sodium independent current was completely inhibited by both drugs, but at a slower rate to that when under anoxic conditions. Thus oxidation was being inhibited more gradually, or the acid secreting mechanism could utilize A.T.P. and its production was being inhibited slowly, or there was an A.T.P. store on which the mechanism could draw. The question then arises, if an energy or A.T.P. store exists, why cannot the tissue use it, under anoxic conditions, to sustain the sodium-free component: there is the possibility that it can only be utilized when oxygen is present, or that oxygenation is required directly for maintainance of the sodium-free component. Perhaps the acid secretion directly needs oxygen, and the amytal is slowly inhibiting respiration. There is about 20 to 25% of the sodium component remaining after maximal inhibition by the drug has been obtained, and this is only slightly affected by anoxia; thus 20 to 25% of the sodium transport can be maintained from anaerobic glycolysis, the rest is dependent on oxidative phosphorylation.

Antimycin A is an inhibitor of the second site of oxidative phosphorylation in the respiratory chain, and has an immediate action on the activity of the foetal gastric mucosa. The rate of decline of the S.C.C. was fairly rapid and in the majority of cases mucosal application was much more effective. This could be

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due to the diffusion rate of the drug through the membrane,
especially if the site of inhibition was placed relatively
nearer to the mucosal surface than the serosal. Again anoxic
conditions have no further effect upon the maximum inhibition
obtained with the drug.

Oligomycin also inhibits oxidative phosphorylation at the
second site of phosphorylation, but represses oxidation steps
only when closely coupled to phosphorylation, but not in an
uncoupled system; though it inhibits A.T.P.-ase and net
phosphorylation in both systems. It seems as though oligomycin
acts on the phosphorylating apparatus rather than on respiration.
Addition of D.N.P. at about 10^{-4}M concentrations, which should
uncouple oxidation from phosphorylation (Racker, 1965), and act
as an electron carrier, has been shown to release respiration
from inhibition caused by oligomycin. However in experiments
performed on the stomach of the foetal rabbit, no release of
inhibition occurred in either the total current, sodium dependent
or sodium independent current; which suggests that in this tissue
oxidative steps in the cytochrome chain are not adequate to
sustain the active transport of sodium, hydrogen or chloride
ions.

Both D.N.P. and azide are well known uncouplers of oxidative
phosphorylation. D.N.P. is a true uncoupler and releases
phosphorylation from respiration, the latter usually being

stimulated. Azide, however, has been reported to stimulate mitochondrial A.T.P.-ase, and then inhibit it (Myers & Slater, 1957); as well as uncoupling oxidative phosphorylation, and it also inhibits respiration. The results obtained on the foetal stomach illustrate this latter point. When nitrogen was bubbled through a system where maximum inhibition with azide had been obtained, no further inhibition occurred under anoxic conditions. However when the system was uncoupled with D.N.P. and maximum inhibition was obtained (about 30% of the initial current remaining) passage of nitrogen through the preparation caused a further decline in the S.C.C., leaving about 5 to 15% of the original current.

Inhibitors of the glycolytic cycle, such as fluoride, also caused a large inhibition of the total S.C.C. of about 60 to 70%, and total inhibition of the sodium independent current. However in both cases there was a time lag before any decline in the current occurred; about 30 minutes in the case of the total current; and a smaller, but still significant, lag of about 10 minutes, when the sodium independent component was measured. Even if the drug was not diffusing almost immediately into the tissue, the experiments with the sodium independent component show that the drug can reach the important metabolic site after 10 minutes; which leaves a further 20 minutes in which the drug is assumed to be present at the specific site but is having no

effect on the total and sodium dependent current. Fluoride prevents the normal functioning of the Krebs' cycle, by both inhibiting the enzyme 'enolase', and hence preventing formation of pyruvate; and by irreversibly forming fluorocitrate and hence any pyruvate present is not oxidized efficiently; and the supplies of reduced Co-enzyme I and II, for the cytochrome chain, are considerably reduced. The only supply available is from substrate level oxidation of glucose and glycogen; hence the production of A.T.P. is considerably reduced. Subjecting the tissue to anoxic conditions after maximal inhibition occurs with the total S.C.C. causes further reduction of the current, to about zero; indicating that no anaerobic, energy producing mechanism is left; and that the fraction of the current that remains after maximal fluoride inhibition is due to substrate level oxidation of glucose and/or glycogen. The sodium independent component is completely inhibited, which could be due to reduction of A.T.P. levels, if A.T.P. is needed, or to the fact that oxidative phosphorylation would be very low, or completely inhibited, in the cytochrome chain, due to the lack of supply of reduced Co-enzyme.

Iodoacetate is an inhibitor which combines with the 'SH' groups in the enzymes of the energy producing mechanisms; and blocks mainly the Krebs' cycle, again at the point of entry of pyruvate, acetyl Co-enzyme A is complexed, and also

glyceraldehyde-3-phosphate dehydrogenase is inhibited by
iodoacetate, thus preventing any reduced Co-enzyme I from
entering the cytochrome chain. Inhibition only commences after
about 30 minutes, in the case of the total current and after
10 minutes with the sodium independent current; so that the
same arguement, about the rate of entry of this drug into the
tissues, applies, as for fluoride.

Experiments with substrate-free bathing solutions have shown
that the foetal stomach can survive at its normal high P.D.
and S.C.C. for periods of 90 to 120 minutes before any decline
occurs and then the rate of fall is slow; and it can be restored
to the maximum values again on the addition of glucose to the
bathing media. It would appear therefore that the foetal tissue
has a definite store of energy in some form, which could
possibly be glycogen, and/or a small reserve supply of A.T.P..
The experiments with both fluoride and iodoacetate, and with
the latter in both substrate-free and substrate containing
media; suggest that there could be an A.T.P. store. If the
energy store was of glycogen, the inhibitory action of these
two drugs would most likely prevent the conversion of glycogen
to glucose and the subsequent oxidation of reduced co-enzymes,
forming A.T.P., long before any decline actually occurs in the
sodium current. With the sodium-free component, the current
declined after about 10 minutes, and allowing a major part of

this for the diffusion of the drug into the tissue (as indicated
by the experiment with iodoacetate in a substrate-free media,
(Fig,35) when the rate of decline of the S.C.C. did not increase
until about 10 minutes after addition of the drug, despite the
fact that the S.C.C. had begun to decline slowly beforehand)
it could suggest that the sodium-free component could not be
energized by stored A.T.P.; or that the co-enzymes were all
oxidized. The idea of an A.T.P. store would also be in good
agreement with the initial time lag and slow rate of inhibition
of the total current with D.N.P..

The experiments with cardiac glycosides do suggest a need
for A.T.P. in the transport of sodium from mucosa to serosa, but
there is no ready explanation for the complete inhibition
obtained with the sodium independent component of the current.
In the $(Na^+ \& K^+)$ stimulated A.T.P.-ases from other tissues ouabain
appears to compete with K^+ ions and at the high doses used there
may have been some interference with the role of K^+ ions in the
secretion of acid.

The residual fraction of 10 to 20% of the total current and
hence about 12 to 25% of the sodium current, (the sodium-free
component being completely inhibited) which remained after
subjecting the tissue to anoxic conditions, can be accounted
for by the A.T.P. that can be formed by the normal functioning
of the anaerobic glycolytic cycle. Ouabain in substrate-free

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media, caused complete inhibition of the total S.C.C. and P.D..

The experiments performed using different substrates indicate that of the exogenous substrates tried the tissue can utilize only glucose, thus they did not answer the question initially posed, of whether the S.C.C. declined, (when inhibitors were used) because of a general lack of A.T.P. or because the oxidative phosphorylation system was not functioning normally. However these results are in some agreement with the observation of Davenport & Chavre, (1952) that glucose, but not fatty acid derivatives, could maintain the normal working of the mammalian gastric mucosa. Alonso et al. (1967) studied the energy sources for gastric metabolism and reached a general conclusion that amphibian gastric mucosae were able to utilize fatty acid derivatives as exogenous substrates; but that the mammalian tissue needed glucose or some glycolytic intermediate in order to sustain its P.D. and S.C.C.. They did however suggest that the normal treatment of the experimental animals before death could have some bearing on the results obtained. Frogs are usually starved for several days before use; and starved tissues are known to utilize fatty acids in preference to carbohydrates. Also the normal frog diet contains little carbohydrate compared with the mammal.

The experiments with the metabolic inhibitors fall into two main groups. Those involving inhibitors which directly affect

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the respiratory chain; (eg. anoxia, amytal, rotonone, azide

and antimycin A), and a second group where the inhibitors act on

the energy producing (oligomycin, iodoacetate, fluoride, D.N.P.)

or utilizing (cardiac glycosides) mechanisms. In the former

group inhibition is rapid and immediate and the maximum

inhibition unaffected by anoxia; in the latter a time lag before

inhibition commences is often seen, (except for cardiac

glycosides) and it is possible that the inhibition only started

because there was a general lack of A.T.P. in the system; and

thus the metabolic breakdown of glucose could not proceed

because there would be no A.T.P. 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. The

experiments with β-hydroxybutyrate did not provide an answer

because in experiments free from drugs this substrate could not

sustain the tissue over long periods.

Section III

The results obtained with the metabolic inhibitors showed

that there could be a possible direct involvement of A.T.P. in

the active transport of sodium ions, especially as in numerous

other tissues A.T.P. has been found to be directly involved

with the suggested carrier mechanism for active sodium transport,

and (Na^+ and K^+) stimulated A.T.P.-ases isolated: (Glynn, 1965).

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It was decided to see if an A.T.P.-ase could be isolated from the gastric mucosa and if it was sensitive to the presence of sodium and potassium ions. The active sodium transport in the gastric mucosa of the foetus responded to the presence of cardiac glycosides, like ouabain (and strophanthin K), in the same way as do red-cells and nerve, and in both of these tissues a (Na^+ & K^+) stimulated, Mg^{++} dependent, A.T.P.-ase has been demonstrated. The results obtained with the sodium independent component are strongly favouring a direct oxygen involvement, but as D.N.P. did cause inhibition, and recently Kasbekar & Durbin, (1965) have claimed to have isolated a halide stimulated A.T.P.-ase from frog gastric mucosa; the A.T.P.-ase enzyme which was isolated from foetal rabbit gastric mucosa was tested to see if it would be stimulated by halide ions.

The gastric A.T.P.-ase was greatly stimulated by the presence of Mg^{++} ions and the amount of activity in the 28 to 30day, and 24 to 26 day foetal stomachs was of the same order, when expressed as /uMoles of phosphate per mg. of protein per minute. However there was a much greater degree of activity in the adult extracts. The foetal mucosa would contain mainly undifferentiated cells and a few oxyntic cells. The adult mucosa has a much more complicated structure, so no relationship can really be deduced between the level of A.T.P.-ase activity and the number of oxyntic cells present in the gastric mucosa. If a

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direct relationship did exist it would have been expected that the 24 to 26 day foetal stomachs would have shown a smaller degree of activity than the stomachs from the 28 to 30 day foetuses.

The observation that the muscle extracts displayed an almost equivalent amount of A.T.P.-ase activity to the mucosal extracts was rather disturbibg. They were both greatly stimulated by the presence of Mg^{++} ions, but the two extracts did differ slightly in their response to the addition of calcium ions. In magnesium-free solutions, calcium ions caused stimulation in all extracts, but in the presence of Mg^{++} ions inhibition occurred to a much greater extent in the muscle fractions, than in the mucosal. Fig.47 demonstrated that competition occurred between the two cations.

The experiments carried out on mucosal enzyme showed no definite stimulation of activity on addition of sodium, potassium, or sodium and potassium together. The possibility of the activity being masked, as shown by Glynn et al. (1965) for the electric organ, was looked into, but after treatment with urea, no activity was revealed. A fraction of the total activity did respond to the presence of cardiac glycosides and a maximum amount of 25% of the total, was inhibited, but these results tended to be variable; also this was not cardiac glycoside inhibition of the activity stimulated by sodium and

potassium. However this did suggest that a fraction of the
A.T.P.-ase activity could be involved with sodium movement; but
as no sizable stimulation was obtained in the presence of sodium
and potassium ions, these results do not justify a conclusion
that a fraction of the A.T.P.-ase enzyme is involved with active
sodium movement. In fact in the isolated gastric mucosa of the
rabbit, ouabain has been demonstrated to inhibit the sodium
independent component, and in the frog to inhibit acid
secretion. However, increasing the potassium ion concentration
does release this inhibition, suggesting that the inhibition is
probably linked with ouabain inhibition of the sodium and
potassium carrier, so the suggested connection between the
A.T.P.-ase and sodium transport is still valid. In the adult
rabbit stomach there is no active transport of the sodium ion,
thus no (Na^+ & K^+) stimulated A.T.P.-ase would be expected; and
the results obtained with the A.T.P.-ase preparation from the
adult stomach shows no stimulation in the presence of sodium
and potassium. In fact the results with both adult and foetal
extracts were the same. The muscle extracts also showed no
response towards the presence of sodium and potassium ions.

Initially the results with the halide ions, and especially
the thiocyanate ion, suggested a very possible link between the
A.T.P.-ase enzyme and acid secretion. The thiocyanate ion
caused a degree of inhibition proportional to the logarithm of

the ionic concentration; in both 28 to 30 day, 24 to 26 day, and in adult extracts. This effect was reversible when chloride or bicarbonate ions were added, provided that the initial inhibition was not greater than about 40%. Chloride caused a degree of stimulation at lower concentrations, up to 20mM, as did bromide ions, in all gastric mucosa extracts. The bicarbonate ion produced the greatest degree of stimulation, at concentrations of up to 40mM. These results were in good agreement with those published by Kasbekar & Durbin, (1965) for frog gastric mucosa, and those for adult rabbit gastric mucosa by Forte, Forte & Bils, (1965). However, when the enzyme extract from the muscle coat, free from mucosal contamination, (as far as could be judged) was subjected to the same ions, almost identical results were obtained. This result, combined with the lack of difference between the 28 to 30 day, and 24 to 26 day foetal extracts, makes the connection between acid secretion and the A.T.P.-ase enzyme, isolated from the foetal rabbit gastric mucosa, much less convincing.

It is suggested that the inhibition produced by the thiocyanate ion might be due to the lipid solubility of the thiocyanate. Due to its electrostatic attraction the ion could then enter the protein and disrupt it. On this basis other anions could also cause inhibition in reverse order to their degree of hydration. However the stimulation obtained with the bicarbonate

ion cannot be explained on this basis, and would instead fit in very well with the suggested link between A.T.P.-ase and acid secretion, as the bicarbonate ion is known to cause stimulation of acid secretion; but this would not explain the stimulating effect the bicarbonate ion has on the muscle extract. Even if there was a slight contamination of mucosal cells in the muscle extract, the degree of stimulation would be expected to be much less than that of the extract comprising total mucosal tissue.

However, despite the unexplained result with the bicarbonate ion, the overall results do not really justify a conclusion that the A.T.P.-ase isolated from the gastric mucosa is directly involved with acid secretion; nor does the A.T.P.-ase seem to be involved in transport of sodium across the membrane; unless there has been a failure to unmask the activity, which seems unlikely considering the various processes used. A small fraction could indeed be indicated from the ouabain experiments, but this would not seem to be adequate to account for all the sodium actively moved in this tissue.

Section IV

There are two main active transport processes in the foetal rabbit stomach. One is the active transport of sodium ions from mucosa to serosa; carried out by the undifferentiated cells, and present in the stomach definitely from the 20th day of

gestation (it has not been possible to measure the transport processes before that time), and responsible for all the P.D. observed until the 23rd day of gestation when acid secretion, the second active transport process, commences.

The various experiments carried out with metabolic inhibitors show a very definite dependence of the sddium independent component, and hence acid secretion; on the presence of oxygen. The relatively rapid response of the sodium independent component to anoxia suggests that there could be a close relationship between it (and hence acid secretion) and electron transport in the cytochrome chain. With all the drugs used, any drug directly inhibiting respiration caused the sodium independent S.C.C. to fall to zero; and no time lag was evident. With inhibitors which affected the metabolic pathways, there was a time lag which was followed by complete inhibition. This might indicate the need for, and a rundown of, some co-enzyme in the oxidized state with consequent interference with the normal functioning of the cytochrome system. Since there would be no hydrogen atoms splitting into H^+ ions and electrons, it can be visualised that there may be no hydrogen ions available for acid secretion. Uncoupling agents, such as D.N.P. and amytal cause inhibition in both the sodium independent component of the gastric mucosa in the foetal rabbit and also in the acid secretion of the frog. Bannister, (1965b)

claims that D.N.P. inhibition does not necessarily mean that
A.T.P. is involved in acid secretion of the frog gastric mucosa,
and favours the hypothesis discussed by Robertson, (1960), (see
page 28). Whittam (1963) also states that D.N.P. causing failure
of active transport processes does not necessarily mean that
A.T.P. is involved, as the site of action of D.N.P. is unknown.
Thus it could well prevent the separation of the negative and
positive charges of the hydrogen atom in the oxidative
phosphorylation chain. Even if D.N.P. does not act in the way
suggested by Robertson, uncoupling of oxidative phosphorylation
would result in a general lack of A.T.P., so no glucose would
be metabolised and hence pyruvate would not be available to
enter the Krebs' cycle, so there would be no further formation
of reduced co-enzymes; and yet again metabolic hydrogen atoms
would not be available for the respiratory chain. Amytal, as
well as uncoupling oxidation from phosphorylation also inhibits
respiration; so the results obtained in the experiments on the
foetal stomach with this drug are still in agreement with the
direct dependence of acid secretion on oxygen.

The experiments performed suggest that the sodium transport
is energised by a different mechanism than is the sodium
independent component. Although it was not possible to isolate
an A.T.P.-ase which showed a definite stimulation in activity in
the presence of sodium and potassium ions, it still appears that

the sodium transport is energised by A.T.P.. The main section
of the experimental evidence in favour of the suggestion is
that about 15-25% of the sodium current still remains during
anoxic conditions, when energy for the process could only come
from some high-energy intermediate of metabolism. The only
chemical reactions common to both aerobic and anaerobic
mechanisms are the oxidation and reduction of NAD and the
synthesis of A.T.P.. However the involvement of NAD in
anaerobic mechanisms in human red cells has been excluded, for
active transport of potassium can take place without any
turnover of NAD, due to the combined operation of the enzymes
lactate dehydrogenase and triose-phosphate dehydrogenase,
(Whittam, 1958); both of which produce some A.T.P.. If this
finding applies to other tissues it must follow that active
transport occurring under anaerobic as well as aerobic
conditions, must depend on A.T.P.. Thus it seems likely that a
tissue would use a form of energy that can be derived from both
anaerobic and aerobic mechanisms for the transport of an ion;
rather than use two different mechanisms for the transport of the
same ion under different conditions. Other tissues where this
type of mechanism has been demonstrated are frog skin, (Leaf &
Renshaw, 1957), and kidney cortex and liver in foetal animals,
(Whittam, 1960; Van Rossum, 1961). Proof of requirement for
A.T.P. has been shown by Caldwell et al. (1960), when A.T.P.,

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injected into a poisoned giant axon of the squid, stimulated efflux of sodium; and in a similar type of experiment, with osmotic entry of A.T.P. into red cell ghosts, (Hoffman, 1960) sodium efflux was stimulated. The inhibitory effect of cardiac glycosides, in the serosal medium, on the sodium transfer is another factor favouring direct involvement of A.T.P., if Shaw's hypothesis (1954) for active sodium transport in red cells can be applied to this tissue. The delay, after addition of such drugs as iodoacetate, fluoride and D.N.P., before inhibition of the sodium current occurred, favours an energy store of possibily A.T.P.. The fact that D.N.P. addition to a tissue partially inhibited by oligomycin, did not reverse the inhibition; suggests that respiration alone is not adequate to sustain sodium transport. In all the experiments using drugs which directly affect the production of A.T.P. and the respiratory chain, the initial rapid fall of current, probably indicates that A.T.P. formation via the oxidative phosphorylation mechanism is more efficient than the anaerobic methods in the case of this particular tissue.

These experiments have shown that the foetal stomach must have some large energy store, possibly glycogen, which is adequate to sustain its P.D. and S.C.C. at normal levels for periods of an hour or longer. This observation would be in agreement with those of other workers showing quite large energy

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stores in the foetal tissues. The fact that the foetal gastric mucosa can sustain about 25% of its normal sodium transport under anoxic conditions could possibly have some physiological bearing in connection with the survival of young rabbits in anoxia, (Dawes et al., 1963). The adult rabbit stomach does not reabsorb sodium ions; so this is one feature of the foetal stomach where a specific foetal mechanism exists. This could be a mechanism assisting in the growth of the foetus by absorbing sodium, so retaining salts; and it could possibly be assisting the intestine and the kidney whose mechanisms alone could be inadequate.

The apparant dependence of the sodium independent current on oxygen is again a mechanism which would favour foetal conditions. Acid secretion would not seem to be of major importance to the foetus in utero (pH of the stomach contents of the foetal rabbit never seem to fall below 5.0, due to buffering by mucus) and hence a process which it could easily do without in cases of emergency. The abolition of acid secretion in anoxic conditions, and yet the retention of a part of the sodium current, is in agreement with this suggestion. Whether the energy source for the secretion of acid is the same in the adult, as it seems to be in the foetus, it is not possible to say as there is some doubt about the energy source which powers acid secretion in the adult tissue. As discussed in the

introduction there are two schools of thought, both with good
supporting evidence, and a possible explanation is the one
forewarded by Kidder III et al. (1966) which acknowledges the
direct link between oxygen and acid secretion, but states that
A.T.P. is also needed indirectly. The results obtained with the
sodium independent component of the foetal rabbit stomach can
be explained on this basis.

The lower level of the gastric mucosal A.T.P.-ase in the
foetus than in the adult (about 20-25% of the adult levels) is
of interest as similar results have been obtained with other
foetal tissues; and could possibly have some bearing on the
survival ability of the foetus. If a foetal tissue and an adult
tissue had an equivalent level of A.T.P., under anoxic conditions
the foetal tissue A.T.P. level would drop more slowly because
its lower A.T.P.-ase level would result in a slower rate of
A.T.P. hydrolysis. Thus a foetal tissue would be able to
maintain its normal activity for longer periods than the adult.
The foetal heart is certainly able to survive up to ten times
longer thab the adult heart when placed under anoxic conditions.

This work has shown that approximately 70% of the total
short-circuit current of the foetal stomach is dependent on the
presence of sodium ions on the mucosal side, and that it is due
to the active transport of sodium from the mucosal to the
serosal solution. A.T.P. seems to be required for the maintain-
-ance of this active transfer, as 20% of it is still maintained

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under anoxic conditions.

The remaining 30% of the total short-circuit current is independent of the presence of mucosal sodium and it has been shown that a serosal to mucosal active chloride flux is 166% of the sodium independent current. The active transport of hydrogen ions from serosa to mucosa is believed to account for for the other fraction of the sodium independent current. This fraction of the total current is much more dependent on the presence of oxygen than the sodium fraction and it is suggested that a redox-type mechanism, with an indirect requirement for A.T.P., could provide the energy for the active transfer of the chloride and hydrogen ions. Although an A.T.P.-ase was isolated from the gastric mucosa, there was not enough evidence to show that it was stimulated by the cations sodium and potassium, or that it was directly involved with the secretion of hydrochloric acid in the gastric mucosa of the foetal rabbit.

Outline of future work.

The measurement of the contribution to the S.C.C. of the non-specific anion transport should be studied by the means of isotopic SO_4^{--}; as this anion seems to be transported in the foetal stomach, as shown by the rise in S.C.C. and P.D. when a sulphate solution (Cl^- free) was used.

The direct relationship between oxygen uptake and the sodium independent component should be determined, in order to see if a redox system explanation of acid secretion is possible for this tissue.

The A.T.P. levels in the foetal gastric mucosa should be determined to see if an A.T.P. store does exist in this tissue; and also to see if there is any difference in the A.T.P. levels in the stomachs of the pre-23 day foetuses and in the stomachs of the 28-30 day foetuses. An interesting comparison could also be made with the A.T.P. levels in the adult gastric mucosa. The firefly luciferase technique could be used.

A determination of the glycogen content in the foetal stomach would also be of interest in comparison to the glycogen concentrations of the stomachs of adult rabbits; and to the glycogen concentrations in other foetal tissues.

Experiments could also be designed to determine quantitativ--ely the role of the foetal gastric mucosa in the salt and water balance between mother, foetus and extra-foetal compartments.

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ACKNOWLEDGEMENTS

ACTIVE TRANSPORT OF IONS BY THE GASTRIC MUCOSA OF THE RABBIT FOETUS

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SUMMARY

1. The short-circuit current and absolute fluxes of Na^+ and Cl^- across the gastric mucosa of the 28-day rabbit foetus have been measured *in vitro*.

2. Substitution of Na^+ in the solution bathing the mucosal surface by choline ion or K^+ resulted in a 70 % decrease in short-circuit current which was reversed when Na^+ was restored to the mucosal solution. The portion of the short-circuit current dependent on the presence of Na^+ in the mucosal solution was found to be equivalent to the net flux of Na^+ from mucosa to serosa.

3. The net flux of Cl^- from serosa to mucosa was compared with the short-circuit current persisting when Na^+ had been replaced in the mucosal solution. Averaged results from sixteen experiments indicated that the net flux of Cl^- was equivalent to 166 % of the Na^+ independent short-circuit current.

4. The results indicated that the component of short-circuit current associated with acid secretion was independent of the presence of Na^+ in the mucosal solution.

5. The small scale of the experiments and the secretion of mucus by the preparation did not permit successful simultaneous measurement of H^+ secretion and short-circuit current.

6. Replacement of Cl^- by SO_4^{2-} or glucuronate in the solutions on both sides did not result in a reversal or decrease in magnitude of the Na^+ independent short-circuit current, even after allowing time for the tissue to become depleted of Cl^-. It is suggested that a non-specific active anion transport was occurring.

INTRODUCTION

Previous *in vitro* work on whole foetal stomach of the rabbit (Wright, 1963) has shown that between 19th and 30th day of gestation Na^+ is transported from the solution bathing the mucosal side across the gastric

mucosa into the serosal solution. Furthermore, this transport was shown to occur against the gradient of electrochemical potential for this ion. On the 23rd day of gestation, concurrent with the appearance of oxyntic cells, H^+ and Cl^- were found to be transported into the mucosal solution against their gradients of electrochemical potential. These transport phenomena were found to be associated with a difference of electrical potential across the mucosa, the serosal side being positive with respect to the mucosal side, even when identical solutions were bathing each side of the stomach.

Ussing & Zerahn (1951) showed that a membrane across which active ion transport was occurring, resulting in the generation of a p.d., even with identical solutions on the two sides, could be short-circuited through a special external circuit and the current so obtained compared with the rate of active ion transport. Explicitly, the short-circuit current is equivalent to the rate of net ionic charge transfer across the membrane. Hogben (1955) applied this technique to the gastric mucosa of the frog and found that the short-circuit current (s.c.c.) was equivalent to the rate of net transport of charge carried by H^+ and Cl^-.

In view of the previous work it would appear that a deeper understanding of the transport phenomena occurring in the foetal stomach could be gained through application of the Ussing–Zerahn technique to this system.

METHODS

Stomachs of rabbit foetuses in the age range 28–30 days gestation age were obtained as described in a previous paper (Wright, 1963).

Some stomachs were used on the same day as the operation whilst others were stored at 2° C in Krebs bicarbonate Ringer solution for up to 5 days. This solution is carefully gassed with a 95 % O_2, 5 % CO_2 mixture to attain a pH of 7·4. The period of storage appeared to have no lasting effect on the electrophysiological properties of the preparation (open-circuit p.d., short-circuit current and resistance) apart from delaying the time of attainment of a maximum short-circuit current and p.d. after being brought to 36° C. After 24 hr storage this delay was about 20 min whilst after 5 days storage the delay was about 6 hr or even longer.

Stomachs were incised through the cephalic surface, opened out to a flat membrane and the *in vivo* contents washed away with Krebs bicarbonate Ringer. The washed membranes were then sandwiched between two half chambers constructed from Perspex and based on the design used by Ussing & Zerahn (1951). The useful area of membrane in these chambers was 0·6 cm² and the volume of each compartment was 5 ml. The solutions were stirred and oxygenated by the oxygen lift incorporated in this type of apparatus.

Measurement of net transport of NaCl. This was determined as the difference of the two absolute fluxes across the membrane. The absolute flux of Na from mucosal solution to serosal solution was measured first in each experiment by adding about 20 μc of ²⁴Na as isotonic ²⁴NaCl solution to the exactly known volume of mucosal solution. Duplicate samples of the musocal solution were taken and diluted 1:10. At the same time duplicate samples of serosal solution were taken and used for background and as a blank. After about 90–120 min a second pair of samples of serosal solution were taken for counting, and also a second pair of samples of mucosal solution which were diluted 1:10. The volume of serosal solution was

accurately dispensed. The concentration of Na in the mucosal solution was determined by flame photometry.

The samples of radioactive solution were dried and counted at infinite thinness on recessed planchettes for 1000 sec. Total counts obtained were greater than 4000 in the weakest samples. Count rates were at least 5 times background. The amount of Na transferred from mucosal solution to serosal solution was calculated from the equation:

$$\text{amount} = [\text{Na}]_m \cdot \frac{C_s}{C_m} \cdot V_s,$$

where $[\text{Na}]_m$ is the Na concentration in the mucosal solution, C_s is the count rate of the sample of serosal solution (corrected for background decay), C_m is the mean count rate of the first and second samples of mucosal solution after correction for dilution, background and decay, and V_s is the volume of serosal solution.

The flux of Na^+ from serosal solution to mucosal solution was then measured using $^{22}\text{Na}^+$ added to the serosal solution and then using analogous procedures to those used for determining the flux from mucosa to serosa. However, all counts for this part of the experiment were obtained 3 weeks later when the activity due to $^{24}\text{Na}^+$ had decayed to insignificant levels.

The net flux of Cl^- across the stomach wall was also measured as the difference of two absolute fluxes. Ideally this could have been carried out using $^{38}\text{Cl}^-$ and $^{36}\text{Cl}^-$ simultaneously or consecutively (as for Na). However, the use of $^{38}\text{Cl}^-$ with its short half life (38 min) was not practicable as no close source of this isotope was available. Instead the experiments were carried out on paired preparations from the same litter which had been stored over the same period, one preparation being used for measurement of $^{36}\text{Cl}^-$ transfer from mucosa to serosa and the other preparation being used for measurement of $^{36}\text{Cl}^-$ transfer from serosa to mucosa. The methods of sampling and counting were similar to those used for radioactive Na^+. $^{36}\text{Cl}^-$ was added as 0·10 M-KCl. These pairs of stored preparations always had similar open circuit p.d.s and short-circuit currents, agreeing to within 4%.

Solutions. The bicarbonate Ringer solution had the following composition (mM): Na^+ 135·7, Cl^- 132·2, K^+ 14·9, Ca^{2+} 3·5, HCO_3^- 25·3, glucose 24·0. Na^+-free solutions were 154 mM choline chloride or 154 mM-KCl, each with 24 mM glucose. There was no difference in the effects of these two solutions. Cl^- free Ringer was made up as above with Ca^{2+} present as the nitrate and all other Cl^- replaced by glucuronate or sulphate. Solutions which were Na^+ and Cl^- free were similar to the Cl^- free solution above, with K^+ replacing all of the Na^+.

Electrical measurements. The integrated s.c.c. was obtained as described previously (Wright, 1965). The Na^+ independent component of the s.c.c. was measured before and immediately after measurement with Na^+ present on the mucosal side, the two values usually being identical. When the preparations were dead, as judged by the absence of a p.d., no diffusion potential greater than 1·0 mV was observed when a Na^+ free solution was placed on the mucosal side.

RESULTS

The manner in which the s.c.c. was dependent on the presence of Na^+ in the mucosal solution is shown in Fig. 1. It was found that about 70 % of the s.c.c. was reversibly dependent on the presence of Na^+ in the mucosal solution, the remaining 30 % appearing to be independent of Na^+. The result shown was obtained with solutions containing Cl^-, but Cl^--free solutions gave the same result. The overshoot seen when Na^+ was returned to the mucosal solution was always observed and is at present unexplained.

The result of sixteen experiments in which the net flux of Na was

measured using isotopes, along with the Na^+ dependent s.c.c., are given in Table 1.

The same sixteen experiments gave the result expressed in Fig. 2. It should be noted that there is no significant intercept of the regression line on either of the axes and that the slope of the line is 45°, indicating that
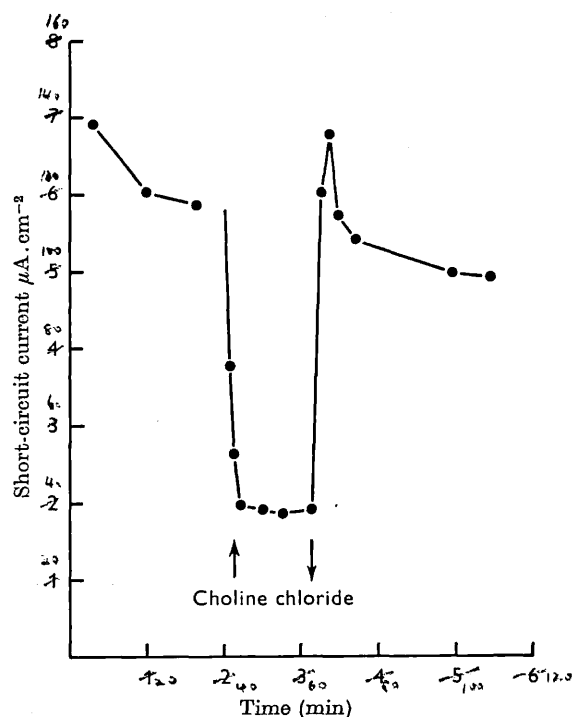

Fig. 1. Short-circuit current of a 28-day stomach showing the dependence of the s.c.c. on the presence of Na^+ in the mucosal solution. During the interval between the lines choline ion replaced Na^+ in the mucosal solution.

TABLE 1. Na^+ fluxes and short-circuit current. Influx is from serosa to mucosa, efflux is from mucosa to serosa. The units of flux are μ-equiv.cm.$^{-2}$hr^{-1}. The short-circuit current is expressed in the same units as the fluxes

Experiment	Influx	Efflux	Net flux	s.c.c.
10. ii. 65	0·46	4·48	4·02	4·47
12. ii. 65	1·17	5·15	3·98	3·98
16. ii. 65	1·93	3·68	1·75	1·36
17. ii. 65	0·44	2·58	2·13	1·49
18. ii. 65	0·54	1·54	1·00	0·91
19. ii. 65	0·47	3·01	2·54	2·48
23. ii. 65	0·57	2·17	1·60	1·50
25. ii. 65	0·40	3·44	3·04	2·28
26. ii. 65	1·73	5·56	3·88	3·87
2. iii. 65	1·08	4·47	3·39	3·07
3. iii. 65	0·92	5·63	4·70	3·10
16. iii. 65	0·46	1·20	0·74	0·99
19. iii. 65	0·10	1·78	1·68	2·00

the next flux of Na^+ from mucosa to serosa was equivalent to the Na current (I_{Na}). The equation of the regression line is $y = 0.98x - 0.01$, $P < 0.001$.
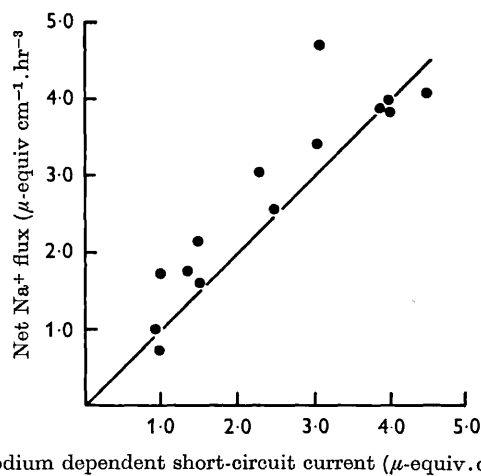

Fig. 2. The relation between net flux of Na^+ from mucosa to serosa and Na^+ dependent short-circuit current for sixteen foetal stomachs. The equation of the regression line is $y = 0.98x - 0.01$, $P < 0.001$.

TABLE 2. Cl^- fluxes and Na^+ independent short-circuit current. Fluxes and units are defined as in Table 1. The net flux is defined as positive when in the direction serosa to mucosa

Experiment	Influx	Efflux	Net flux	s.c.c.
15. iii. 66	4·37	6·56	2·19	1·37
4. iv. 66	3·81	5·86	2·05	1·27
4. iv. 66	7·31	4·00	−3·31	0·64
5. iv. 66	5·62	7·58	1·95	1·15
6. iv. 66	2·08	4·91	2·83	0·69
25. iv. 66	5·39	8·20	2·81	0·78
26. iv. 66	5·94	6·95	1·01 .	2·14
27. iv. 66	6·44	7·72	1·28	2·58
28. iv. 66	5·95	7·81	1·86	1·89
2. v. 66	9·14	6·12	−3·02	1·46
3. v. 66	5·30	9·06	3·76	1·64
3. v. 66	4·53	7·00	2·47	1·32
9. v. 66	4·41	6·14	1·73	0·79
10. v. 66	3·97	8·28	5·31	1·25
10. v. 66	4·56	6·06	1·50	1·19
16. v. 66	4·27	6·57	2·30	1·72
16. v. 66	3·37	5·31	1·94	1·24
17. v. 66	4·78	6·33	1·55	1·01

Determinations of the s.c.c., in the absence of Na^+ on the mucosal side, and the radioactive Cl^- absolute fluxes produced the results shown in Table 2.

The use of two separate preparations for each experiment gave rise to a greater variation of results. The over-all result obtained from this second

34-2

series of sixteen experiments, shown in Fig. 3, indicated that the mean net flux of Cl^- from serosa to mucosa was 166 % of the Na^+ independent s.c.c. The statistical significance of this result was investigated using the t test for related means, which gave $t_{n-1} = 2.24$, $0.01 < P < 0.025$: the difference of the means is considered to be significant.
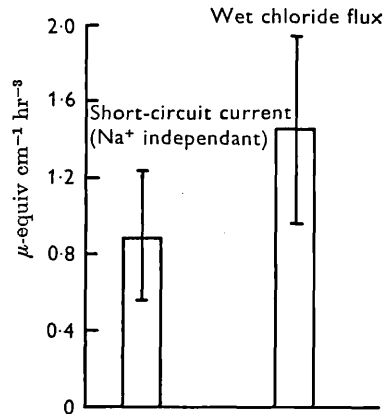

Fig. 3. The left-hand column shows the mean Na^+ independent short-circuit current and s.d. for sixteen experiments. The right-hand column shows the mean net flux of Cl^- from serosa to mucosa and σ for the same sixteen experiments. The t test for related means gives $t_{n-1} = 2.24$, $0.01 < P < 0.025$ which shows that the difference of the means is significant.

Since the net flux of Cl^- from serosa to mucosa was found to exceed the Na^+ independent component of the s.c.c., it was necessary to determine the extent to which active transport of H^+ into the lumen contributed to this component of the s.c.c. Attempts to measure directly the rate of appearance of H^+ on the mucosal side, simultaneously with s.c.c., using a glass electrode and 0.01 N-KOH, were unsuccessful owing to the high degree of frothing occurring when the volume of this solution was reduced to a level at which measurable pH changes would be detected. Oxygenation of the serosal solution alone proved to be inadequate.

An attempt was made to determine indirectly the contribution of H^+ secretion to the s.c.c. by replacing Cl^- by an anion species that would not be expected to be subject to active transport. Under this condition, with Na^+ free solution on the mucosal side, the gastric p.d. and s.c.c. should be reversed in direction if H^+ transport was the only active process occurring: results of this nature have been obtained from frog gastric mucosa when Cl^- was replaced by SO_4^{2-} (Heinz & Durbin, 1959; Rehm, Davis, Chandler, Gohmann & Bashirelahi, 1963). Neither of the anions used in the present series of experiments (SO_4^{2-} or glucuronate) produced a reversal of p.d.

and s.c.c. when used to replace Cl⁻: in fact there was always a large increase in p.d. and s.c.c. as shown in Fig. 4 (compare Durbin, 1964). It is noteworthy that even after 4 hr, there was no fall in the s.c.c. observed in these experiments.

TABLE 3. Effect on Na^+ independent s.c.c. of replacing Cl^- by SO_4^{2-} or glucuronate

Experiment	Anion	p.d. mV		s.c.c. μA	
		Before	After	Before	After
17. v. 66	SO_4^{2-}	5·5	17·5	30	140
18. v. 66	SO_4^{2-}	4·2	34·0	22	90
23. v. 66	Glucuronate	12·5	21	56	100
24. v. 66	Glucuronate	10·0	26	54	100
25. v. 66	Glucuronate	12·0	30	52	100

Mucosal solutions were Na^+ free. The values of p.d. and s.c.c. are those existing immediately before replacement of Cl^- and 2 hr after replacement.
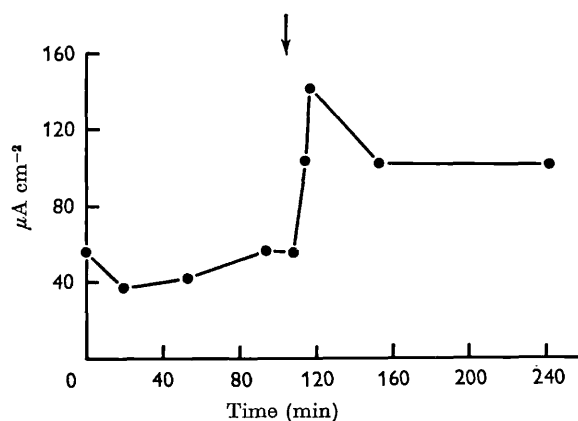

Fig. 4. The effect on short-circuit current of a 28-day stomach of replacing Cl^- by glucuronate in mucosal and serosal solutions. The mucosal solution was Na^+ free at all times. Area of membrane = 1·15 cm².

Previous work has shown that replacement of Cl^- by methyl sulphate or ethyl sulphate also fails to reverse the p.d. and s.c.c. (Wright, 1964).

DISCUSSION

The measurements of net flux of Na^+ and s.c.c. have shown that I_{Na} can be used as an exact measure of the net flux of Na^+ from mucosa to serosa. Although this result might have been anticipated from Fig. 1, there would still have remained the possibility that the value of the Na^+ independent component of the s.c.c. was in fact altered when Na^+ was present in the mucosal solution: the results presented in this paper have shown that this possibility is not realized.

It was shown in an earlier paper (Wright, 1963) that the active transport of Na^+ from mucosa to serosa was a function performed by the non-differential cells. In the same paper it was shown that the onset of HCl secretion into the mucosal solution coincided with the appearance of oxyntic cells in the mucosa (on the 23rd day of gestation). Before the appearance of the oxyntic cells there was no p.d. across the mucosa when Na^+ was absent from the mucosal solution: under this condition no s.c.c. would be obtained. It is concluded that the Na^+ independent component of the s.c.c. arises from oxyntic cell activity associated with acid secretion.

The results presented in this paper have shown that the Na^+ independent component of the s.c.c. and the net flux of Cl^- are in opposite directions and that the latter is 166 % of the former. If H^+ and Cl^- were secreted at the same rate there would be no s.c.c. associated with acid secretion. The following explanations of this result may be considered:

(a) Active transport of Cl^- is electrogenic and active transport of H^+ is non-electrogenic and no other electrogenic process is occurring. H^+ and Cl^- may or may not be secreted in equivalent amounts.

(b) Active transport of both H^+ and Cl^- is electrogenic, but Cl^- is secreted in excess of H^+. No electrogenic transport of any other ion species is considered to occur.

(c) Equivalent electrogenic secretion of H^+ and Cl^- is occurring and some other cation is being electrogenically transported from mucosa to serosa.

(d) Electrogenic transport of H^+ and Cl^- in equivalent amounts is occurring and some other anion is being transported electrogenically from serosa to mucosa.

(e) H^+ and Cl^- secretion is non-electrogenic and some other cation is subject to electrogenic active transport from mucosa to serosa.

(f) H^+ and Cl^- secretion is non-electrogenic and some other anion is subject to electrogenic active transport from serosa to mucosa.

Previous work (Wright, 1964) has shown that K^+ only passes across the foetal gastric mucosa in either direction, down its gradient of electrochemical potential; and also in experiments with Na^+ free solution on the mucosal side there was no alteration in s.c.c. when $choline^+$ was substituted for K^+. The Na^+ independent s.c.c. reported in this paper would not therefore have received any contribution from choline ion or K^+ transport, thus ruling out alternatives (c) and (e). It has also been shown (Wright, 1963, 1964 and unpublished) that Cl^- secretion always occurs in excess of H^+ secretion, hence ruling out alternatives (c) and (d). Alternative (a) would only apply if the Na^+ independent s.c.c. was exactly equivalent to the rate of net transport of Cl^-; this was shown not to be the case. Since net flux of Cl^- was found to bear a definite relation to Na^+ independent

s.c.c. it would appear that active transport of Cl⁻ is electrogenic, thus ruling out alternatives (e) and (f). It could appear then that alternative (b) is the correct one to apply to this preparation.

If alternative (b) was correct, then replacement of Cl⁻ by non-actively transported anions should have reversed the Na^+ independent s.c.c. The failure to obtain this reversal showed that electrogenic active transport of H^+ could not be demonstrated by these means and that the s.c.c. which persists is presumably due to a non-specific active transport of anions: this point requires direct verification using various species of labelled anions. This s.c.c. might have been due to extrusion of an intra-cellular pool of Cl⁻ from the oxyntic cells. However, calculation of the maximum amount of Cl⁻ which could have been contained within these cells at the beginning of an experiment could not provide the amount of charge carried by the s.c.c. over the experimental period. Considering the result shown in Fig. 4, an s.c.c. of 100 μA over a period of 4 hr is equivalent to the charge carried by 14·8 μ-equiv. of Cl⁻. A typical value for the wet weight of the preparation is 0·14 g and 20 % of this is generously assumed to be intracellular water of oxyntic cells in which Cl⁻ is assumed to be present at a concentration of 150 m-equiv/l., then the amount of Cl⁻ which could have been contained in these cells initially was 4·2 μ-equiv.
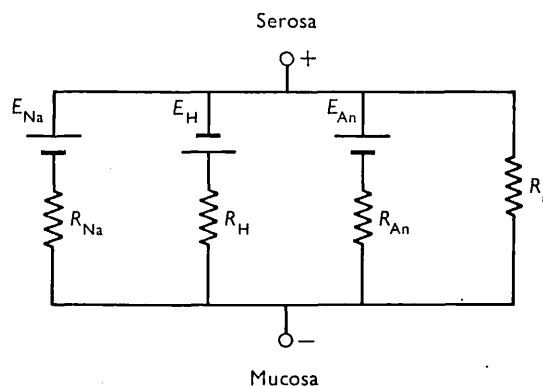

Fig. 5. An equivalent circuit describing the properties of the gastric mucosa of the late rabbit foetus. E_{Na}, E_H and E_{An} represent the E.M.F.s of the Na^+, H^+ and anion transport systems respectively whilst R_{Na}, R_H and R_{An} represent the internal resistances of these systems. R_L is the 'passive' leakage resistance of the mucosa to all ions.

The results presented in this paper, considered with earlier results, lead to the conclusion that the major part of the s.c.c. of the foetal rabbit stomach of 28–30 days gestation age is accounted for by a specific active transport of Na^+ from mucosa to serosa. The remainder of the s.c.c. is associated with HCl secretion from serosa to mucosa, the Cl⁻ perhaps

being transported by a non-specific anion transport mechanism. An equivalent circuit which describes the results obtained is shown in Fig. 5. E_{Na}, E_{An} and E_H are the E.M.F.s of the Na^+, anion and H^+ transport systems. R_{Na}, R_{An} and R_H are the internal resistances of these systems and R_L is the leakage pathway through the mucosa. This circuit is similar to one proposed by Bornstein, Dennis & Rehm (1959) to describe the transport phenomena occurring in the resting stomach of the dog, in which active transport of Na^+ was observed.

The calculation of E_{Na} and E_{Cl} from the flux ratio (Ussing, 1949) was not made as it has been shown by Kedem & Essig (1965) that the value obtained is only valid when the resistance of parallel leakage pathways is very high, as indicated by a high flux ratio ($> 100:1$). The Na^+ flux ratios obtained in these experiments of about $10:1$ are low and give a value of E_{Na} which is perhaps only 25 % of the true value (see Fig. 1 of Kedem & Essig, 1965). The flux ratios for Cl^- were even lower and subject to greater variation.

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