Involvement of cyclic AMP in sodium transport across the gastric mucosa of the rabbit fetus

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by

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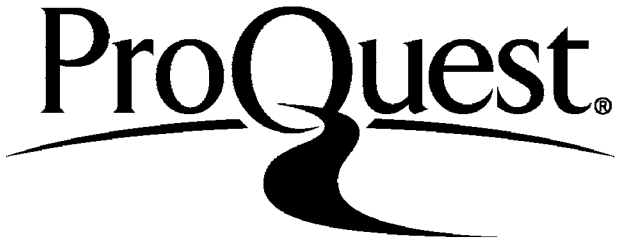

ABSTRACT

Transepithelial ion transport across the gastric mucosa of the 28 day old rabbit fetus was studied in vitro using the short-circuit current (s.c.c.) technique of Ussing and Zerahn (1951).

Addition of adenosine 3',5'-monophosphate (cyclic AMP) or its dibutyryl derivative to the short-circuited membrane preparation was immediately followed by an increase in ion transport across this membrane: administration of methyl xanthines also resulted in a similar increase. The application of adrenaline, vasopressin, glucagon or pentagastrin was also followed by an increase in the s.c.c.

Mucosal cell suspensions, obtained by the method of Wright and Malinowska (1978), were used to study the possible effect of various agents on the intracellular cyclic AMP level. Experiments have shown that the addition of theophylline, adrenaline or vasopressin resulted in an increase in the intracellular cyclic nucleotide level which was followed (but not led) by an increase in the s.c.c. These results indicate the involvement of cyclic AMP in the regulation of ion transport across the fetal stomach.

Application of prostaglandin synthetase inhibitors, acetyl salicylic acid and similar non-steroidal anti-inflammatory drugs resulted in an inhibition of ion transport across the membrane, whereas the addition of prostaglandin precursor, arachidonic acid was followed by an increase in the s.c.c. These results indicate (1) the ability of fetal stomach to synthesize prostaglandins and (2) the

involvement of prostaglandins in the regulation of active ion transport across this membrane.

Radioactive isotopes, $^{22}Na^{+}$ and $^{36}Cl^{-}$ were used to investigate the effect of theophylline on the undirectional transport of sodium and chloride ions across the 28 day old gastric mucosa. Experiments have shown that the addition of theophylline resulted in a large increase in net transport of sodium in the direction of mucosa to serosa, whereas chloride transport was unaffected. Furthermore such experiments have indicated that some other ion(s) besides sodium was also stimulated by these drugs.

Further experiments were carried out on intact membrane preparations to determine whether the gastric mucosa of the fetus possesses seperate adenylate cyclase system each being responsive to a single hormone only or whether both adrenaline and vasopressin increase ion transport by stimulating a single cyclase which is common to both hormones. The response of the gastric mucosa to adrenaline was blocked by β-receptor blockers, propranolol and timolol but not by α-receptor blocker, phentolamine. α- or β-receptor blockers had no effect upon the response to vasopressin. The addition of adrenaline together with vasopressin did not result in an additive increase in the s.c.c. From these results it is concluded that the fetal gastric mucosa posseses a single common adenylate cyclase system which is stimulated by both adrenaline and vasopressin each acting on separate receptor sites of the enzyme.

To my parents

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CHAPTER 1

INTRODUCTION

1.1. Potential difference and active transport across biological membranes

Early appreciation of the existence of an electric potential in living systems was demonstrated towards the end of the 18th century by Galvani in 1786. Following the development of the first galvanometer after 1820, it became possible to measure electric currents in animal tissues. This was first shown by Matleucci (1838) and then by Reymond (1843). They discovered that current flowed along the longitudinal surface of a nerve or muscle to the cut end, but if the two electrodes were placed anywhere along the outside surface of an intact resting nerve or muscle, no electrical potential was observed. The potential difference (p.d.) was stated to result from injury at the cut end (Hermann, 1879): it was further established that the completely dead tissue does not generate a current flow. Bernstein (1902) showed the uninjured surface to be the seat of an electromotive force, the injured region just provides a low resistance pathway between the outside and the inside of the membrane.

Du Bois Reymond (1848) first demonstrated that the frog skin was a site of electromotive potential and could produce a current flow. Galleotti (1904) extended these findings to show that this p.d. depended upon the presence of sodium or lithium ions in the bathing solutions, and persisted even when the skin was bathed in identical solutions on both sides of the membrane. It was therefore concluded that the p.d. was not due to diffusion alone.

Mond (1927) observed that the mucosal surface of the frog skin was electrically negative with respect to the serosal surface. Francis (1933) found that the partially short-circuited frog skin produces an electric current. This was the first experiment using a short-circuited membrane preparation, however it was not possible to totally short circuit the membrane due to the high resistance between the electrodes used. Huf (1935) observed that isolated frog skin, in contact with Ringer, will transport chloride ions from mucosal to serosal side. He did not however analyse for sodium ions assuming the process to be an active transport of sodium chloride inwards as cyanide or bromoacetate inhibited this process.

Hevesy et al. (1935) found osmotic permeability of frog skins to be three to five times greater than the diffusion permeability of water. Krogh (1937) working on whole amphibian, observed that frogs in need of salt will take up chloride ions through the skin even from very dilute solution of sodium chloride.

Lund and Stapp (1947) using an improved technique of Francis (1933) and electrodes of a lower resistance almost completly short-circuited the skin. They, however, failed to correlate the current flowing through the circuit with ionic movements.

Such studies continued until in 1949(a), when Ussing devised an experiment using an isolated frog skin placed so as to seperate two Ringer solutions of identical composition; upon measuring the p.d. found it to be around 100mV; inside electrically positive with respect to the outside. On measuring the exchange rates of isotopic sodium and chloride ions across the skin, he found that the rate of

sodium influx could not be accounted for by diffusion alone. It was therefore suggested that an active transport of sodium from mucosal to serosal side is involved and this gives rise to a p.d. across the skin.

Ussing (1949b) deduced the following equation to describe the behaviour of free ions diffusing through a membrane under the combined influence of both concentration and potential gradient:-

$$\frac{J_{1\rightarrow 2}}{J_{2\rightarrow 1}} = \frac{A_1}{A_2} = \frac{C_1}{C_2} \cdot \frac{F_1}{F_2} \exp^{-\left[\frac{ZF}{RT}(\psi_2 - \psi_1)\right]}$$

$J_{1\rightarrow 2}$ = absolute flux of ions from side 1 of the membrane to side 2

$J_{2\rightarrow 1}$ = absolute flux of ions from side 2 of the membrane to side 1

A_1 and A_2 = electrochemical activities of the ions on the two sides of the membrane

C_1 and C_2 = concentrations of the ion species on the two sides of the membrane

F_1 and F_2 = activity coefficients of the ions on the two sides of the membrane

$\psi_2 - \psi_1$ = difference of electrical potential between side 1 and side 2 (p.d.)

Z = net charge of the ion species (valency)

F = Faraday constant (96500 coulombs)

R = gas constant

T = absolute temperature

This equation was stated to be independent of membrane structure; it also assumes independent movement of particles.

With identical solutions on both sides of the membrane, the above equation reduces to:

$$\frac{J_{1\rightarrow 2}}{J_{2\rightarrow 1}} = \exp^{-\left[\frac{ZF}{RT}(\psi_1 - \psi_2)\right]}$$

i.e. the flux ratio $[J_{1\rightarrow 2}/J_{2\rightarrow 1}]$ should be a function of the p.d. only. Deviation from this equation would indicate that the ion species is subject to active transport and therefore does not diffuse in the free state but as a part of some complex moving system. The equation can, therefore, be used to distinguish between ion species that diffuse passively in a free state from those which do not. Another term was introduced into the equation to account for the discrepancy between $J_{1\rightarrow 2}/J_{2\rightarrow 1}$ and A_1/A_2, i.e. the action of the transporting energy on ion species in the membrane. This transporting energy was considered to be equal to E_{Na}, and appears in the equation:-

$$\frac{J_{1\rightarrow 2}}{J_{2\rightarrow 1}} = \exp - \left[\frac{ZF}{RT}\,\frac{(\psi_2 - \psi_1)}{E_{Na}}\right]$$

This equation was tested by Koefoed-Johnson, Levi and Ussing (1952); they measured the p.d. across the skin together with the inward and outward fluxes of isotopic chloride. The theoretical flux ratios were found to agree with those obtained experimentally suggesting that the transfer of chloride ions from serosal to mucosal side was passive.

Ussing's hypothesis stated that the p.d. across the frog skin arose mainly from the active and specific transport of Na^+ ions inwards, associated with a passive diffusion of Cl^- ions; the net result being a transfer of NaCl from the outside to the inside of the skin, and possibly a small exchange of mucosal Na^+ with the serosal K^+ ions. However there still remained the possibility of ions such as H^+ and HCO_3^-, contributing to the potential difference. Ussing and Zerahn (1951) stated that there should be an equivalence between electrical and chemical measurements in such a system. They argued that if one short-circuited the p.d. to zero by applying a counter EMF (electromotive force) and also reduced the chemical potential gradient to zero by bathing the skin with identical solutions on both sides, then under these conditions, no net transfer of passive ions should take place. However the ion species which are actively

transported should continue to flow as under in vivo conditions. The rate of charge running through the circuit should be equal to the net active transport of all ion species. They further suggested that if under these conditions the amount of current passing through the circuit is larger than the net rate of sodium flow inward, then this would indicate that some other ion species are also subject to active transport and thereby contribute to the total current and the p.d. across the membrane in the non short-circuited skin. If however the current is less than or equal to the net influx of Na^+ ions then this would support their hypothesis, i.e. Na^+ is the main ion species actively transported and therefore is the main cause of a potential difference across the frog skin.

Experiments showed that the short-circuit current was exactly equal to the rate of net active transport of sodium ions from mucosal to the serosal side. This relationship was found to hold true even when the current was increased to over 150% of its normal value by the application of neurohypophyseal extracts.

1.2. Ion transport across the gastric mucosa

1.2.1. Amphibian gastric mucosa

In the resting frog gastric mucosa, the mucosal surface is electrically negative with respect to serosal surface (Mond, 1927). Rehm (1946) by inserting "crude" electrodes into various layers of the gastric mucosa reported that the muscle wall has no influence on the p.d. across this tissue. These observations were later confirmed by Hogben (1951,1955). He applied the Ussing short-circuit

current technique and found that the p.d. persisted even after the muscle layer had been removed from the gastric mucosa. The s.c.c. across the amphibian stomach, was found to be equal to the net rate of chloride ions transported from serosa to mucosa, minus the rate of net hydrogen ions transported in the same direction. No evidence was found for the active transfer of sodium or potassium ions; these ions were therefore suggested to traverse the membrane by passive diffusion. The active transport of chloride ions was suggested to create a potential which accelerates the passive transfer of cations. Replacement of chloride ions in the bathing solution by sulphate ions resulted in complete abolition of the p.d. (Heinz and Durbin, 1959; Rehm et al., 1963). Variation of mucosal sodium concentration (Rehm, 1962) and serosal and mucosal sodium concentration (Harris and Edelman, 1964) had no effect on the gastric potential or hydrogen ion secretion. However when a weak solution of sodium chloride was placed on the mucosal side, a net movement of sodium from mucosal to serosal side was observed (Dennis et al., 1956). Substitution of sodium in the bathing solution by choline resulted in severe reduction of the gastric p.d. and hydrogen ion secretion (Sachs et al., 1966). Similar results were also obtained for the early tadpole stomach (Forte et al., 1969). These observation suggested that an active transport of sodium ions contribute towards the total gastric potential.

Machen and Mclennan (1980) reported that the s.c.c. but not hydrogen ion secretion was rapidly reduced when serosal sodium concentration is reduced in the Ringer solution. It was suggested that chloride transport (but not hydrogen) was very sensitive to

concentration of sodium in the bathing solution; removal of serosal sodium but not mucosal, resulted in reduced p.d. and s.c.c.

Schwartz et al. (1981) demonstrated that if HCO_3^- ion concentration gradient is eliminated it is possible to show the presence of a small amount of active transport of sodium ions from the mucosal to the serosal side i.e. elimination of HCO_3^- ion gradient appears to unmask a small active transport of sodium ions: in this case the potential difference across the gastric mucosa recovers when choline is once again replaced by sodium. These observations are consistent with the earlier findings of Flemstrom (1971) where an active transport of Na^+ from mucosal to serosal side was only observed when the mucosa was gassed with about 40% O_2 and 5% CO_2 but not when it was gassed with 95% O_2 and 5% CO_2.

1.2.2. Mammalian gastric mucosa

Wright (1962, 1964) reported the existence of a p.d. across the isolated rabbit fetal gastric mucosa: serosal surface being electrically positive with respect to the mucosal surface. The p.d. was found to be present from at least the 20th day to full term of gestation (30 days). The existence of a p.d. before the 20th day was not shown due to the difficulties involved in handling the small size stomach (volume of the lumen on the 20th day of gestation being less than 0.1cc). Up to the 22nd day, replacement of mucosal sodium by choline resulted in complete abolition of the p.d. and therefore the s.c.c. However after the 23rd day until birth, removal of mucosal sodium and its replacement by choline or potessium, no longer completely abolished the p.d. or s.c.c. but left a small fraction

of about 20-30%. These findings were related to a small amount of acid secretion which also commenced on the 23rd day. The rate of acid secretion remains steady and very low with a sudden increase occurring on the 27th day. Cytological studies (Menzies, 1958) of the fetal stomach reported the appearance of parietal cells on the 23rd day of gestation which suddenly increase on the 27/28th day; prior to the 23rd day only non-differentiated epithelial cells are present. Wright (1962), therefore concluded that an active transport of sodium ions is a function of the non-differentiated epithelial cells and that parietal cells are associated with gastric acid secretion (as is true for all other mammalian gastric mucosae studied so far).

From studies of the net flux of ions (using isotopes of sodium and chloride) across the 28th day old fetal gastric mucosa, the sodium-dependent component of the short-circuit current (70%) was found to be an exact measure of the net flux of sodium from mucosa to serosa; the second component of the short-circuit current or the sodium-independent short-circuit current was related to non-specific anion transport in the opposite direction (Kendall and Wright, 1967). The sodium-independent fraction of the short-circuit current was not precisely characterized because of the difficulties involved in directly measuring the rate of acid production. However from the work of Wright (1962 and 1964) and Kendall and Wright (1967), the sodium independent short-circuit current (30%) can be associated with the secretion of H^+ and Cl^- ions from serosa to mucosa. The transport of potassium and bicarbonate ions was suggested to be passive, down the electrochemical potential gradient, and do not

contribute towards the s.c.c.

Prior to Wright most of the work on mammalian stomachs was carried out under in vivo conditions; the measurements of ion transport was, therefore, not possible (for review see Rice and Ross, 1947). However the work under in vitro conditions was limited to short survival period of adult mammalian preparations in contrast to the amphibian gastric mucosa. Patterson and Stetten (1949) found that the rat stomach can survive in vitro: although the p.d. was not measured, acid secretion was found to increase over a period of 3 - 6 hours. Davenport and Chavre (1951, 1953) reported that the mouse stomach in vitro, could be made to exhibit maximum acid secretion but only if it was subjected to hyperbaric oxygen tensions (320mmHg); this they concluded was necessary in order to enhance the chemical gradient for the diffusion of oxygen to the tissue. Rehm (1945, 1953) and Bornstein et al. (1959) found that the dog stomach could survive only if the blood supply was left intact.

The main difficulties encountered in obtaining functional mammalian, in vitro gastric mucosal preparations were (1) inadequate oxygenation due to the thickness of the underlying muscle coat if not removed, or on the thickness of the separated mucosa and (2) damage to the epithelial structure when mucosa is separated from the thick muscle coat. In the stomach of the fetal rabbit the muscle coat is very thin and therefore adequate oxygenation is achieved (Wright, 1962). This is also true for the guinea pig (Holton and Spencer, 1976) and the immature mouse stomach (Wan, 1977). In both cases the preparations remain viable for up to 6-8 hours with their serosal muscle coat intact, which is relatively thin compared to the adult

stomach.

However techniques have now been devised which allow the successful and satisfactory isolation of mucosal layer from the underlying muscle coat. These include separation of the mucosal layer from the serosal muscle coat by careful dissection, using scissors and forceps: a procedure used for the preparation of dog gastric mucosa (Kuo and Shanbour, 1978). A similar procedure has been used for the adult rabbit stomach (Fromm, Schwartz and Quijano, 1975a); here the stomach piece is tightly clamped to a half Ussing-type chamber and the serosal muscularis (muscle coat) is sharply stripped away to the level of the muscularis mucosa.

A technique widely employed is the so called blistering technique, introduced by Forte et al. (1972). This technique involves injection of a small amount of saline buffer between the mucosa and the thick serosal muscle layer, which results in blister formation. The thick muscle coat can then be readily dissected away.

Such techniques have enabled the study of ion transport across other mammalian stomachs. However studies have been mostly carried out on post natal up to adult stomachs only where the active transport of sodium, chloride or both ions have also been reported for a variety of other mammalian stomachs, including the gastric mucosa of the dog (Bornstein et al., 1959; Kitahara et al., 1969), rat (Cummins and Vaughan, 1965; Sernka and Hogben, 1969; Wan et al., 1974), piglet (Forte et al., 1975), monkey and man(Kitahara et al., 1969), guinea pig (Shoemaker et al., 1966; Sernka and Hogben, 1969; Holton and Spencer, 1976) and cat (Kitahara et al., 1969). In the

cat gastric mucosa, active transport of both sodium and chloride ions have been demonstrated (Kitahara, 1967). Replacement of either sodium by choline or chloride by sulphate ions results in a 50% fall in the potential difference across this tissue; hence active transport of both ions contribute to the generation of a p.d. However the transport of Na^+ and Cl^- ions are independent of each other, as is also true for the fetal rabbit (Wright, 1962, 1964). In the dog gastric mucosa, replacement of sodium by choline results in great reduction of the p.d. across the tissue; replacement of chloride or potassium has only a very slight effect. Therefore in the canine gastric mucosa, the potential difference is primarily due to an active transport of sodium from mucosa to serosa (Kuo and Shanbour, 1979). In the immature piglet gastric mucosa, replacement of sodium leads to a 50% fall in the tissue potential difference, whereas the active transport of sodium contributes towards about 60% of the total short-circuit current (Forte and Machen, 1975; Machen et al., 1978). However in monkeys, sodium and chloride contribute to an equal extent towards the short-circuit current (Tripathi and Rangachari, 1980). Hence in all mammalian stomachs studied so far, transepithelial transport of sodium and chloride contribute significantly towards the short-circuit current, however the exact percentage appears to depend upon the particular species.

1.3. Cyclic adenosine 3',5'-monophosphate

1.3.1. Discovery of cyclic AMP

Cyclic adenosine 3',5'-monophosphate (cyclic AMP) was discovered by Rall et al., (1957). They studied the effects of adrenaline and glucagon on the breakdown of glycogen and the production of glucose by homogenates of liver cells. The response to these hormones was lost if the homogenate was centrifuged. When the particulate fraction, which contained the plasma membrane, was added back to the original supernatant and incubated with adrenaline and glucagon in the presence of adenosine triphosphate (ATP) and Mg^{2+}, a heat-stable factor was formed. This observation of hormone action in plasma membrane-free homogenate provided a landmark in the history of hormone action; it suggested that hormonal response was separated into two parts: the interaction of the hormone with the particulate fraction, containing plasma membrane, to produce a heat-stable factor, followed by its effect on the supernatant to activate the relevent enzymes. The heat-stable factor was crystallized and found to contain adenine, ribose, and phosphate in the ratio 1:1:1, suggesting that it arose from ATP. Mild hydrolysis resulted in the formation of adenylic acid. It was purified and identified as the cyclic adenosine 3',5'-monophosphate, an unusal derivative of adenylic acid (Rall and Sutherland, 1958). Fig. 1A shows the structure of cyclic AMP.

A

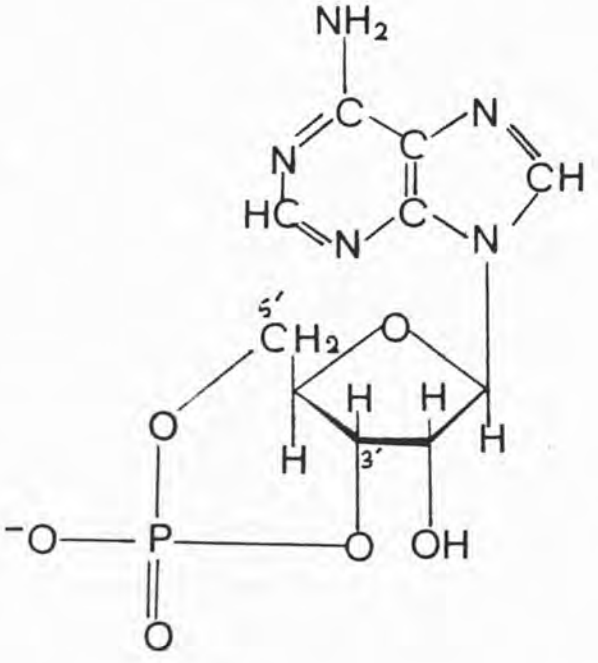
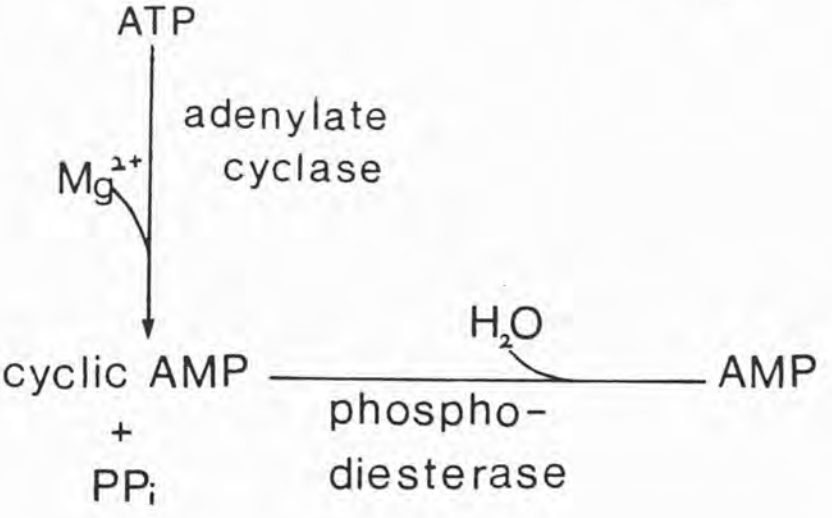


Fig.1

A, cyclic adenosine 3',5'-monophosphate (cyclic AMP), and B, synthesis and the degradation of cyclic AMP.

B



It was soon established that this nucleotide serves as the intracellular mediator in glycogenolylic action of adrenaline and glucagon. It has now become clear that this nucleotide functions as an intracellular second messenger which mediates many of the actions of a number of other hormones (first messenger) in mammalian and non-mammalian tissues. Table 1 shows some examples of hormones whose actions are mediated by cyclic AMP. Cyclic nucleotide also plays a versatile role in controlling the rate of a number of key cellular processes in bacteria, euglena, yeast and amoeba. Cyclic AMP has been implicated in the regulation of many diverse processes such as enzyme activation and inactivation, DNA, RNA and protein synthesis, cell aggregation, cell growth and differentiation, melanosome dispersion, aggregation of blood platlets, muscle cell contraction and relaxation, sensory and neural excitation, cellular permeability and secretion, labour onset in man; it is even involved in the synthesis of the first messenger (hormones) and their release into the circulation. It is also becoming increasingly evident that cyclic guanosine 3',5'-monophosphate, (cyclic GMP), the only other cyclic nucleotide known to occur in nature, may also play a role as a second intracellular messenger. Cyclic GMP mediates effects which are antagonistic to those caused by cyclic AMP in certain biological tissues. In fact cyclic AMP and cyclic GMP have been reported to act in an antagonistic manner to provide intracellular control.

Table 1 Example of some hormones whose actions are mediated by cyclic AMP

Adrenaline
Noradrenaline
Glucagon
ACTH
LH
Vasopressin
Histamine
Insulin

1.3.2. Adenylate cyclase and cyclic AMP synthesis

Further work by Sutherland et al. revealed the presence of a Mg^{2+}-dependent enzymatic reaction in the plasma fraction; this enzyme is stimulated by adrenaline or glucagon which then converts ATP to cyclic AMP with a loss of inorganic pyrophosphate (Fig. 1B). The enzyme was identified as adenylate cyclase (Sutherland et al., 1962); it is specific for ATP as its substrate and will not form 3',5'-cyclic nucleotide from adenosine diphosphate or from other nucleotide tri- phosphates.

Adenylate cyclase is localized in the plasma membrane (Robison et al., 1968.; Sutherland and Robison, 1969.; Pastan and Perlman, 1971) or in certain membrane-bound components of the cytoplasm such as the sarcoplasmic reticulum (Rabinowitz et al., 1965) and microsomes (Sutherland et al., 1962; Entman et al., 1969). Cyclic AMP is produced on the inner surface of the cell membrane, where it brings about the required response. One of the unique features of adenylate cyclase found in mammalian cells is its ability to respond to hormones. In some cases only a single hormone may activate this enzyme in a given tissue such as the adrenocorticotropic hormone (ACTH) in the adrenal gland (Taunton et al., 1969); in others a number of hormones may activate a single adenylate cyclase. This is true for the fat cells, where glucagon, ACTH, thyroid stimulating hormone (TSH), secretin and adrenaline are effective in activating a single adenylate cyclase (Birnbaumer and Rodbell, 1969).

A model has been proposed to explain the structure of the membrane localized adenylate cyclase (Rodbell et al., 1971). The model assumes that the enzyme has multiple components; the specific receptor sites of this enzyme are located on the external membrane surface and the catalytic site on the interior surface of the cell membrane which has access to ATP and Mg^{2+} and which catalysis the hydrolysis of ATP to cyclic AMP. There is also an intermediate coupling or transducer mechanism which "translates" the hormone binding to cyclase activation. In addition there is probably a regulatory site which modifies the binding of the hormone to the receptor and the response of the system to the bound hormone (for a detailed study of the various sites of this enzyme see Bradhan and Cheung, 1982).

1.3.3. Cyclic AMP phosphodiesterase and cyclic AMP degradation

The duration of adenylate cyclase stimulation by the first messenger (hormone) is important in determining the nature and intensity of cyclic nucleotide response in any given tissue; the cyclase system would not provide responsive control if the cyclic nucleotide persisted in cells beyond the period of hormonal-activated synthesis. An enzymatic activity which was capable of destroying the biological activity of cyclic AMP in the presence of Mg^{2+}, was detected in the extracts of heart, brain and liver (Sutherland and Rall, 1958); it could be inhibited by methyl xanthines such as caffeine and theophylline. The enzymatic activity was found to be present in all tissues which possess cyclic AMP. The enzyme was purified and identified as the Mg^{2+}-dependent cyclic AMP

phosphodiesterase. It deactivates the cyclic nucleotide by cleaving off the phosphodiester bond at the 3'- position producing the inactive 5'-AMP and pyrophosphate (Fig. 1B).

Pyrophosphate, ATP and other nucleotide triphosphate and citrate act as inhibitors of phosphodiesterases in vitro (Cheung, 1966, 1967; Gulyassy, 1971). It is likely that these agents inactivate the phosphodiesterase by acting as Mg^{2+} chelators (Kimberg, 1974). In addition to methyl xanthines, other compounds such as adenosine (Gulyassy, 1971), puromycin (Appleman and Kemp, 1966), diazoxides (Schultz et al., 1966) and papaverine (O'Dea et al., 1970) also inhibit cyclic AMP phosphodiesterase but their mechanism is unknown.

In summary the interaction between the first messenger (hormone) and the receptor sites of the adenylate cyclase results in stimulation of the enzyme which then increases the production of the second messenger (cyclic AMP) on the cytoplasmic side of the membrane (Fig. 2).

1.3.4. The involvement of cyclic AMP in ion transport

In subsequent years, following the discovery of cyclic AMP, increasing evidence has been obtained regarding its involvement in the regulation of a number of gastrointestinal ion transport processes. Before considering the role which this nucleotide may play in the regulation of gastric ion transport, it is of importance to review the criteria which Sutherland et al. have set forth as those which should be satisfied in order to confirm that cyclic AMP is a mediator of hormonal effects in any given tissue (Sutherland et

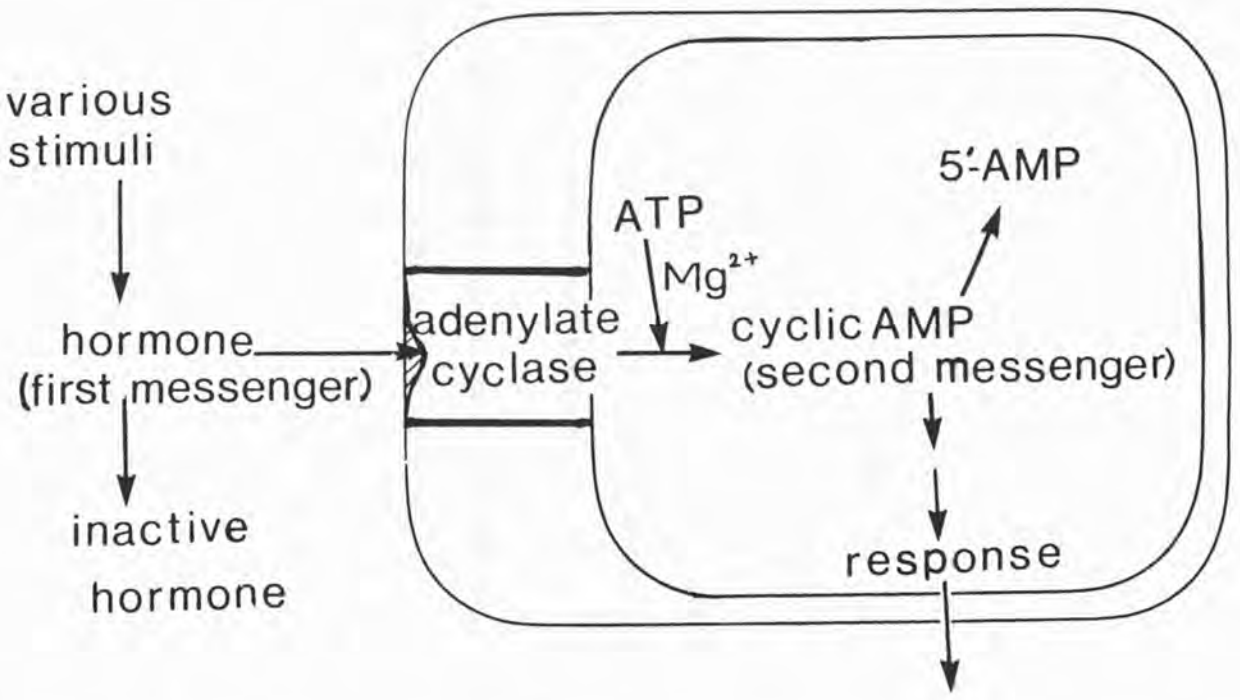


Fig.2

Fig.2 An illustration of the second messenger concept. The interaction between the first messenger (hormones) and the receptor sites of adenylate cyclase results in stimulation of the enzyme. The enzyme then increases the production of the second messenger [cyclic AMP] on the cytoplasmic side of the membrane.

al., 1968). Thus (1) adenylate cyclase in broken cell preparation should respond to the same hormone which is effective in the intact tissue. Hormone analogues should have the same order of potentiality in vitro as in vivo preparations; (2) cyclic AMP concentration, in the intact tissue should change in response to the hormone; the change in nucleotide level should preceed but not follow the physiological response; (3) it should be possible to mimic hormonal effects with exogenous cyclic AMP, or one one of its derivatives, which in this respect is $N^{6'}$, $O^{2'}$-dibutyryl cyclic AMP (dibutyryl cyclic AMP), and (4) the effects of hormones which activate adenylate cyclase should be potentiated by drugs or substances which inhibit cyclic nucleotide phosphodiesterase activity, if the hormone is present in submaximal concentration.

It has long been known that vasopressin increases both the transport of Na^+ ions and the permeability changes in the frog skin (Ussing and Zerahn, 1951 and Koefoed-Johnson and Ussing, 1953) and the toad bladder (Bently, 1958; Leaf et al., 1958). Vasopressin also increases both the oxygen consumption and the rate of glycogenalysis (Leaf, 1960). Such reports provoked Orloff and Handler (1962) to investigate the possibility that vasopressin might also stimulate the production of cyclic AMP in the toad bladder and that this nucleotide may be modifying the permeability of membrane structure to water. Experiments showed that the addition of cyclic AMP and theophylline (a phosphodiesterase inhibitor) to the serosal side of the toad bladder resulted in an increased water permeability and Na^+ transport which resembled in all aspects to the increases caused by vasopressin. They concluded that since cyclic AMP, theophylline and

vasopressin elicited similar effects in the toad bladder it is consistant with the view that vasopressin induces its effects on permeability and Na^+ transport by stimulating the production of cyclic AMP in this tissue.

After the demonstration by Orloff and Handler, Alonzo and Harris (1965) and Alonzo et al. (1965) became interested in the role which this cyclic nucleotide might play in the regulation of gastric acid secretion. Using isolated frog gastric mucosa, they demonstrated that histamine and methyl xanthines could stimulate acid secretion whereas the imidazole ring, the only structure common to both, caused an inhibition. The imidazole ring was shown to activate cyclic nucleotide phosphodiesterase. Harris et al. (1965 and 1969) and Way and Durbin (1969) demonstrated more directly that the addition of cyclic AMP to the medium bathing the amphibian gastric mucosa increased acid secretion, furthermore the time course and the magnitude of cyclic nucleotide level exactly corresponded to the secretory response of the amphibian gastric mucosa exposed to methyl xanthine (Harris et al., 1969). In addition to methyl xanthines and exogenously applied cyclic AMP, the application of gastric pentapeptide to in vitro gastric mucosa of the frog increased acid secretion which was accompanied by a rise in mucosal cyclic nucleotide level (Rosen et al., 1971; Charters et al., 1973). Furthermore Ray and Forte (1973) have demonstrated the presence of a histamine – sensitive adenylate cyclase activity in homogenates of frog gastric mucosal cells. The presence of a cyclic AMP and cyclic GMP phosphodiesterases in gastric mucosa of the mud-puppy (Necturus), dog, rat and the guinea pig has also been reported (Sung et al.,

1972).

Studies with amphibian gastric mucosa have provided strong evidence in favour for a role of cyclic AMP as an intracellular mediator of gastric acid secretion in these species.

Large numbers of similar studies have also been carried out on mammalian gastric mucosae. The work has mainly centered on the action of histamine. However, earlier results were less clear and conflicting in some respects. For example, exogenously applied cyclic AMP and theophylline were reported to cause a decrease in H^+ secretion in the in vivo rat stomach (Taft and Sessions, 1972) but an increase in the in vitro rabbit stomach (Fromm et al., 1975b). Mao et al. (1972, 1973) failed to obtain evidence in favour for a role of cyclic AMP in gastric acid secretion in the in vivo dog stomach. They observed that whilst theophylline inhibited gastric mucosal cyclic nucleotide phosphodiesterase activity when added in vitro, it was nevertheless incapable of initiating gastric acid secretion when administered in vivo; it could however potentiate the secretory response to histamine or pentagastrin. The failure of methyl xanthines to initiate acid secretion in dogs had also been reported previously (Robertsons et al., 1950).

Ramwell and Shaw (1968) demonstrated an increase in acid secretion when an in vivo mucosa of the rat stomach was perfused with cyclic nucleotide; this effect could also be elicited by perfusing the membrane with theophylline. Perrier and Laster (1970) have also reported stimulation of this nucleotide by histamine in the guinea pig gastric mucosa, but Rosenfield et al. (1976) did not. Amer

(1972) suggested that acid secretion is associated with a decrease in the cyclic AMP level whereas Kimberg (1974) concluded that in some species at least, it is associated with an increase.

Thompson et al. (1981) explained such confliction to be related to methodological and species difference and also to cellular heterogeneity of the gastric mucosa in which the acid secreting parietal cells may account for different proportions of the total cell population in different species. They further suggested that the presence of an enzyme such as adenylate cyclase, which has a universal cellular distribution also makes studies of this kind very difficult.

However, recent reports favour the stimulation of cyclic AMP production by hormones, followed by an increase in ion transport across the mammalian gastric mucosa. For example Ekblad et al. (1978) reported that the initial response to histamine by the in vitro guinea pig gastric mucosa is an increase in the intracellular cyclic AMP level followed by stimulation of H^+ ion secretion. However while H^+ ion stimulation remains steady, the changes in cyclic nucleotide level are only temporary. Thus also explaining the conflictions observed in earlier experiments. Wollin et al. (1979) have demonstrated similar increases in cyclic AMP level in parietal cell preparation from dog gastric mucosa. Other groups of workers, studing isolated parietal cell preparation have also found equivalent results (Batzri and Gardner, 1978; Major and Scholes, 1978 and Sonnenberg et al., 1978). The activation of adenylate cyclase following stimulation with histamine in rabbit gastric gland preparation has also been reported (Chew et al., 1980) where direct

measurements also showed increased cyclic AMP levels following histamine addition. Similar increases in cyclic nucleotide levels was also observed if theophylline or histamine was applied to the serosal side of piglet gastric membrane (Machen et al., 1982).

Recent work in this field have shown that not only is acid secretion affected by cyclic AMP but transepithelial transport of cation is also affected. However the ion species stimulated by increased cyclic AMP level appears to depend upon the particular gastric mucosa. For example Kuo and Shanbour (1980) demonstrated that cyclic AMP stimulated the net flux of Na^+ ions from mucosal to serosal side when applied to serosal side of the isolated dog gastric mucosa. However it did not stimulated H^+ or Cl^- secretion. Similar effects have also been reported earlier for the frog skin (Hall et al., 1976) and the toad urinary bladder (Mendoza et al., 1970). Theophylline was shown to have similar effects to histamine, i.e. it increased H^+ and Cl^- ion secretion but had no effect on Na^+ ion transport; similarly dibutyryl cyclic AMP was also found to increase H^+ and Cl^- secretion but not Na^+ absorption (Kuo and Shanbour, 1980). Stimulation of H^+ secretion by dibutyryl cyclic nucleotide has also been reported for the in vitro rabbit gastric mucosa (Fromm et al., 1975b) and earlier for the in vivo rat stomach (Jawaharlal and Berti, 1972). However the dibutyryl derivative of cyclic AMP failed to stimulate acid secretion in the in vivo dog stomach (Mao et al., 1972). Canfield et al. (1977) suggested that the difference in response to dibutyryl cyclic AMP in different species could be due to species difference in metabolising this nucloetide or that the rate of entry of cyclic nucleotide could be very low in some species so

that it is largely destroyed by the phosphodiesterase before it reaches the respective cells. A similar explaination was provided by Kuo and Shanbour (1980). The presence of a histamine-sensitive adenylate cyclase in not only parietal cells but also in the surface epithelial cells has also been demonstrated. Hence it appears that the stimulation of adenylate cyclase by the hormone may result in increased cyclic AMP production which would in turn stimulate ion transport across mammalian membranes. However the ion species affected by increased cyclic nucleotide level appears to depend upon the animal in question.

1.4. Prostaglandins

1.4.1. General

Prostaglandins (PG) are a family of fatty acid derivatives which have a variety of potent biological activities of a hormonal or regulatory nature. The name prostaglandin was initially derived by the Swedish physiologist Von Euler (1934) for a lipid soluble acidic substance found in the seminal plasma, the prostate gland and the seminal vesicles. In very small amounts this compound was found to lower blood pressure and to stimulate certain smooth muscles to contract.

Although readily synthesized in mammalian tissues, only little endogenous prostaglandins are detected in pheripheral blood because they are destroyed enzymatically by the lungs (Samuelsson et al., 1975 and Bukhave and Rask-Hadsen, 1979). There is no evidence for the storage of prostaglandins (Piper and Vane, 1971 and Anggard and

Larsson, 1971) and thus their release is considered to reflect de novo synthesis, which may be affected by nervous or hormonal stimulation, mechanical damage or chemical irritants (Collier, 1971 and Rask-Hadsen and Bukhave, 1979).

The molecular basis of many of the major activities of prostaglandins is not yet fully clear. Some of the effects of prostaglandins are the stimulation of the regulation of blood flow to particular organs, the control of ion transport across membranes and the modulation of synaptic transmission. There is much clinical interest in prostaglandins. For example prostaglandins are thought to participate in parturition: infusion of PGE_2 induces delivery within a few minutes in laboratory animals. Prostaglandins seems to modulate the action of hormones rather than act as hormones themselves. They often alter the activities of the cells in which they are synthesized. The nature of their effect may vary from one type of cell to another in contrast to the uniformity found in the action of hormones.

1.4.2. Structure of prostaglandins

The structure of prostaglandins was established by Bergstrom and his collegues. They initially reported the isolation of two prostaglandins. The compounds were named PGE and PGF, depending upon their solubility in buffer as apposed to ether solvents. The full chemical structure of these and related naturally occurring prostaglandins were elucidated in 1962 and 1963 by Bergstrom et al.

Prostaglandins are 20-carbon fatty acids that contain a cyclopentane (5-carbon) ring, the basic skeleton of which is named prostane and the corresponding monocarboxylic acid (Fig. 3A), from which the chemical names of all prostaglandins are derived.

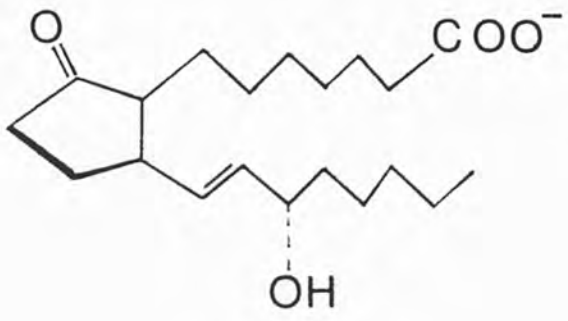
Six major series of natural prostaglandins have so far been described, depending upon the difference in the cyclopentane ring. These parent prostaglandins are designated by the letters E, F, A, C, B and I.

The biosynthetic pathway for prostaglandins was elucidated by Von Dorp et al. (1964) and Bergstrom et al. (1964) independently. Both workers found that the polyunsaturated fatty acid, arachidonic acid, is a precursor of prostaglandins. The enzyme responsible for the conversion of the polyunsaturated fatty acid to prostaglandins was named the prostaglandin synthetase (Fig. 3B).

1.4.3. The relationship between prostaglandins and cyclic AMP

That prostaglandins might mediate some of their effects through alteration of cyclic AMP level was initially suggested by Steinberg et al. (1964). They reported that prostaglandin E_1 inhibited the effects of catecholamines, ACTH, glucagon and TSH on both lipolysis and phosphorylase activity in rat epidermal fat pad. Butcher et al. (1965) suggested that PGE_1 might be acting by lowering the intracellular cyclic AMP level. This view was further supported by Orloff et al. (1965); they reported that PGE_1 antagonized the effects of arginine vasopressin and theophylline on water and ion movement in the toad bladder, thereby suggesting the possible effect of prostaglandins on phosphodiesterase. Conversely the effects of

A



B

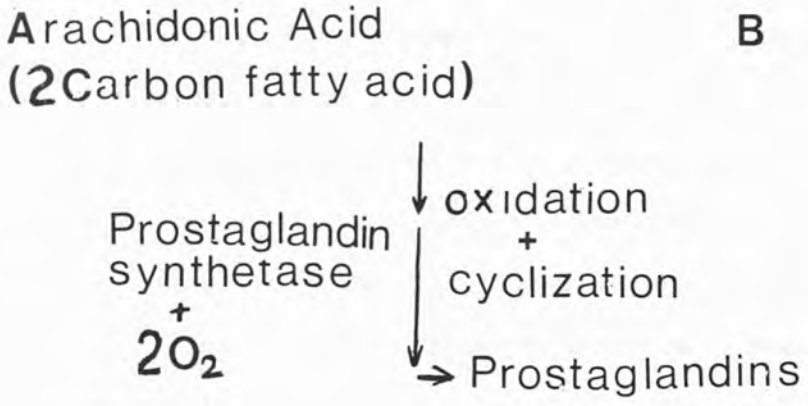


Fig.3

Basic skeleton of prostaglandins, A and the synthesis of prostaglandins from arachidonic acid, B.

exogenously applied cyclic AMP on these parameters were not antagonized by PGE_1. These findings led Butcher el al., (1967) and Butcher and Baird (1968) to determine whether prostaglandins exerted their anti-lipolytic effects on fat pads through lowering cyclic nucleotide level. They consequently found that PGE_1 antagonized the effects of adrenaline on cyclic AMP level but when applied by itself, it significantly increased the cyclic nucleotide level. The stimulatory effect of PGE_1 on cyclic AMP level was lost when isolated fat cells were used. Hence their work with intact fat pads rather than with isolated fat cells, showed that prostaglandins could increase as well as decrease cyclic AMP level depending upon the cell type involved. Since the anti-lipolytic action of PGE_1 was exerted aganst all the above mentioned hormones, it seemed likely that it either inhibits the enzyme system, adenylate cyclase, or prevents cyclic AMP from activating the enzyme lipase (catalytic enzyme for lipids) by increasing nucleotide phosphodiesterase activity. Steinberg and Vaughen (1967) reported that prostaglandins did not antagonize the lipolytic effect of dibutyryl cyclic AMP and thereby suggested that prostaglandins were acting to inhibit the adenylate cyclase system rather than activating the nucleotide phosphodiesterase in adipose tissues.

1.4.4. Involvement of prostaglandins in ion transport

Various investigators have reported the involvement of prostaglandins on the transport of water and electrolyte in frog cornea (Beitch et al., 1974), rabbit kidney thick ascending limb and collecting tubules (Stockes, 1979), canine trachea (Al-bazzaz et al.,

1981), and on both water and sodium transport across the toad bladder and the frog skin (Orloff et al., 1965; Barry and Hall, 1969; Fassina et al., 1969; Ramwell and Show, 1970; Lipson and Sharp, 1971; Ozer and Sharp, 1972). The involvement of prostaglandins in the regulation of transepithelial ion transport, in particular Na^+ ion transport, has been largely studied in epithelial membranes such as the frog skin and the toad urinary bladder. It is clear that cyclic AMP plays an important role in sodium transporting properties of the epithelial membranes. It has long been recognised that vasopressin stimulates the active transepithelial transport of sodium ions in the amphibian skin mediated by an increased production of cyclic nucleotide (Baba et al., 1967; Rider and Thomas, 1969; Rajerson et al., 1972 and Barry et al., 1975). That the maintainance of active sodium transport depends upon the biosynthesis of the prostaglandins was first reported by Barry and Hall (1969); they found that exogenous PGE_1 stimulated sodium transport across the isolated frog skin. Ramwell and Show (1970) observed that E-type prostaglandins are released from the frog skin. Lipson and Sharp (1971) reported that applied PGE_1 stimulated sodium transport across the isolated urinary bladder of the toad by increasing the intracellular cyclic AMP level. Hall (1973) observed that arachidonic acid also stimulated sodium transport across the skin of the frogs. These findings suggested that endogenous prostaglandins may play a role in the regulation of sodium transport across the epithelial membranes. The discovery that anti-inflammatory drugs such as acetyl salicylic acid, indomethacin and phenylbutazone can inhibit prostaglandin biosynthesis by inhibiting prostaglandin synthetase activity (Vane, 1971; Ferreira et al., 1971; Smith and Willis, 1971; Flower et al.,

1972) provided a means by which it was possible to block the cellular biosynthesis of prostaglandins and observe the effect on sodium transport. Since alteration of cellular PGE biosynthesis and exogenous PGE are reflected in intercellular cyclic AMP level, it became apparent that prostaglandins, like vasopressin, may exert their effects on active transepithelial sodium transport via cyclic AMP. Hall et al. (1976) reported that the anti-inflammatory drugs not only decreased transepithelial sodium transport, as measured by the short-circuit current, but that these drugs also reduced the release of prostaglandin-like material from the amphibian skin. They further found that exogenously applied prostaglandins increased the intracellular cyclic AMP level two minutes prior to the increase in the short-circuit current, thus confirming that prostaglandins exert their effect on cation transport through elevation of cyclic AMP in the frog skin. Similar observation were also made by Gerencser (1978).

Prostaglandins, especially the E and F series, have also been reported to influence sodium transport across other biological membranes. However the effects are diverse depending upon the nature of the epithelia involved. For example PGE_1, E_2 and $F_{2\alpha}$ have been shown to inhibit sodium transport as well as the s.c.c. in the gall bladder (Leyssac et al., 1974), rabbit kidney collecting tubules (Iino and Amai, 1978) canine treachea (Al-Bazzaz et al., 1981) and intestinal mucosa (Al-Awqati and Greenough, 1972).

As far as the author is aware, the involvement of prostaglandins in the transepithelial transport of Na^+ ions across the gastric mucosa has not been reported so far. However, a large number of reports can be found in the literature regarding the involvement of prostaglandins in acid secretion by the gastric mucosa. For example Rabert and his associates (1967) demonstrated that the infusion of PGE_1, PGE_2 and PGA could in a dose dependent manner effectively inhibit food or histamine stimulated gastric acid secretion in dogs. These findings were confirmed by Nezamis et al., (1971). Wilson and Levine (1972) and Jacobson (1970) similarly observed that PGE_1 could diminish the secretory response in dogs. They concluded that the anti-secretory effect of PGE_1 was likely mediated directly at the parietal cell level.

Although a large number of reports are in favour of the inhibitory effect of prostaglandins on gastric acid secretion or secretagogue-stimulated acid secretion, the secretory inhibitor PGE_2 has been reported to increase both the adenylate cyclase activity and therefore the level of cyclic AMP in the gastric mucosa of several species and in different animals (Simon and Kather, 1977; Thompson et al., 1977; Wollin et al., 1976 and 1979; Soll and Wollin, 1977).

The findings that histamine, a potent stimulator of acid secretion and prostaglandins, its inhibitor, both stimulate the production of cyclic AMP in the gastric mucosa (Sonnenberg et al., 1978) provided more confusion to the mechanism employed by prostaglandins in inhibiting acid secretion. Whilst a large number of studies on intact membrane systems have indicated that histamine stimulates adenylate cyclase of parietal cells leading to an

increased production of cyclic AMP, it was however unclear as to the action of prostaglandins at the cellular level. A number of investigators have explained the difference between stimulation of cyclic AMP by acid secretory hormones and prostaglandins and their opposite effects on acid secretion by their action on different cell populations within the gastric mucosa (for example Dousa and Dozois, 1977).

Most of the earlier studies were carried out on whole intact gastric mucosae, the cellular heterogeneouity of which made the association between cyclic AMP response and the acid secretory response difficult to interpret. However the use of isolated gastric mucosal cells have allowed the situation to become more clear. A large number of investigators have reported that prostaglandins stimulate cyclic AMP production by the non-parietal cells and even cause an inhibition of its production by the parietal cells (for example Major and Scholes, 1978; Wollin et al., 1979; Glick, 1974 and Soll, 1980). Why prostaglandins increase cyclic nucleotide production by the non-parietal cells is at present not known, but whether this could be associated with sodium transport has yet to be determined.

1.5. Present work

Since its descovery in 1958, cyclic AMP has been associated with the regulation of a wide variety of cellular functions including ion transport across biological membranes. It has been identified in all animal species studied so far, including bacteria as well as in unicellular organisms. In those cells in which it occurs, cyclic AMP

seem to play largely a regulatory role. So far cyclic AMP has not been shown to be essential in the same sense as for example ATP; cellular functions would come to a halt in the absence of ATP. Cyclic AMP seems to act in most cases to either increase or decrease the rates of cellular processes.

The involvement of cyclic AMP in the regulation of the active transport of Na^+ ions and in the secretion of acid across amphibian skin and the gastric mucosa is well established. There is also stronge evidence in favour of the involvement of cyclic AMP in the regulation of the active transport of Na^+ ions (Kuo and Shanbour, 1980) and in acid secretion (Chew et al., 1980 and Rutten and Machen, 1981) across mammalian gastric mucosa. Where as acid secretion in mammallian stomach, has been shown to be a function of parietal cells (see for example Ito and Schofield, 1978 and Forte et al., 1981), the cell type which give rise to net Na^+ transport has not yet been specified. As mentioned previously, because the active transport of Na is present in the stomach of the fetal rabbit which possesses a large population of non-differentiated epithelial cells and in which parietal cells have not yet developed (Menzies, 1958) it was concluded that the active transport of Na^+ ions is a function of non-differentiated epithelial cells (Wright, 1962, 1964).

The gastric mucosa of the 28 day old fetal rabbit carries out the active absorption of Na^+ ions from the mucosal to the serosal side which constitutes a major proportion of the total s.c.c. (approximately 70%) across the membrane. It was of interest to investigate the possible involvement of cyclic AMP in the regulation of Na^+ transport across this membrane. As the 28 day old gastric

mucosa of the rabbit fetus still contains a large proportion of non-differentiated epithelial cells and relatively few parietal cells the effect of cyclic AMP on Na^+ ion transport would also indicate its effect on non-parietal cells.

The effect of methyl xanthines and non-steroidal hormones on the s.c.c. across the 28 day old fetal gastric membrane was studied as was the effect of prostaglandin precursor, arachidonic acid, and its inhibitors, the anti-inflammatory drugs.

The effect of theophylline on the unidirectional transport of both Na^+ and Cl^- ions was also studied. Alterations in the intracellular cyclic AMP content of mucosal cells with respect to the application of theophylline, adrenaline and vasopressin was determined.

C H A P T E R 2

MATERIALS AND METHODS

2.1. MATERIALS

All laboratory chemicals used were of Analar grade and obtained from the British Drug House Chemicals Ltd., (BDH). Other materials were obtained as follows:-

Chemical	Supplier
Acetyl salicylic acid [powder]	May and Baker Ltd.
Adenosine 3',5'-monophosphate (cyclic)	Sigma Chemical Co.
Adrenaline	"
Agar	Hopkin and Williams Ltd.
Arachidonic acid (supplied as free acid which is a clear colourless liquid, 99% pure)	Sigma
Caffeine	BDH
Cellulose ester millipore filters (diameter = 25mm, pore size = 0.45μm)	Millipore Corp.
$^{36}Cl^-$ (half life = 10^6 years)	Amersham

Cyclic AMP assay kit (radioisotope dilution test with A-3',5'-MP binding protein: filter technique)	Boehringer Mannhein GmbH. Biochemics
Dibutyryl cyclic AMP	Sigma Chemical Co.
Glucagon	"
Indomethacin	May and Baker Ltd.
$^{22}Na^{+}$ (half life = 2.6 years)	Amersham
Pentagastrin	Sigma Chemical Co.
Phentolamine	Ciba
Phenylbutazone	May and Baker Ltd.
Propranolol	ICI
Rabbits (New Zealand white doe)	Morton Commercial Rabbits
Saffan	Glaxo
Silicone antifoam emulsion	Hopkins and Willians Ltd.
Theophylline	BDH
Timolol	Merk, Sharp and Dohme

Vasopressin (arginine and lysine) (supplied in 0.9% sodium chloride solution, containing 0.5% chlorobutanol)	Sigma Chemical Co.

2.2. METHODS

2.2.1. Operative Procedure

On the 28th day of pregnancy, New Zealand white doe were anaesthetized by the administration of an intravenous dose of Saffan (9mg/kg of rabbit weight), via the marginal ear vein. A mid line incision was made through the abdomen to expose the uterus. The fetuses, of which there were usually 8-10, were removed from the mother, killed immediately by a blow to the head followed by dislocation of the cervical vertebrae. The whole fetal stomach was rapidly removed within 20 seconds and gently dropped into a conical flask containing 200 ml of pre-oxygenated ice-cold bathing medium. Approximately 4-5 stomachs were placed in each 200 ml of the medium thereby increasing the thermal mass around the stomachs so that any sudden change in the temperature of the surrounding medium was kept at minimum. The stomachs were further cooled by storing the conical flasks at 2-3^{o}C until required. Under these conditions, stomachs remained viable for up to 5 days and even up to 8 days in some cases, as judged by their ability to develop a potential difference of a magnitude similar to those observed for fresh and 24 hours stored gastric mucosae.

Fresh and 24 hours stored stomachs reached maximum p.d. within 20 minutes of the experiment being set up, whereas stomachs which had been stored for more than 24 hours required a longer period of time (up to 1 hour) to reach the maximum p.d.

2.2.2. Mounting Procedure

Fetal stomach was cleared of adherent tissue and cut open through the cephalic surface, in vivo contents were washed away with ice-cold bathing solution. The stomach was opened out to a flat membrane and gently spread over a coarse cotton gauze, care being taken to avoid damage to the membrane through instrumental contact. Membrane plus the cotton gauze was then sandwiched between two perspex chambers (Fig. 4) of the type used by Kendall and Wright (1967) based on the design of Ussing and Zerahn (1951). A membrane with a useful area of $0.6cm^2$ was obtained. The volume of each chamber was 5 ml.

Each chamber was provided with an oxygen lift which oxygenated and stirred the solution. A small quantity of silicone antifoam emulsion was thinly spread around the inner top circumference of each chamber to prevent frothing. The solutions in each chamber were brought into contact with reversible calomel electrodes through the use of salt bridges (thin polythene tubes of 2mm diameter, filled with 3M KCl containing 2% (w/v) agar). Calomel electrodes were used for measuring the p.d. across the membrane, the tips of the salt bridges were inserted at point A in the chambers and lying 1mm from the membrane (Fig. 4). Current was passed through the preparation using large area Ag/AgCl electrodes, salt bridges from which entered the chambers perpendicular to the membrane (point B in Fig. 4) and about 1cm from it.

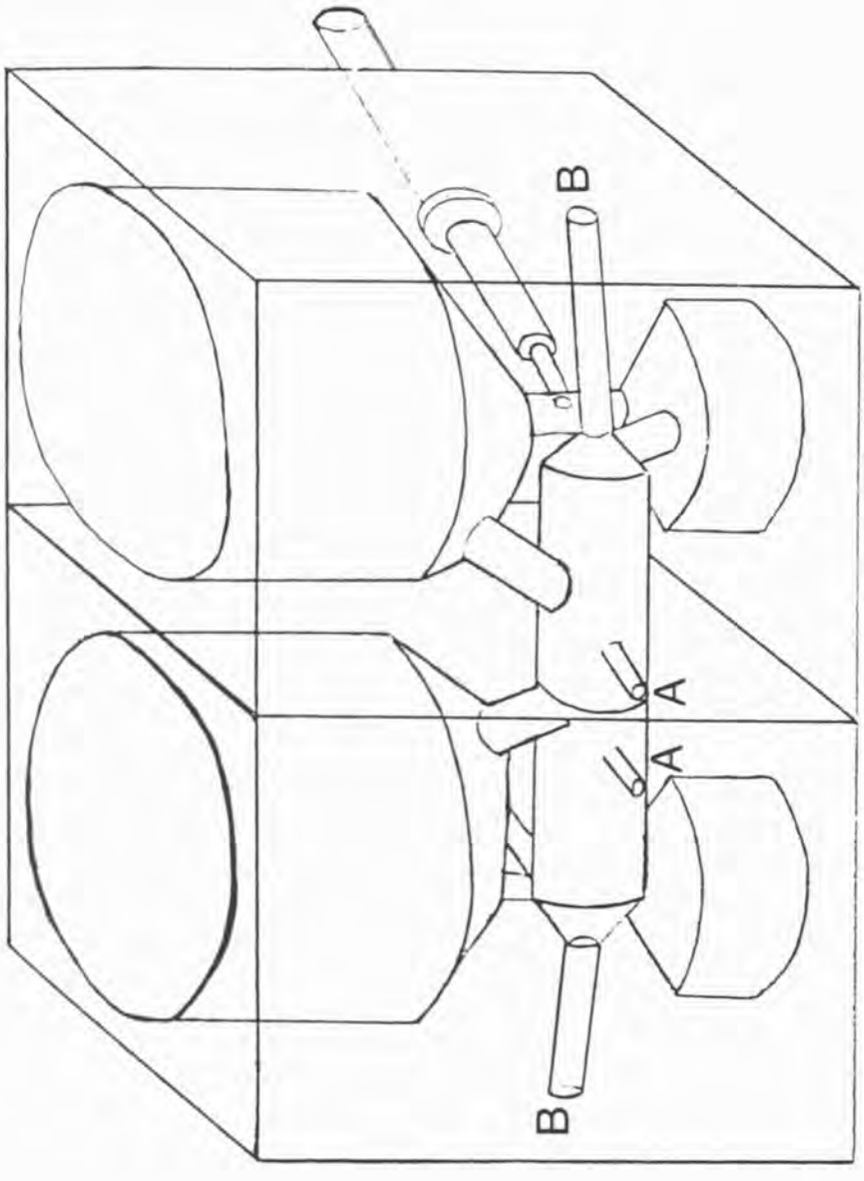


Fig. 4 The perspex chambers used for clamping the gastric mucosa.

2.2.3. Measurement of the s.c.c. and open-circuit p.d.

A feed back device was used to voltage clamp the preparation to zero transmembrane p.d., allowing for inter-electrode solution resistance and difference in single electrode potential of the calomel half-cells. The calomel electrodes were connected to the input of the device and the current output passed through the preparation via the Ag/AgCl electrodes and salt bridges. The output was monitored using a Bryans 27000 chart recorder. With the voltage clamped to zero, this current was now the Ussing - Zerahn s.c.c. Open circuit p.d. could be observed by breaking the current output circuit. Fig. 5 shows the feedback circuit used to measure the s.c.c. and the open circuit p.d. across the membrane.

Both the salt bridges and the electrodes were checked periodically so that residual p.d., if any, could be recorded and the feedback system adjusted accordingly.

The chambers were placed in a water bath, the temperature of which was thermostatically controlled to $\pm$ 0.2°C. The bathing medium within the chambers was maintained at a constant temperature of 35°C. The chambers were partially covered with rubber bungs to prevent evaporation and to preserve Po_2 and Pco_2.

2.2.4. Bathing medium

A buffered solution was used as the experimental bathing medium.

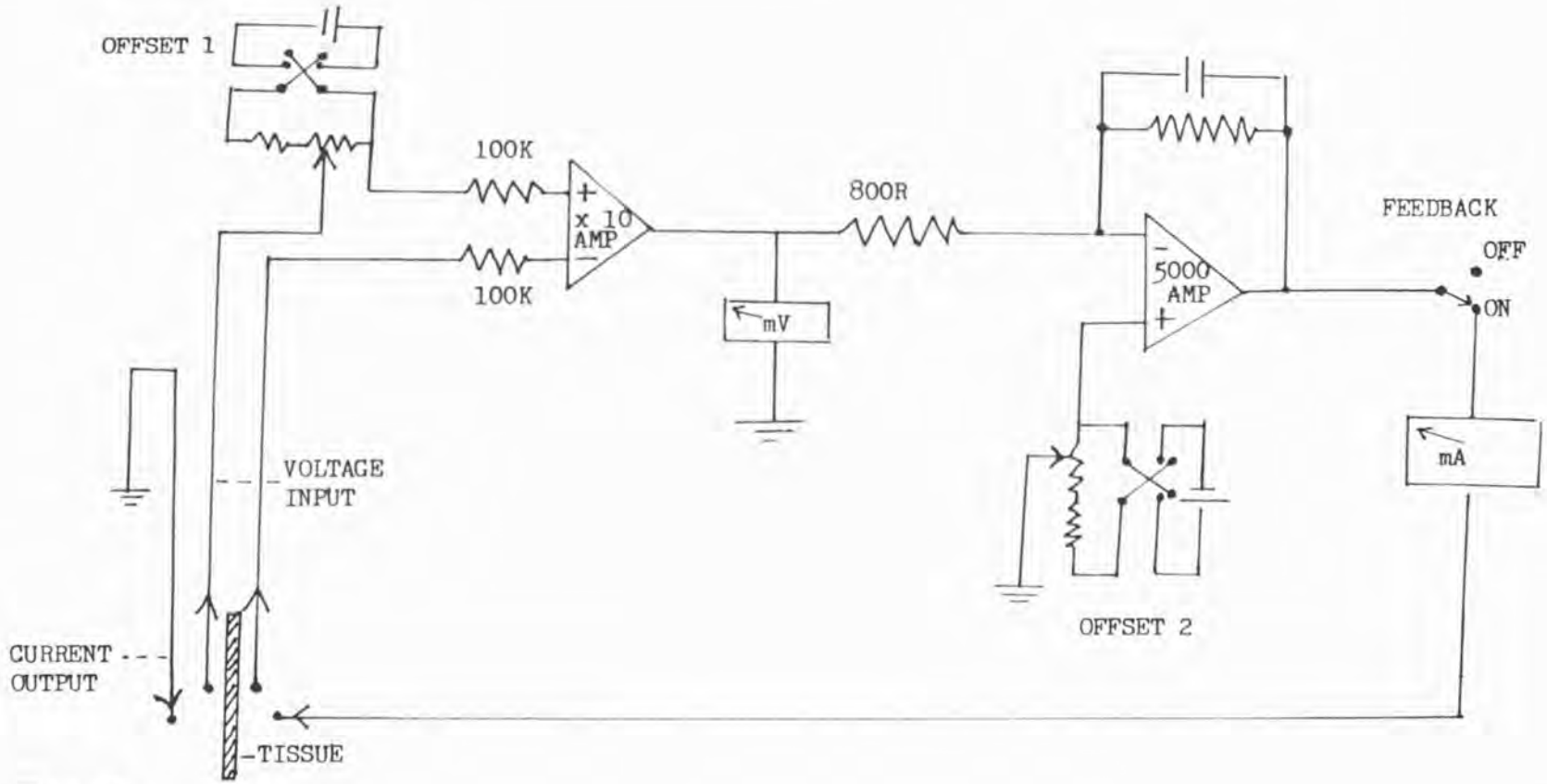


Fig.5

The integrated circuit used to measure the s.c.c. and open circuit p.d.
Offset 1 corrects the difference in potentials of calomel half cells.
x10 AMP senses potential across the tissue (input impedance 10^{12}).
x500 AMP detects "error" signal and developes a proportional current.
Offset 2 enables the system to clamp to a voltage other than zero.
Feedback ON/OFF displays open circuit voltage (OFF); or current necessary to clamp tissue to chosen voltage (ON position)

This solution had the following composition (mM):-

Na^+	=	133.91
Cl^-	=	147.29
K^+	=	13.39
Ca^{2+}	=	6.69
HCO_3^-	=	13.39
Glucose	=	24.00

The solution was gassed with 95% oxygen and 5% carbon dioxide for at least 45 minutes before use and continuously during the experiment. The pH of this solution was 7.4 after gassing.

2.3. Measurements of the unidirectional transport of sodium and chloride ions

Further experiments were carried out to investigate whether the stimulation of the s.c.c. and hence the net ion transport across the fetal gastric mucosa resulted from an increase in that part of the s.c.c. which is dependent on the presence of Na^+ ions on the mucosal side or whether Cl^- ions also contributed to this increase and if so, to what extent?

In the experiments described below only theophylline was used to stimulate ion transport since within the present work it was found that this methyl xanthine is one of two agents which maximally stimulated ion transport across the gastric mucosa of the rabbit

fetus, the other being dibutyryl cyclic AMP (see Chapter 3, section 3.1 and 3.2). In each experiment theophylline was applied to the solution bathing the serosal surface at a maximum effective concentration [10^{-4}M] (again see Chapter 3).

Only the absolute unidirectional fluxes were measured in any one preparation, that is, a series of mucosal to serosal flux were measured across one set of gastric mocosae, the flux in the direction serosa to mucosa were measured in another set of preparations.

Gastric mucosae were removed from the 28 day old fetus and the membranes set up under short circuit conditions as described in Section 2.2.2 and 2.2.3 of this Chapter. An Integrating Ammeter was inserted in the current passing circuit which enabled the direct measurement of the total area under the s.c.c. curve and therefore the net ion transport. Known volumes (5.0 ml) of the bathing medium were placed in the serosal and mucosal chambers. Once the s.c.c. had stabilized, 0.1 ml samples were taken from the mucosal and serosal chambers (at t_o) and these were used as background and blank. 2μC of $^{22}Na^+$ (half life Of 2.6 years) as isotonic sodium chloride, were carefully dispensed into the mucosal chamber. About 60 to 90 minutes later (t_1), a second sample was taken from the serosal chamber ("cold" side) which was immediately followed by the addition of theophylline (10^{-4}M) to the serosal solution. After allowing for maximum stimulation of ion transport, a third sample was taken from the serosal chamber and another from the mucosal chamber ("hot" side) and the time accurately recorded (t_2).

The absolute unidirectional serosal to mucosal sodium flux was measured using exactly the same procedure as described above but in this case 2μC of $^{22}Na^{+}$ were added to the serosal chamber ("hot" solution). The radioactivity in each sample was measured using a Scintillation Counter (see Counting below). The count rate in the weakest samples was always at least ten times greater than the background count (which ranged from 80 to 100 cpm).

The absolute unidirectional chloride fluxes across the membrane in the direction serosa to mucosa and mucosa to serosa were determined by analogous procedures using $^{36}Cl^{-}$ (half life of 10^{6} years).

The amount of ion transferred from "hot" to "cold" chamber from time 0 (t_o) to time 1 (t_1) was calculated from the equation:-

$$\text{Unidirectional flux}_{t_o \to t_1} = X_{hot} \times \frac{cpm_{cold}}{cpm_{hot}} \times \frac{V_{cold}}{M} \times \frac{60}{t_1 - t_o}$$

$(\mu M cm^{-2} hr^{-1})$

where X_{hot} = concentration of the ion species in the "hot" solution.

cpm_{cold} = counts per minute on the "cold" side

cpm_{hot} = counts per minute on the "hot" side

V_{cold} = volume of the bathing medium in the "cold" chamber

M = exposed area of the membrane (0.6 cm^2)

Throughout the experiments care was taken to prevent contamination of the two solutions through splashing. The volume of the bathing medium in each chamber was maintained constant at 5.0 ml; removal of each sample was immediately replaced by the addition of fresh and prewarmed bathing medium. Glassware such as pipettes etc. used for the "cold" and "hot" sides were always kept well separate.

In carrying out the above experiments, it was assumed that the specific activity of $^{22}Na^{+}$ and $^{36}Cl^{-}$ ions remained constant in the "hot" solution; losses of Na^{+} or Cl^{-} ions through their transport to the "cold" side was assumed to be negligible in comparison to the large amount present in the "hot" solution. This assumption has been supported by the fact that the count rate from the "cold" side was small relative to that obtained from the "hot" side; flux measurements have shown that less than 1% of the "hot" counts were transported to the "cold" side.

2.3.1. Counting

The scintillation solution used for counting $^{22}Na^{+}$ and $^{36}Cl^{-}$ had the following composition:-

2, 5-diphenyloxazole (PPO)	=	4 gms
toluene (as the solvent)	=	500 ml
triton X100 (as the detergent)	=	500 ml

The concentration of the scintillant was 0.4% (w/v). 0.1 ml of the sample was directly dispensed into 10 ml of the scintillation solution contained in vials. The resulting mixture in each vial was allowed to stand for two hours with regular shaking. After this period, the vials were placed in the scintillation counter (Beckman type LS-3133P), the window of which was adjusted to admit the energy of radiation over a pre-determined specific range. This range was from 0.50 to 0.62 Mev for $^{22}Na^{+}$ and 0.60 to 0.70 Mev for $^{36}Cl^{-}$, respectively. Each of the two isotopes was counted in the complete absence of the other in order to prevent the radioactivity from one isotope being added to that of the sample under measure. Each sample was counted over a period of 10 minutes.

2.4. Cellular Studies

2.4.1. Measurement of intracellular cyclic AMP level

Intracellular cyclic AMP concentration of fetal gastric mucosal cell suspensions were measured. Only fresh stomachs and those which had been stored for no more than 24 hours were used for cyclic AMP estimation since without electrical measurements one had no means of determining whether a stomach which had been stored for longer than 24 hours was viable or not.

2.4.1.1. Experimental procedure

Fetal stomachs were cut open through the cephalic surface and the in vivo contents washed out with ice-cold bathing medium. Mucosal cells from 3 to 4 stomachs were gently scraped off the underlying tissue with a blunt wide edged spatula, using not more than two strokes to prevent the cells from being damaged. Cell aggregates were immediately transferred to 20 ml ice-cold bathing medium contained in a 50 ml conical flask and kept on ice until required.

Cell aggregates were gently shaken loose and divided to provide a control and a test sample. A small quantity of silicone antifoam emulsion was finely spread around the inner circumference of each flask to prevent frothing. The system was incubated (at 35°C) with shaking and continuously flushed with 95% oxygen and 5% carbon dioxide (Fig. 6). Effectors were added to test samples (and control samples where stated) as required.

2.4.1.2. Extraction procedure

2 ml samples were withdrawn from each flask at known time intervals and transferred to a pre-cooled centrifugal tube. Samples were centrifuged at 1700g for 10 minutes in the cold; centrifugal tubes and their holders were pre-cooled by keeping them in ice so as to reduce the reaction rate of the enzymes as best as possible since liquid nitrogen was not used. The supernatant was discarded and the pellet of cells (which weighed between 17 - 55 mg) was dropped into a test tube containing 1 ml boiling cyclic AMP assay buffer. Cyclic

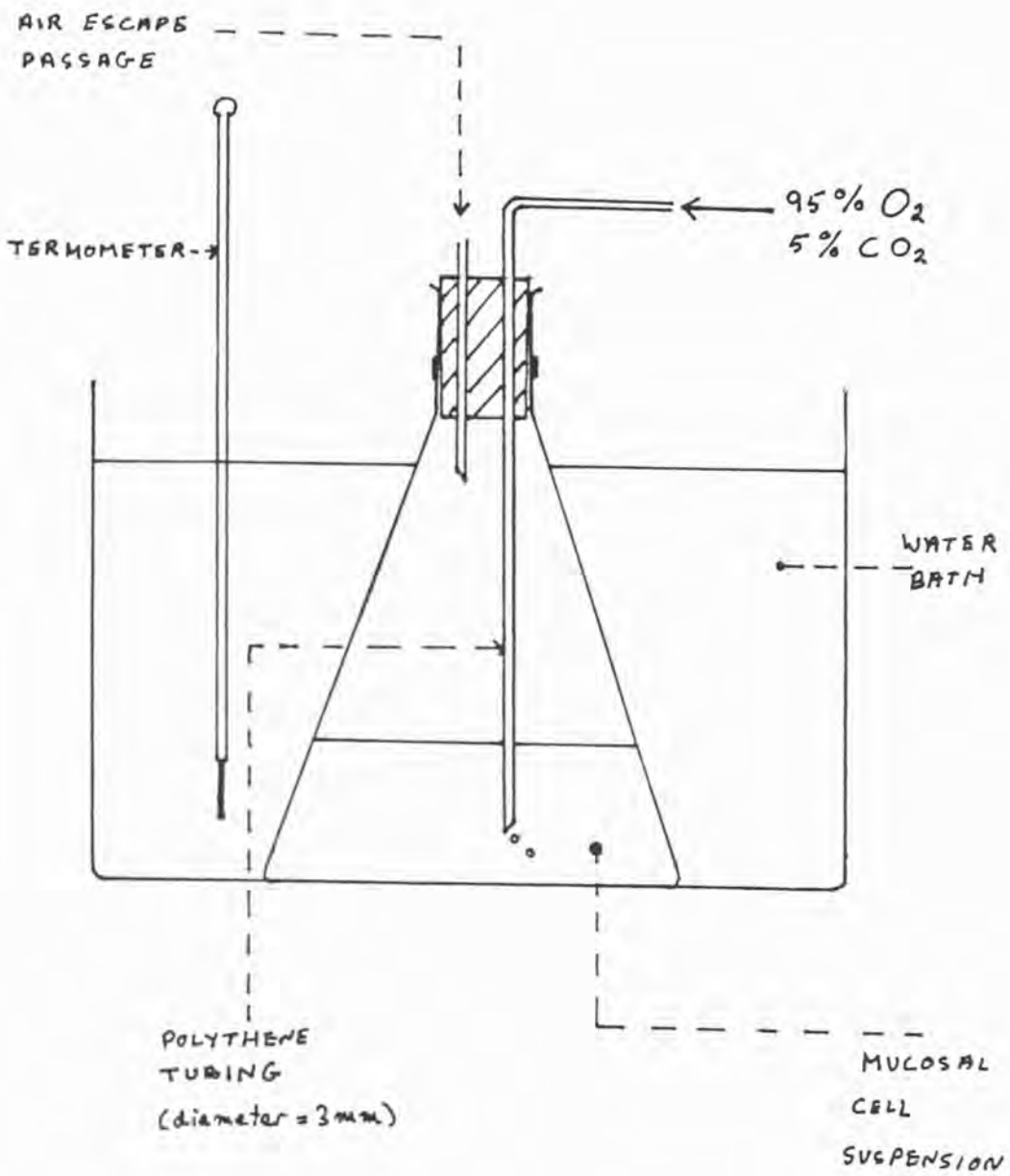


Fig.6

The experimental set-up used for incubating gastric mucosal cells.

AMP assay buffer (pH 7.4) contained Tris (hydroxymethyl) methylamine (50mM), theophylline (8mM) and 2-mercaptoethanol (6mM). The tubes were covered to prevent evaporation and boiled for 5 minutes. Mucosal cell suspension were sonicated for 2 minutes at 15KHz/minute using a 3mm titanium probe connected to an MSE model 300W Ultra-Sonicator. The test tubes were surrounded by ice to prevent the mixture from reaching the boiling point and thus evaporating. The denatured protein was removed by centrifugation at 25000g for 10 minutes; dehydrated over phosphorous pentoxide over a period of 4 days and finally weighed. The supernatant was freeze dried using an Edward Centrifugal Freeze Drier.

2.4.1.3. Assay procedure for cyclic AMP

Cyclic AMP content of mucosal cells was determined by the method of Gilman (1970). This method depends on the molecular events involved in the activation of cyclic AMP-dependent protein kinase by cyclic AMP i.e. it depends on the specific and tight binding between the regulatory or the binding protein subunit of the enzyme and cyclic AMP (see Appendix for the Mechanism of cyclic AMP action). The cyclic AMP-protein complex thus formed is then selectively adsorbed onto the cellulose ester millipore filter (Nirenberg and Leder, 1964; Kuwano and Schlessinger, 1970 and Gill and Garren, 1970).

The high affinity of cyclic AMP for the binding protein subunit of the kinase and therefore its ability to displace other cyclic nucleotides and ^{3}H-cyclic AMP is further used as a means of determining the unknown quantity of cyclic AMP present in samples.

Hence to a known amount of binding protein ^{3}H-cyclic AMP is added at saturating concentration. The added unlabeled cyclic AMP (present in samples or standard) then competes and displaces ^{3}H-cyclic AMP from the binding site of the enzyme; the quantity of ^{3}H-cyclic AMP displaced depends upon the quantity of unlabelled cyclic AMP added. Radioactivity of remaining bound ^{3}H-cyclic AMP is then determined. The effect of added unknown cyclic AMP is then evaluated from a linear decrease in the total bound ^{3}H-cyclic AMP (method often termed the Radioisotope Dilution technique).

A heat stable protein, an inhibitor of cyclic AMP-dependent protein kinase (Posner et al., 1964, 1965; Appleman et al., 1966 and Walsh et al., 1971) is also included in the assay mixture which further increases the affinity of cyclic AMP for the enzyme (Posner et al., 1964; Appleman et al., 1966). The reaction is conducted at pH 4 (in sodium acetate buffer) as the binding constant approaches 10^{-9}M at this pH.

In summary the assay procedure depends on the following:-

(i) competition between ^{3}H-cyclic AMP (present at saturating concentration) and unlabelled cyclic AMP for the binding protein

(ii) the formation of cyclic AMP-binding protein complex

(iii) isolation of the complex through its retention on ester millipore filter

(iv) evaluation of cyclic AMP content from a linear decrease in total bound ^{3}H-cyclic AMP

The assay is conducted in a small volume (50µl) of sodium acetate buffer, pH 4. The only other reaction components are ^{3}H-cyclic AMP, unknown or standard cyclic AMP, binding protein and a maximum effective concentration of protein kinase inhibitor. The binding reaction is initiated by the addition of binding protein after chilling of microtubes etc. and is allowed to continue for 1 hour and 40 minutes at 0°C. At equilibrium the mixture is diluted to 1 ml with cold potassium phostate buffer (pH 6). The bound cyclic AMP is separated from free by passage through the cellulose ester millipore filter (previously rinsed by the same potassium phosphate buffer). Unbound cyclic AMP is further removed by washing the filter with more phosphate buffer. Bound cyclic AMP is then brought out into solution by dissolving the filter in an organic solvent. Radioactivity of remaining bound ^{3}H-cyclic AMP is determined and cyclic AMP content (from samples) is evaluated by the radioisotope dilution technique.

A series of simultaneous standard experiments are also carried out in which known amount of cyclic AMP (standards) is added in place of the sample. A graph is ploted of cpm of zero standard (containing only ^{3}H-cyclic AMP and binding protein but no unlabelled cyclic AMP) over cpm of standards (known quantity of added cyclic AMP) against quantity of standards. Amount of cyclic AMP present in samples is read off from the standard line.

Although other cyclic nucleotides are capable of interfering with the binding between cyclic AMP and the kinase, the most effective being cyclic GMP (Kuo and Greengard, 1969a; Miyamato et al., 1969 and Gilman, 1970) mammalian tissue cyclic GMP levels are

nevertheless not sufficiently high to interfere (Goldberg et al., 1969 and Ishikawa et al., 1969). Also as the binding reaction is conducted at 10 fold dilution of the samples this further reduces the concentration of other cyclic nucleotides.

Boehringer cyclic AMP kit (binding protein radioisotope dilution technique) was used to measure cyclic AMP content of mucosal cells. This kit further simplifies the procedure involved in measuring cyclic nucleotide level and is based entirely on the method developed by Gilman. Infact the only simplification provided by the Boehringer kit is that it not only contains a ready-to-use supply of cyclic AMP-binding protein and binding protein inhibitor but also contains a supply of ^{3}H-cyclic AMP, unlabelled cyclic AMP (for carrying out standards) together with the two buffers required for the assay. Depending on the kit purchased the kit can contain enough material for up to 150 tests.

2.4.1.4. Cyclic AMP assay

The freeze dried supernatant was redissolved in 1 ml of ice-cold 50mM sodium acetate (pH 4) and assayed for its cyclic AMP content.

The solutions plus the samples were pipetted into dry

siliconized micro-reagent tubes in the following order:-

	blank	zero standard	standards			sample
			2pmol	10pmol	20pmol	
soln.1	0.05ml	0.05ml	0.05ml	0.05ml	0.05ml	0.05ml
soln.2	---	---	0.01ml	0.05ml	0.10ml	---
sample	---	---	---	---	---	0.10ml
soln.3	0.02ml	0.02ml	0.02ml	0.02ml	0.02ml	0.02ml
soln.4	0.02ml	---	---	---	---	---
redist. water	0.11ml	0.11ml	0.10ml	0.06ml	0.01ml	0.01ml
mixed well						
soln.5	---	0.02ml	0.02ml	0.02ml	0.02ml	0.02ml

where

solution 1 Acetate buffer, 0.2mol/l, pH 4

Solution 2 Adenosine 3', 5'-monophosphate (cyclic), 200nmol/l

solution 3 [^{3}H] - adenosine 3', 5'-monophosphate, 40nmol/l

solution 4 γ- glubulin, 22.5mg/ml (protein inhibitor of cyclic AMP-dependent protein kinase)

Solution 5 Cyclic AMP binding protein and γ- glubulin

Solution 6 Potassium phosphate buffer, 20nmol/l, pH 6

Additional reagents required but not supplied with the test kit were:

Cellulose ester millipore filters	diameter of filter = 25mm pore size = 0.45μm
Scintillation solution	1 part of 2-(methoxy)-ethanol to 4 parts of toluene containing 0.03M of PPO

Equipment used included micropipettes with interchangeable plastic tips, filtration apparatus with porous plate (25mm diameter) with a detachable top for filtering off radioactive precipitates and scintillation counter.

Redistilled water, included in all standards and samples was used to determine the radioactivity of the blank.

The reaction was initiated by the addition of the binding protein (solution 5), the tip of each pipette was rinsed with the test mixture so as to remove any traces of solution 5 which may have adhered to the plastic tip. A new tip was used for each addition. The solutions were then very carefully mixed by tilting the microreagent tubes thereby allowing the test mixture to gently run along the sides of the tubes. Shaking was avoided as this causes the formation of foam which may destroy the binding protein. Test

mixtures were allowed to stand for 1 hour and 40 minutes at 0°C. At equilibrium the mixtures were diluted to 1 ml with ice-cold 20mM potassium phosphate buffer (solution 6). Cellulose ester filters were placed on the porous plate and moistened with solution 6. Test mixtures were applied to the filters. Filters were rinsed with 10 ml of ice-cold solution 6, microreagent tubes were also rinsed with this solution as was the detachable top of the filtration apparatus. Filters were dried by suction then placed into scintillation vials and dissolved in 1 ml of 2-(methoxy)-ethanol with gentle agitation. 10 ml of the scintillation solution was added and the firmly closed vials were vigorously shaken for 2 minutes and then placed into the scintillation counter (Tri-carb liquid scintillation spectrometer; model 3375) and allowed to stand for 2 hours with occational shaking. The counts per minutes (cpm) for the standards and the samples were determined over a period of 10 minutes. The blank was also counted for 10 minutes.

All glassware used was siliconized before use to minimise adsorption of solutes onto the glass surface. When dissolving the filters, care was taken to prevent the filters from sticking to the wall of the vials; this would have made quantitative dissolving difficult. Solution 5 (binding protein) was prevented from shaking as was the test mixture after the addition of solution 5.

2.4.1.5. Method of calculation: evaluation of cyclic AMP content

From the resulting cpm values the following values were calculated:-

Co = C zero standard - C blank

Cx = C standard - C blank

Cs = C sample - C blank

where C = count per minute [cpm]

A calibration curve was plotted using Co/Cx values of the standards against the corresponding cyclic AMP [pmols] values of the standards. A straight line of the type shown in Fig. 7 was obtained.

Co/Cs values were determined for samples. Cyclic AMP content of the samples were read off from the calibration curve, and multiplied by a factor of 10 to obtain the total content of cyclic AMP in each sample of the supernatant. The calibration curve was newly established at each analysis.

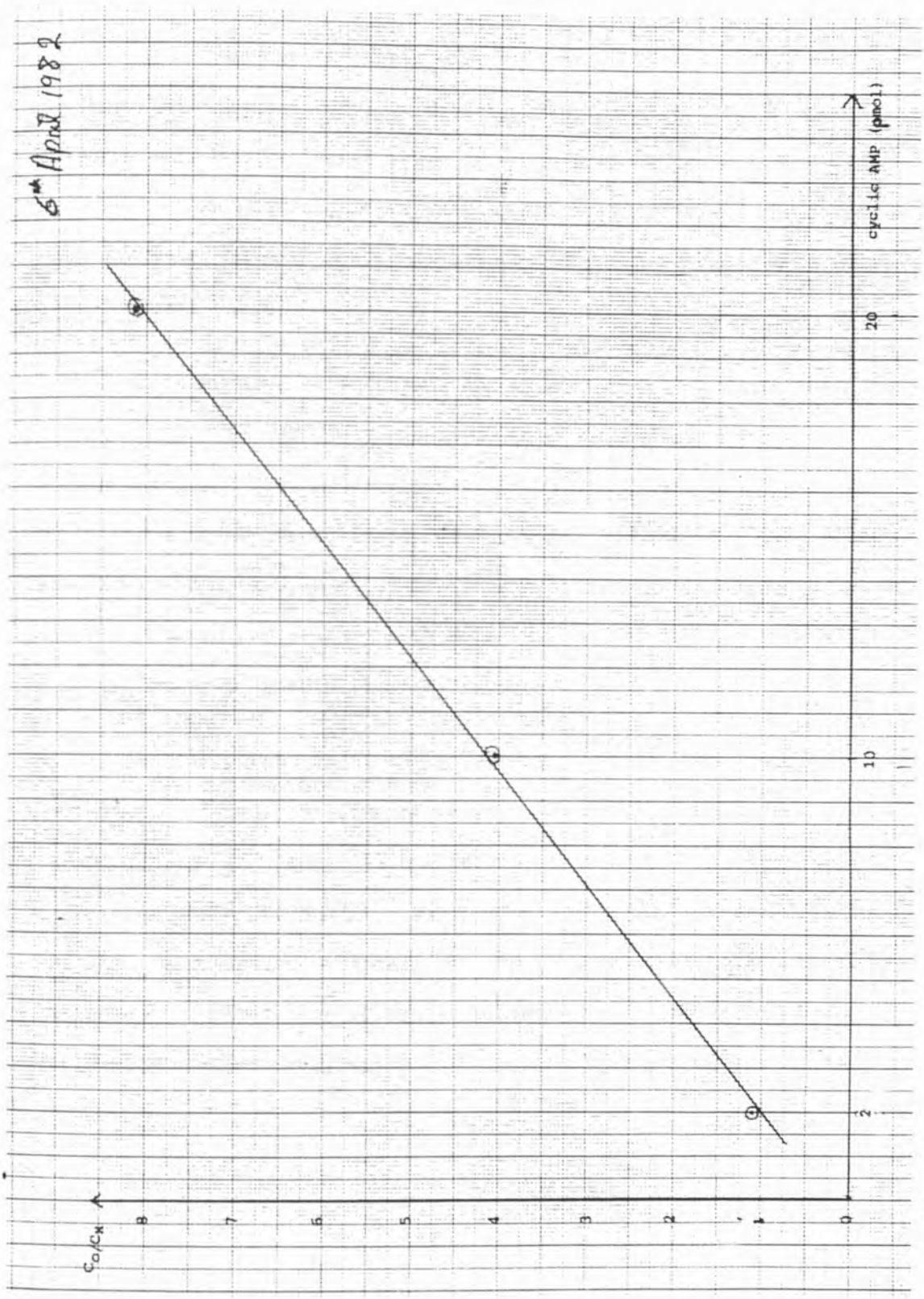


Fig. 7 An example of the type of Calibration curve obtained on 6th April 1982

2.4.1.6. Light microscopy and eosin exclusion test:test of viability of cells

Light microscopy and eosin exclusion tests were carried out on mucosal cell suspensions to determine whether the cells removed by scraping were metabolically alive or not and also to acertain that the cell suspensions contained mainly epithelial cells. These tests were conducted on both the fresh and 24 hours stored gastric mucosae of the rabbit fetus.

Gastric mucosal cell suspensions were prepared as described in section 2.4.1.1. of this chapter. Cell suspensions were incubated with shaking and continuously gassed at 35°C. After 3 hours, cell suspensions were centrifuged at 1700g for 10 minutes. Cell pellet was resuspended in 0.05 ml of the supernatant. Smears of cells were made, air dried and stained with haematoxylin and eosin. Smears were finally observed under a light microscope.

Eosin exclusion test (Blum et al., 1971) was used as an indicator of the viability of cells. Mucosal cell suspensions were incubated in an equal volume of glucose-free bathing medium containing eosin (0.05gm/ml.) for 1 minute at 35°C. A haemocytometer was used to determine whether the cells had taken up eosin or excluded it. Exclusion of eosin was taken as an indication that the cells were metabolically alive.

C H A P T E R 3

RESULTS

In all experiments gastric mucosa preparations were allowed to reach and maintain a steady value of s.c.c., p.d. and resistance for at least 40 minutes prior to agent addition unless stated otherwise. Control experiments have shown that this tissue can maintain a steady s.c.c. p.d. and resistance over a period of 12 hours (Kendall, 1968).

All agents with the exception of vasopressin, pentagastrin and arachadonic acid (which were supplied in liquid form, see Materials) were made up to the required molarity in pre-oxygenated (95% O_2 and 5% CO_2) bathing medium. The resulting mixtures were pre-warmed to 35 C before application to the preparation thereby preventing any sudden fall in the temperature of the bathing medium.

It should be made clear at this point that agent concentrations quoted in the text refer to their final concentration in the bathing medium (5 ml). Agents were applied at minimal concentration to elicit the maximum response. Increasing the concentration further did not result in much greater increases in the response; the concentrations used within the present work are in line with those used by others (see for example Hall et al., 1976; Kuo and Shanbour, 1979;). Maximum response was observed only if the agents were applied to the serosal surface of the gastric mucosa. Therefore unless stated otherwise, concentrations given refer to the minimum concentration required to elicit a maximum response when applied to the chamber bathing the serosal surface of the gastric mucosa.

The magnitude of the s.c.c. was found to vary from one membrane preparation to another. Therefore for comparison purpose the control s.c.c. at the point of agent application was taken to represent a value of 100%. Electrical parameters, i.e. s.c.c. (p.d. and conductance, where given) are represented as those values displayed across a total membrane area of 0.6cm^2 (unless stated otherwise).

It is pointed out that although p.d. and the s.c.c. were directly measured across the membrane preparations as described in Chapter 2, the resistance or the total membrane conductance was however calculated using Ohm's law. It has has been previously reported that the gastric membrane of the rabbit fetus obeys this law (Wright, 1974).

3.1. Effect of cyclic AMP on the s.c.c.

3.1.1. Adenosine 3',5'-monophosphate

From a total of twenty five gastric mucosa preparations used only 14 displayed an increase of or greater than 4% in the s.c.c. i.e. only 56% displayed a response to the exogenous application of cyclic AMP (when applied at a final concentration of 10^{-3}M), In each case cyclic AMP was applied to the serosal surface.

Reference to Table 2 shows that the membrane preparations used to test the effect of cyclic AMP were not only taken from different rabbits over a varying period of time but also that out of the total membranes which displayed an increase equal to or greater than 4%, 5 from a total of 6 were those which had been stored for 72 hours,

Table 2 The effect of cyclic AMP on the s.c.c.

Table shows the effect of exogenous cyclic AMP [10^{-3} M] on the s.c.c. response of the gastric mucosa. The s.c.c. at the point of cyclic AMP addition and at maximum response are shown together with per cent increase in s.c.c.

Date of experiment	storage time (hours)	s.c.c. (μA)/M		% increase in s.c.c.
		at point of cAMP addition [10^{-3}M]	at maximum response	
01.11.79	72	32.00	80.00	150
05.11.79	0	51.00	58.00	14
06.11.79	24	144.00	146.00	3
08.11.79	72	89.00	91.00	2
13.11.79	24	50.00	50.00	0
14.11.79	48	79.00	80.00.	1
14.11.79	48	49.00	55.00	12
15.11.79	72	38.00	49.00	29
19.11.79	0	75.00	76.00	1
19.11.79	0	79.00	80.00	1
03.12.79	0	24.00	24.00	0
06.12.79	72	54.00	56.00	4
10.12.79	1	42.00	93.00	121
12.12.79	48	16.00	14.00	0
21.02.80	72	51.00	56.00	10
26.02.80	24	108.00	165.00	53
26.02.80	24	108.0	135.00	25
04.03.80	24	126.00	129.00	2
04.03.80	24	159.00	246.00	55
06.03.80	72	24.00	31.00	29
18.03.80	0	42.00	44.00	5
19.03.80	24	16.00	20.00	25
25.03.80	24	41.00	41.00	0
25.03.80	24	69.00	87.00	26
26.03.80	48	58.00	58.00	0

M = s.c.c. values are shown as per 0.6 cm^2 of the total membrane area.

another 5 out of 9 consisted of 24 hours stored stomachs, only 1 from a total of 4 were those which had been stored for 48 hours and 3 out of a total of 6 fresh membranes responsed to applied cyclic nucleotide.

3.1.2. $N^{6'}$, $O^{2'}$- dibutyryl adenosine 3',5'-monophosphate

Unlike the naturally occurring cyclic AMP all gastric mucosa preparations used (n = 9), displayed a response to its dibutyryl derivative. Application of dibutyryl cyclic AMP [10^{-4}M] to the serosal solution resulted in a simultaneous increase in the s.c.c. which reached a maximum value of 39.40 $\pm$ 6.08% (s.e.m.) in approximately 40 minute (Fig. 8). The gastric mucosae used for this analysis varied in their storage time ranging from 0 to 72 hours (Table 3).

Dibutyryl cyclic AMP also increased the p.d. and the total conductance across the membrane. However these changes were smaller and less consistent in comparison to the changes observed in the s.c.c. (Table 3).

The effect of dibutyryl cyclic AMP was reversible upon removal of the nucleotide by thoroughly washing out the serosal chamber and the addition of fresh and prewarmed bathing medium i.e. once the cyclic nucleotide was removed from the chambers and the membrane preparation was surrounded by fresh bathing medium, the s.c.c. shortly (within 2-4 minutes) returned to original or just below the original baseline observed prior to dibutyryl cyclic AMP addition.

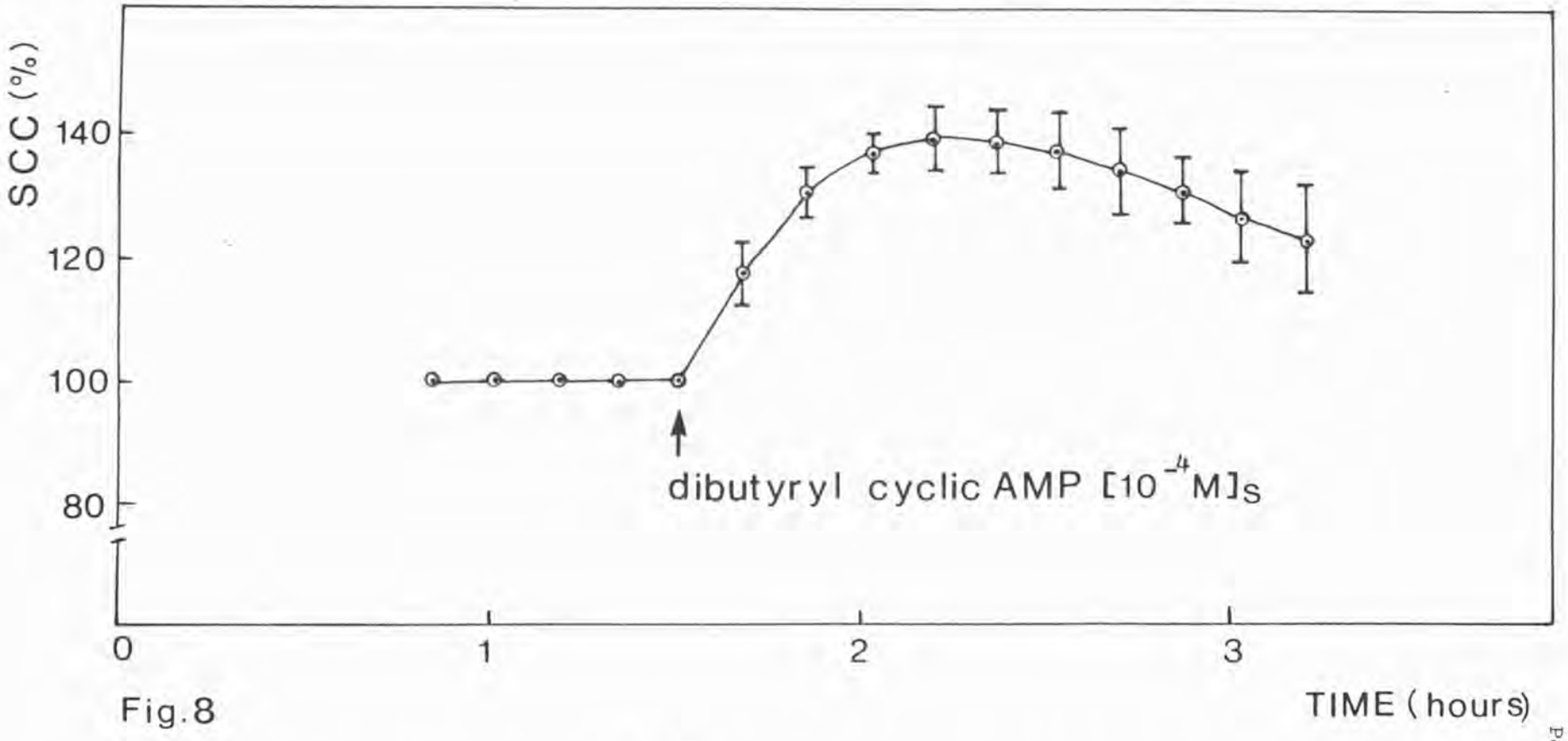


Fig.8

The effect of dibutyryl cyclic AMP $\left[10^{-4}M\right]$ on the s.c.c. across the fetal gastric mucosa. Each point represents the mean $\pm$ s.e.m.; n =8 . s = represents the addition of dibutyryl cyclic AMP to the chamber bathing the serosal surface of the gastric mucosa.

Table 3 The effect of dibutyryl cyclic AMP on the electrical parameters across the gastric mucosa

Table shows the s.c.c. (μA) at the point of dibutyryl cyclic AMP [10^{-4}M] addition and at maximum response together with per cent increase. Dibutyryl cyclic AMP was applied to the serosal surface. The p.d and total membrane conductance (where taken) are also shown.

		s.c.c. (μA/M)			p.d. (mV/M)		conductance (x 10^{-3} mho/M)	
Date of experiment	Storage time (hours)	at point of agent addition	at maximum response	% increase in s.c.c.	at point of agent addition	at maximum response	at point of agent addition	at maximum response
14.05.80	48	138.00	175.50	27.17	-	-	-	-
19.05.80	0	198.00	247.50	25.00	11.00	18.00	18.00	13.75
28.05.80	24	49.00	60.00	22.44	2.50	3.00	19.50	20.00
28.05.80	24	114.00	145.50	27.63	9.00	11.00	12.67	13.23
11.06.80	48	69.00	121.50	76.09	5.50	9.50	12.50	12.78
27.06.80	0	156.00	219.00	40.38	11.00	14.00	14.18	15.64
24.07.80	72	99.00	153.00	54.54	11.00	14.50	9.00	10.55
24.07.80	72	75.00	106.50	42.00	-	-	-	-
mean $\pm$ s.e.m (n = 8)		112.25 $\pm$16.46 p < 0.005	153.56 $\pm$20.01 p < 0.005	39.40 $\pm$ 6.08				

M = represents a total membrane area of 0.6 cm^2.

3.2. Effect of methyl xanthines on the s.c.c.

3.2.1. Theophylline (1,3-dimethyl xanthine)

The application of theophylline [10^{-4}M] to the serosal chamber was also followed by an increase in the s.c.c. Like the response to dibutyryl cyclic AMP the increase following the addition of theophylline reached a maximum value of 38.15 ± 1.67% (s.e.m., n = 8) in about 40 minutes (Fig. 9).

Table 4 shows the storage time of the stomachs used and also the values of s.c.c. (p.d. and total membrane conductance, where taken) at the point of theophylline addition and at maximum response.

3.2.2. Caffeine (1,3,7-trimethyl xanthine)

In a series of five experiments the effect of caffeine [10^{-4}M] was very similar to that seen following the application of theophylline. The time course of the response was also very similar (Fig. 10).

Both theophylline and caffeine were also found to be capable of eliciting a response when applied to the mucosal side of the membrane. However the response was of a smaller magnitude in comparison to that observed when methyl xanthines were applied to the serosal surface.

Methyl xanthines caused similar increases in the p.d. and the total conductance across the membrane but again these changes were less consistent.

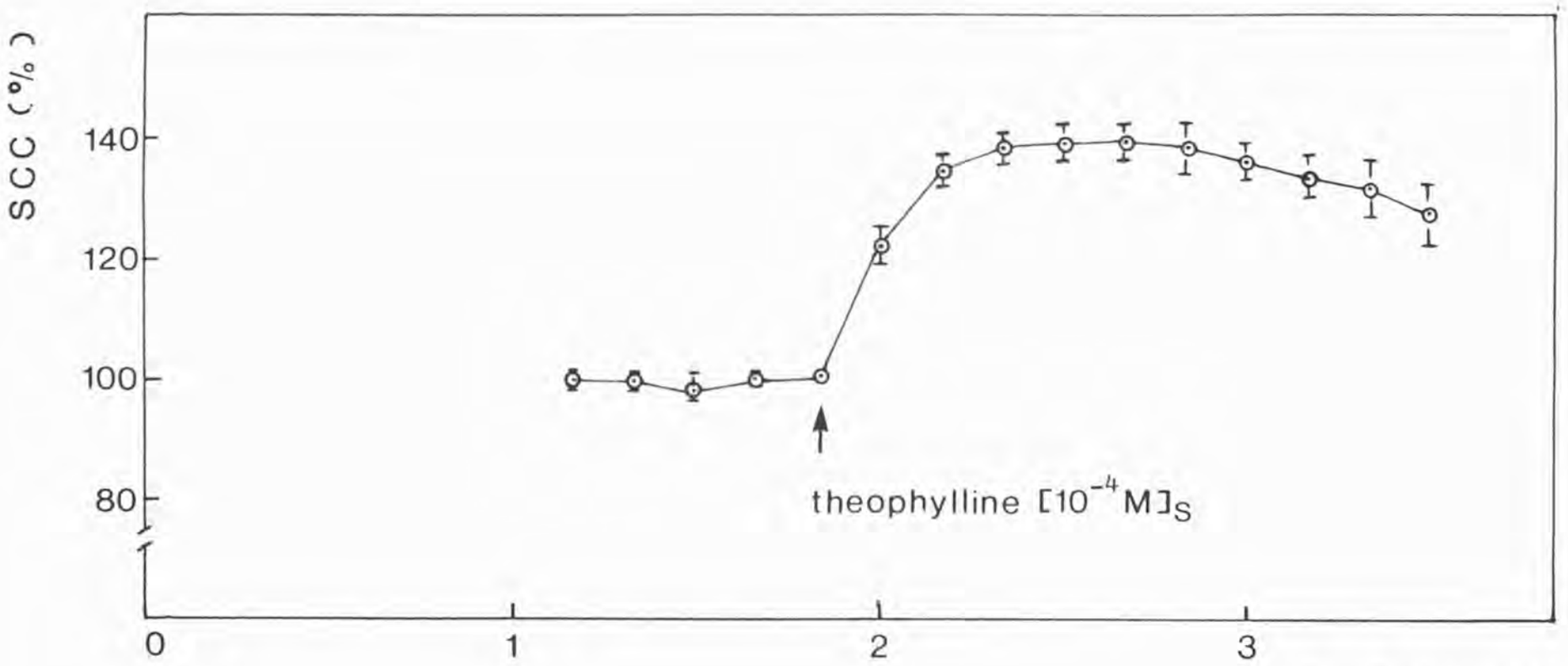


Fig.9

The effect of theophylline [10^{-4}M] on the s.c.c. across the fetal gastric membrane. Each point represents the mean ± s.e.m.; n = 8. s = addition of theophylline to the chamber bathing the serosal surface of the stomach.

Table 4 The effect of theophylline on the electrical parameters across the gastric mucosa

The s.c.c., p.d. and total membrane conductance (where taken) are shown at the point of theophylline [10^{-4}M] addition and at maximum response. Theophylline was applied to the chamber bathing the serosal surface.

		s.c.c. (μA/M)			p.d. (mV/M)		conductance (x 10^{-3}mho/M)	
date of experiment	storage time (hours)	at point of theophylline addition	at maximum response	% increase in s.c.c.	at point of theophylline addition	at maximum response	at point of theophylline addition	at maximum response
28.07.80	0	185.00	255.00	37.84	-	-	-	-
30.07.80	48	203.00	270.00	33.00	-	-	-	-
11.05.82	0	126.00	170.00	34.92	13.00	16.00	9.69	10.62
12.05.82	24	97.00	129.00	32.99	8.50	12.00	11.41	10.75
25.05.82	24	109.00	153.00	40.37	9.50	12.50	11.47	12.24
26.05.82	48	164.00	245.00	48.48	14.00	18.50	11.71	13.24
15.12.82	72	148.00	207.00	39.86	-	-	-	-
31.01.83	0	135.00	186.00	37.78	10.00	13.00	13.50	14.31
mean $\pm$ s.e.m. (n = 8)		145.87 $\pm$12.10 $p < 0.005$	201.87 $\pm$16.91 $p < 0.005$	38.15 $\pm$ 1.67				

M = a total membrane area of 0.6 cm^2.

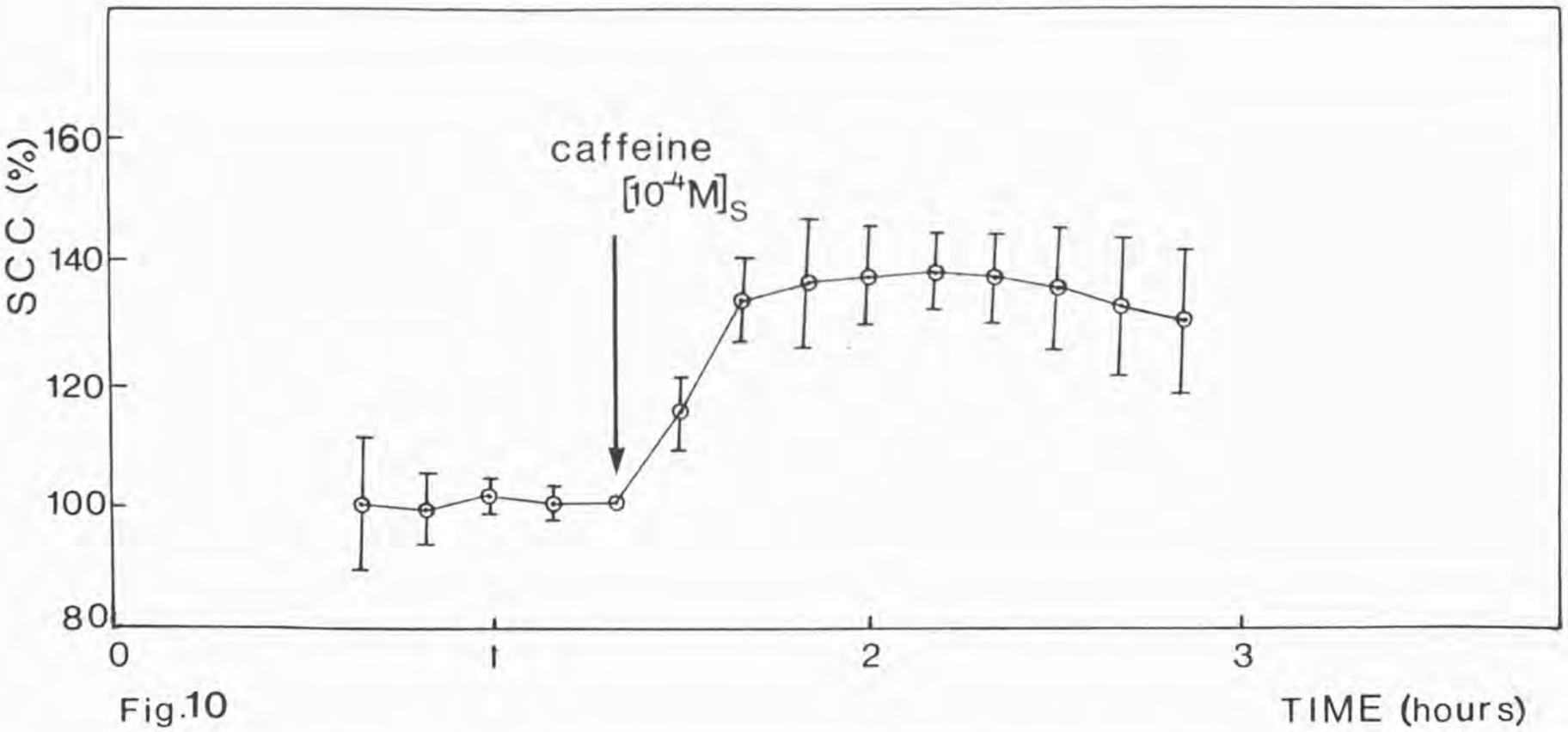


Fig.10

The effect of caffeine on the s.c.c. across the gastric mucosa of the rabbit fetus. Each point represents the mean ± s.e.m.; n = 5. s = represents the addition of caffeine to the serosal surface of the gastric mucosa.

The stimulatory effect of both methyl xanthines used was reversible upon their removal from the serosal chamber.

3.3. Effect of non-steroid hormones on the s.c.c.

3.3.1. Adrenaline

In a series of fifteen experiments, the application of adrenaline [10^{-5}M] to the serosal chamber resulted in a rapid increase in the s.c.c. This increase reached a maximum value of 21.02 $\pm$ 1.88% (s.e.m.) within about 20 minutes following the application of the hormone (Fig. 11).

Reference to Table 5 shows that the storage time of the stomachs used ranged from 0 to 48 hours; the values of the s.c.c., p.d. and total membrane conductance (where taken) at the point of adrenaline application and at maximum response are also shown.

3.3.2. Vasopressin

Arginine vasopressin is the naturally occurring hormone found in most animals (Frieden, 1976 and Barrington, 1979). In a series of three experiments the application of lysine vasopressin resulted in a response similar to that observed with arginine vasopressin (Table 6). However the results presented below are for arginine vasopressin only.

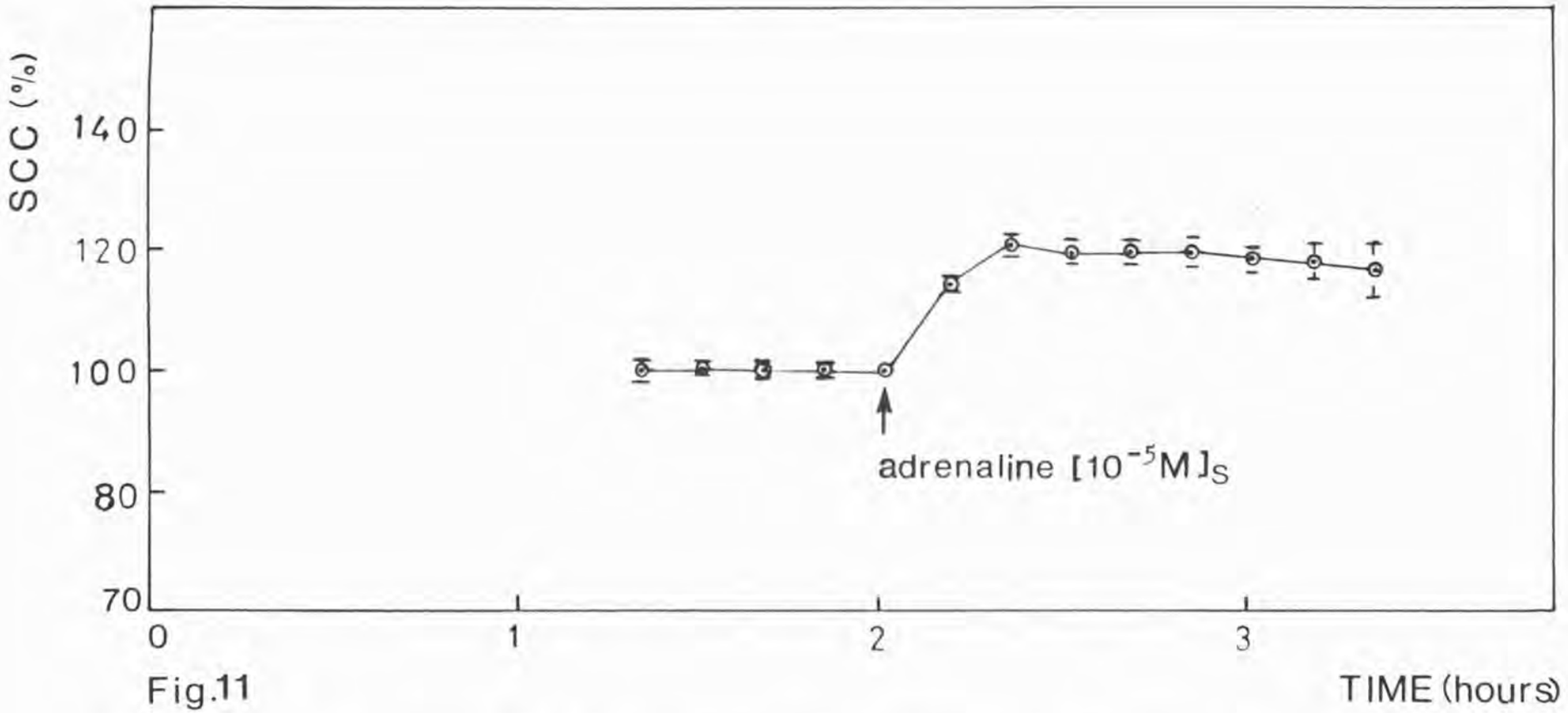


Fig.11

The effect of adrenaline $\left[10^{-5}M\right]$ on the s.c.c. across the fetal gastric mucosa. Each point represents the mean $\pm$ s.e.m.; n = 15. s = represents the addition of adrenaline to the chamber bathing the serosal surface of the gastric mucosa.

Table 5 Effect of Adrenaline on the electircal parameters across the gastric mucosa

Table shows the s.c.c., p.d. and the total conductance (where recorded) at the point of adrenaline [10^{-5}M] addition and at maximum response. Adrenaline was applied to the serosal chamber.

Date of experiment	storage time (hours)	s.c.c. (μA /M)			p.d. (mV /M)		conductance (x 10^{-1} mho /M)	
		at point of adrenaline addition	at maximum response	% increase in s.c.c.	at point of adrenaline addition	at maximum response	at point of adrenaline addition	at maximum response
12.11.80	24	179.00	195.00	8.93	10.50	12.00	17.05	16.25
12.11.80	24	129.00	168.00	30.23	8.00	10.00	16.20	16.80
13.11.80	48	232.00	273.00	17.67	19.50	27.00	11.89	10.11
17.11.80	0	189.00	231.00	22.22	13.00	16.00	14.50	14.44
18.11.80	24	230.00	276.00	20.00	17.00	20.00	13.53	13.80
25.11.80	24	183.00	222.00	21.31	-	-	-	-
28.01.80	24	125.00	156.00	24.80	7.00	8.50	17.86	18.35
05.02.81	48	150.00	174.00	16.00	16.00	17.00	9.37	10.23
26.05.82	48	141.00	174.00	23.40	11.00	13.00	12.82	13.38
02.06.82	24	102.00	141.00	38.23	8.00	10.50	12.75	13.43
17.06.82	48	119.00	133.00	11.76	8.00	9.00	14.87	14.78
15.06.82	17	150.00	192.00	28.00	12.00	15.00	12.50	12.80
14.07.82	48	120.00	135.00	12.50	12.00	13.00	10.00	10.38
13.12.82	0	99.00	120.00	21.20	7.00	8.50	14.14	14.12
18.01.83	24	100.00	119.70	19.00	9.00	11.00	11.11	10.88
mean (n=15)		149.87 $\pm$10.96 $p<0.005$	180.65 $\pm$12.72 $p<0.005$	21.02 $\pm$ 1.88				

M = represents values displaced per 0.6cm^2 of the total membrane area

Table 6 The effect of lysine vasopressin [1i.u./ml] on the s.c.c. response of the gastric mucosa

The actual s.c.c. (μA) at the point of vasopressine addition and at maximum response are shown together with the per cent increase in the s.c.c. Lysine vasopressin was applied to the serosal surface of the gastric mucosa.

date of experiment	storage time (hours)	s.c.c. (μA)/M		% increase in s.c.c.
		at point of hormone addition	at maximum response	
25.11.80	24	76.00	87.50	15.13
13.12.82	0	117.00	147.00	25.64
16.06.82	25	130.00	155.00	19.23
mean ± s.e.m. (n = 3)		107.67 +13.30 $p<0.025$	129.83 +17.40 $p<0.025$	20.00 ±2.50

M = total membrane area of 0.6cm^2.

From Fig. 12 it can be seen that the application of vasopressin [1i.u/ml] to the serosal solution resulted in an increase in the s.c.c. by 24.22 $\pm$ 4.41% (s.e.m., n = 8).

Unlike the response to adrenaline, the response to this hormone was short-lived, i.e. once the maximum increase in the s.c.c. was reached, it was closely followed by a gradual decline. Like adrenaline, the response to vasopressin could only be elicited if the hormone was applied to the serosal surface.

Membrane preparations used to test the effect of vasopressin were also taken from different rabbits over a varing period of time. The storage time of gastric mucosae used also ranged from 0 to 72 hours (Table 7).

3.3.3. Pentagastrin

Pentagastrin is a synthetic peptide with the same four terminal amino acids as the naturally occuring gastrin. In was used in place of gastrin to test its effect on the s.c.c. across this membrane.

Exogenously applied pentagastrin [10^{-5}M] resulted in an increase in the s.c.c. However an increase was only observed in three out of a total of five membrane preparations where an average increase of 11.13 $\pm$ 1.20% (s.e.m.). was reached and maintained over a period of several hours (Fig. 13). Again an increase in the s.c.c. was only observed if the hormone was applied to the serosal surface.

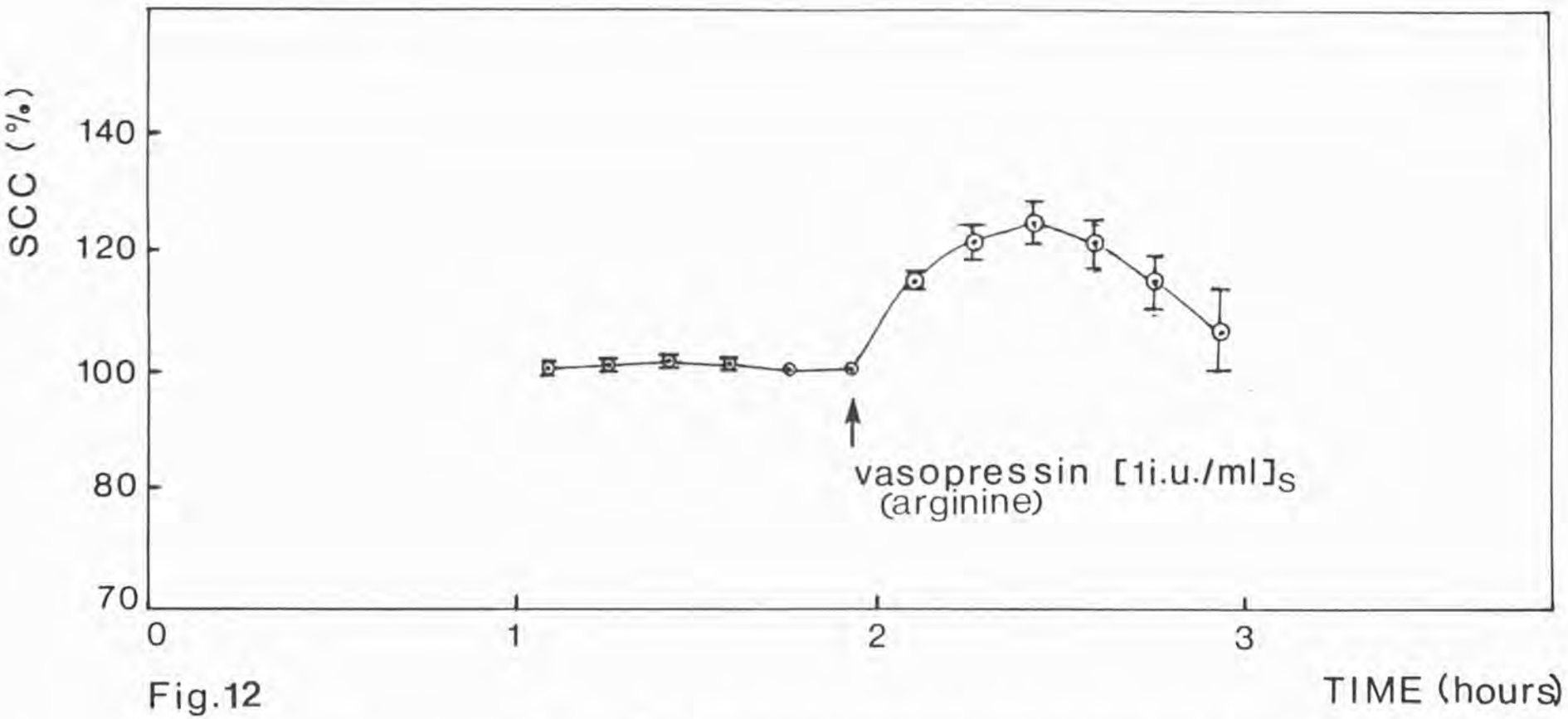


Fig.12

The effect of vasopressin [li.u./ml] on the s.c.c. across the gastric mucosa of the rabbit fetus. Each point represents the mean ± s.e.m.; n = 8. s =represents the addition of vasopressin to serosal surface.

Table 7 The effect of arginine vasopressin on the electrical parameters across the gasteric mucosa

The s.c.c., p.d. and the total membrane conductance (where taken) across the fetal gastric mucosa at the point of arginine vasopressin [1i.u./ml] and at maximum response. Vasopressin was applied to the chamber bathing the serosal surface of the membrane.

		s.c.c. (μA/M)			p.d. (mV/M)		conductance (x 10^{-3}mho/M)	
date of experiment	storage time (hours)	at point of hormone addition	at maximum response	% increase in s.c.c.	at point of hormone addition	at maximum response	at point of hormone addition	at maximum response
25.01.80	24	66.00	70.00	6.06	-	-	-	-
25.01.80	24	71.00	82.00	15.49	8.00	8.00	8.87	10.25
28.01.80	0	141.00	174.00	23.40	12.00	14.00	11.75	12.42
25.05.82	24	201.00	225.00	11.94	16.00	18.00	12.56	12.50
17.06.82	48	156.00	193.00	23.71	12.00	16.00	13.00	12.06
15.07.82	72	99.00	139.00	40.40	6.50	8.50	15.23	16.35
06.12.82	0	90.00	130.00	44.44	8.00	11.00	11.25	11.82
25.01.83	0	120.00	154.00	28.33	12.00	14.00	10.00	11.00
mean $\pm$ s.e.m. (n = 8)		118.00 $\pm$15.26 $p < 0.005$	145.87 $\pm$17.43 $p < 0.005$	24.22 $\pm$ 4.41				

M = values are represented as displayed across a total membrane area of 0.6 cm^2.

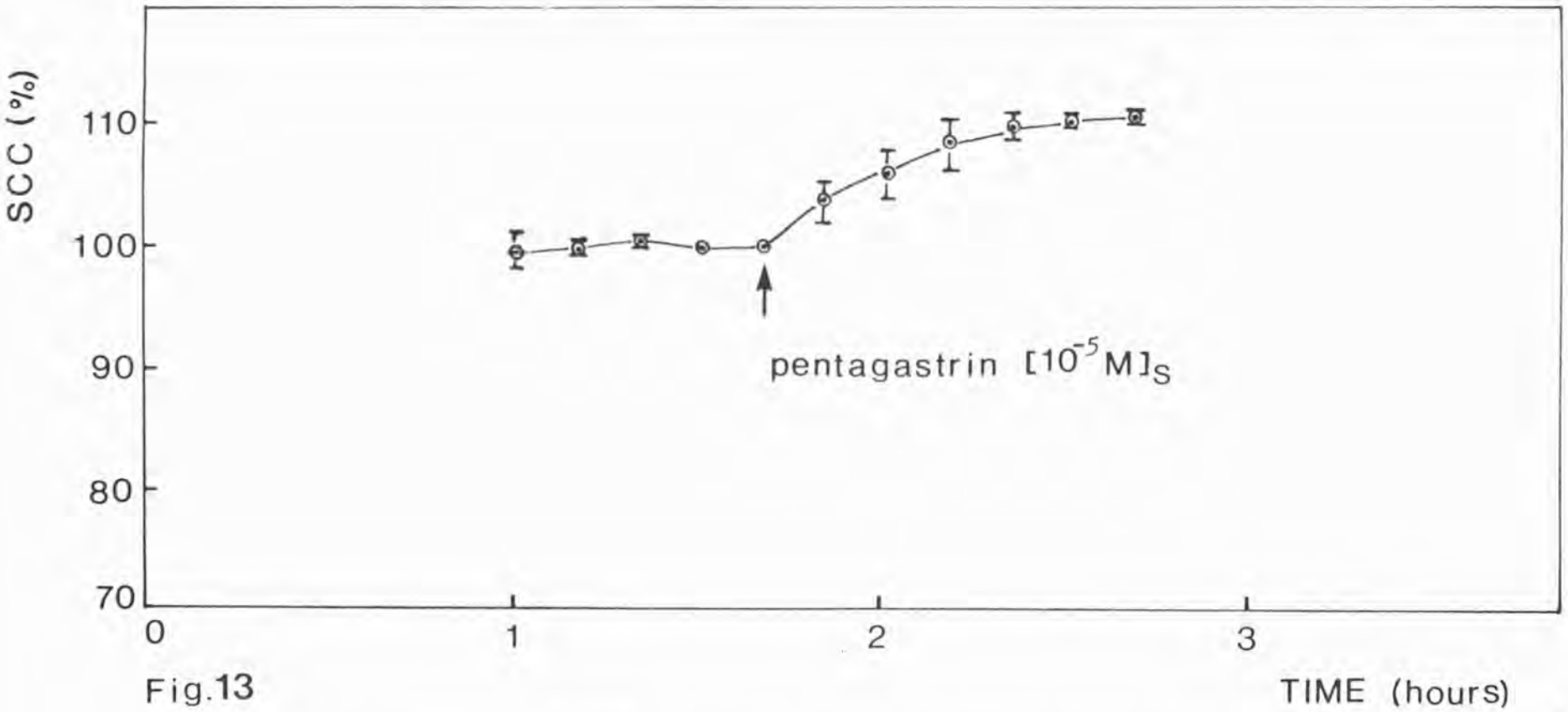


Fig.13

The effect of pentagastrin [10^{-5}M] on the s.c.c. across the gastric mucosa of the rabbit fetus. Each point represents the mean ± s.e.m.; n = 3. s = addition of the hormone to the chamber bathing the serosal surface of the membrane.

Table 8 shows the storage time of the stomachs used and the s.c.c. [μA] at the point of hormone addition and at maximum response.

3.3.4. Glucagon

Fig. 14 shows the response obtained following the application of glucagon [10^{-6}M]. Regretfully the cost of this hormone limited its use to two experiments only.

All four hormones above also caused an increase in the p.d. and a less consistent increase in the total conductance across the membranes.

In all cases the response observed following the addition of each of the four hormones was reversible upon washing out the particular bathing medium to which the hormone was applied and its replacement with fresh and pre-warmed bathing medium.

3.3.5. Effect of the consecutive application of adrenaline and vasopressin on the s.c.c.

Hormones were applied at a concentration which is known to elicit the relevent maximum response, reported in section 3.3 above.

Rabbit fetal gastric mucosa is capable of responding to both adrenaline and vasopressin even when one is applied immediately after the response to the first hormone had declined to its control or just below the control value of the s.c.c. (Fig. 15). The response curve to each hormone was also very similar to that observed when the

Table 8 The effect of pentagastrin on the s.c.c. across the gastric mucosa

The s.c.c. at the point of pentagastrin [10^{-5}M] addition and at maximum response are shown together with mean and per cent increase in the s.c.c. Pentagastrin was applied to the serosal surface of the membrane.

		s.c.c. (μA/M)		
date of experiement	storage time (hours)	at point of hormone addition	at maximum response	% increase in s.c.c.
15.12.82	48	145.70	159.00	9.13
16.12.82	72	150.00	171.00	14.00
18.01.83	24	117.00	129.00	10.25
mean ± s.e.m. (n = 3)		137.57 ± 8.47 $p \leqslant 0.005$	153.00 ±10.20 $p \leqslant 0.005$	11.13 ±1.20

M = membrane area of 0.6 cm^2.

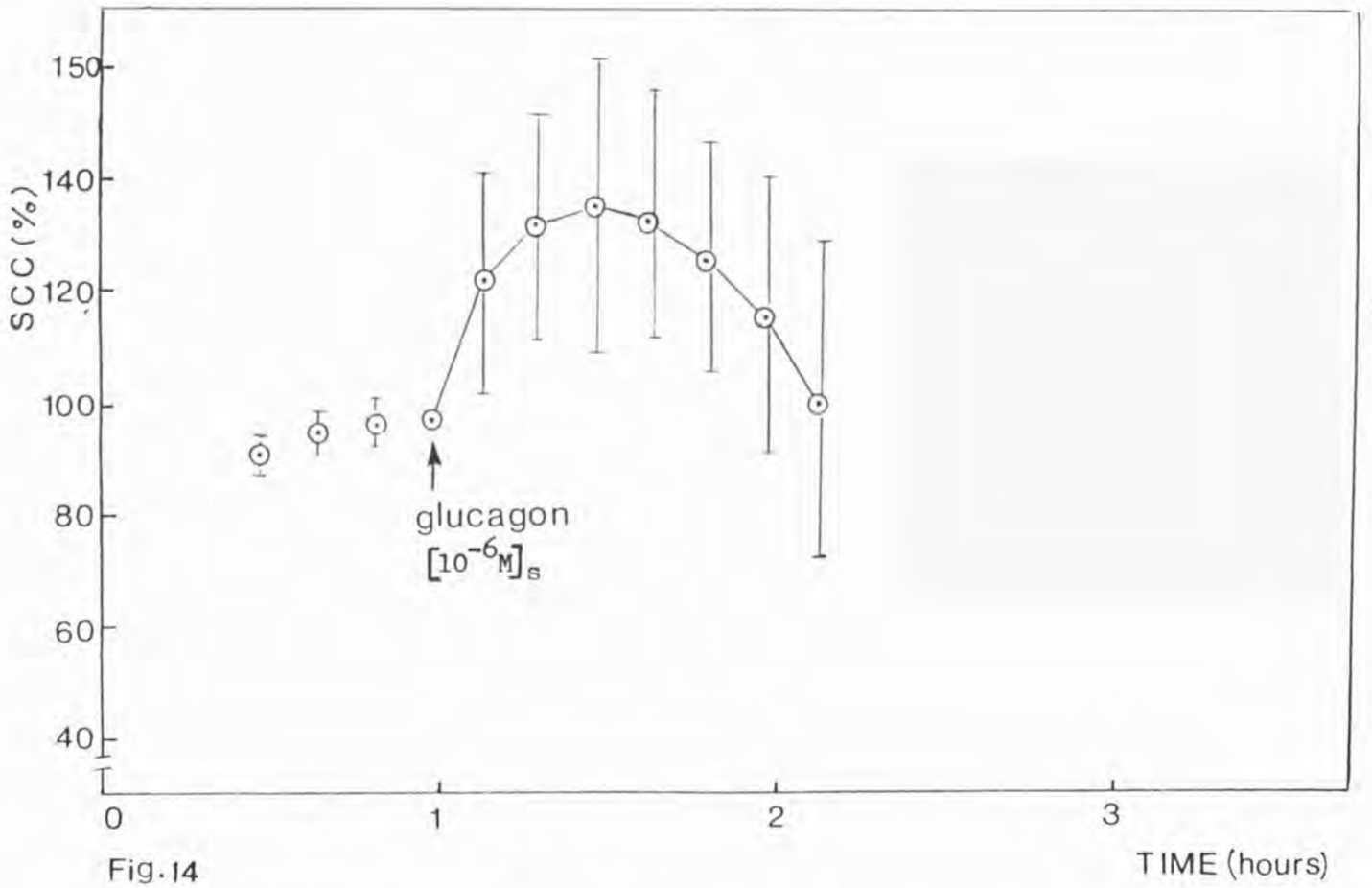


Fig.14

The effect of glucagon $[10^{-6}M]$ on the s.c.c. Each point was calculated from a total of two experiments only. s = the addition of pentagastrin to the serosal chamber.

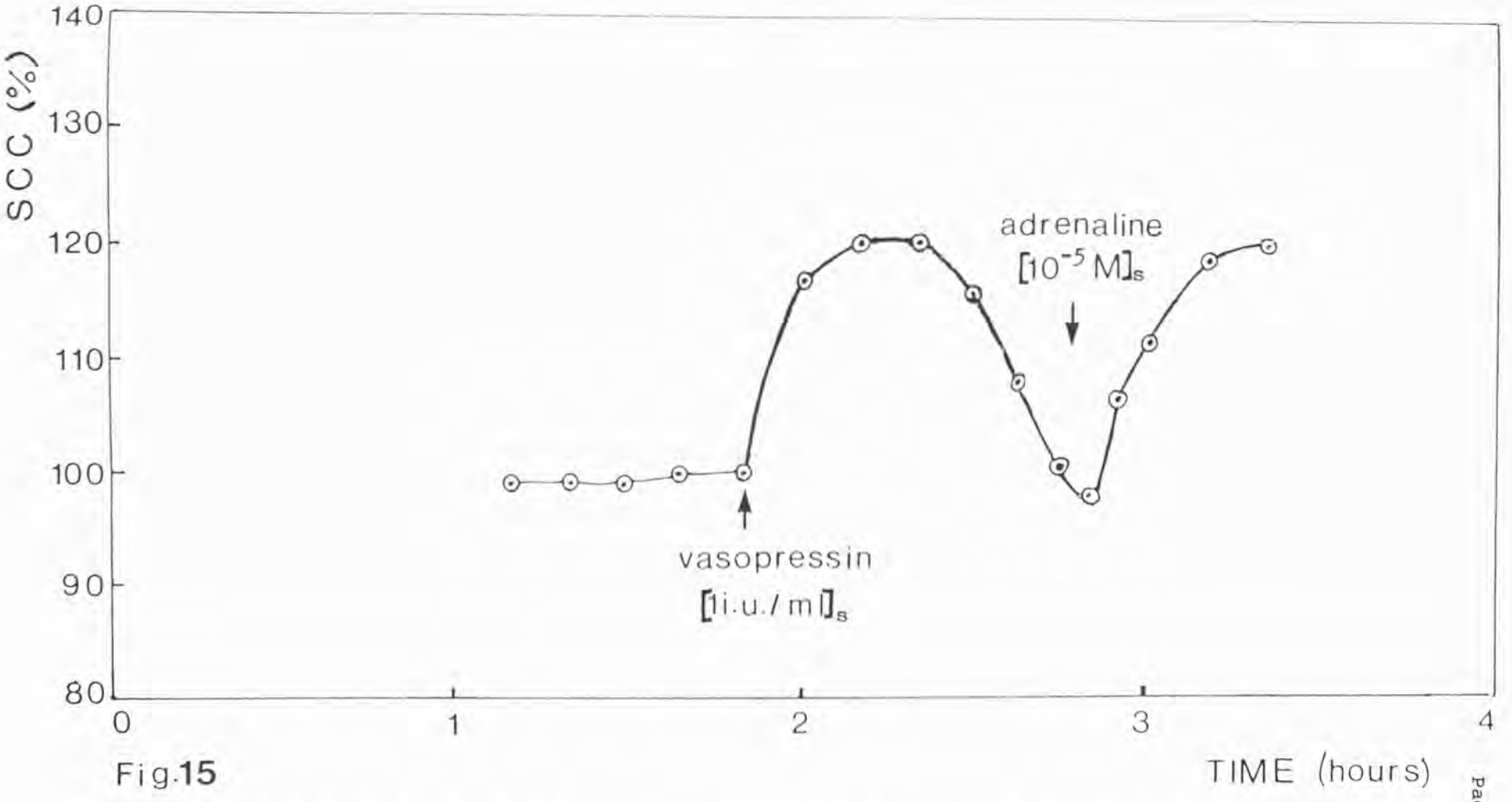


Fig.15

The effect on the s.c.c. of the consecutive application of vasopressin and then adrenaline. The above is an example of the type of response observed in three experiments. The order of hormone addition appeared to be irrelevant to the response.

membrane was treated with a single hormone (Figs. 11 and 12, above).

However, if the second hormone was applied during the maximum response to the first, i.e. at point A in Fig. 16, no further increase in ion transport was observed. But the application of the second hormone at a point when the response to the first hormone just began to decline, at point B in Fig.16, then a further increase in the s.c.c. was observed (n = 4). The second increase was non-additive to the first. The order of hormone addition appeared to be irrelevent to the response observed. In each case the addition of theophylline was followed by a further increase in ion transport which was relatively larger compared to the increases resulting from the addition of the two hormones. Fig. 16 shows an example of the type of response observed in four experiments.

3.3.5.1. Effect of α-receptor blocker on the s.c.c. response to adrenaline and vasopressin

Phentolamine is an α-receptor blocker of adenylate cyclase. The drug possesses high affinity for α-receptors with a negligible action on β-receptors (see chapter 4). Pre-treatment of gastric membranes with this drug [10^{-4}M] (applied to the serosal surface) had no observable effect on the response to either adrenaline or vasopressin (Fig. 17).

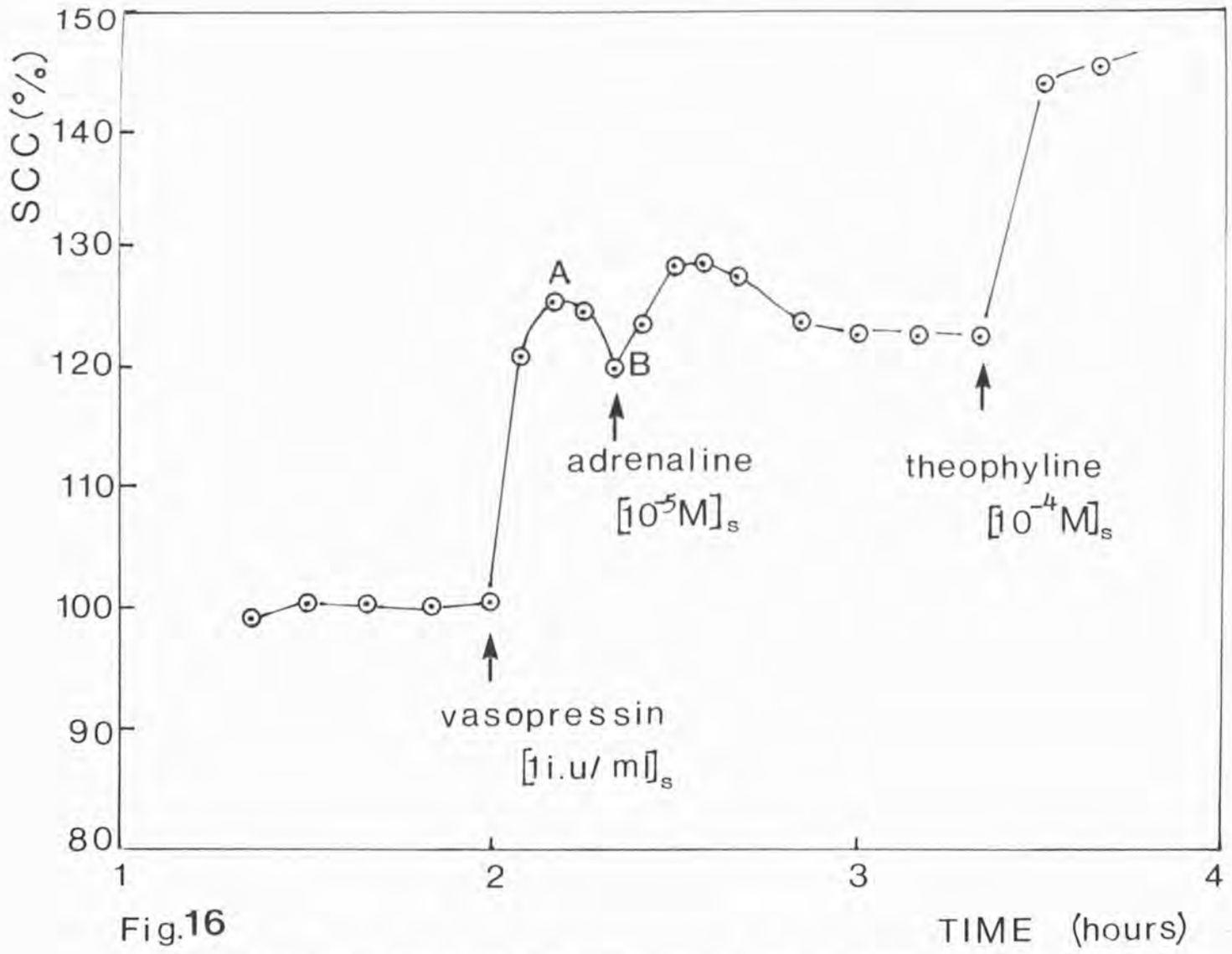


Fig.16

The effect on the s.c.c. of the application of a second hormone during the response to the first applied hormone; this is typical of the response observed in a series of four experiments.

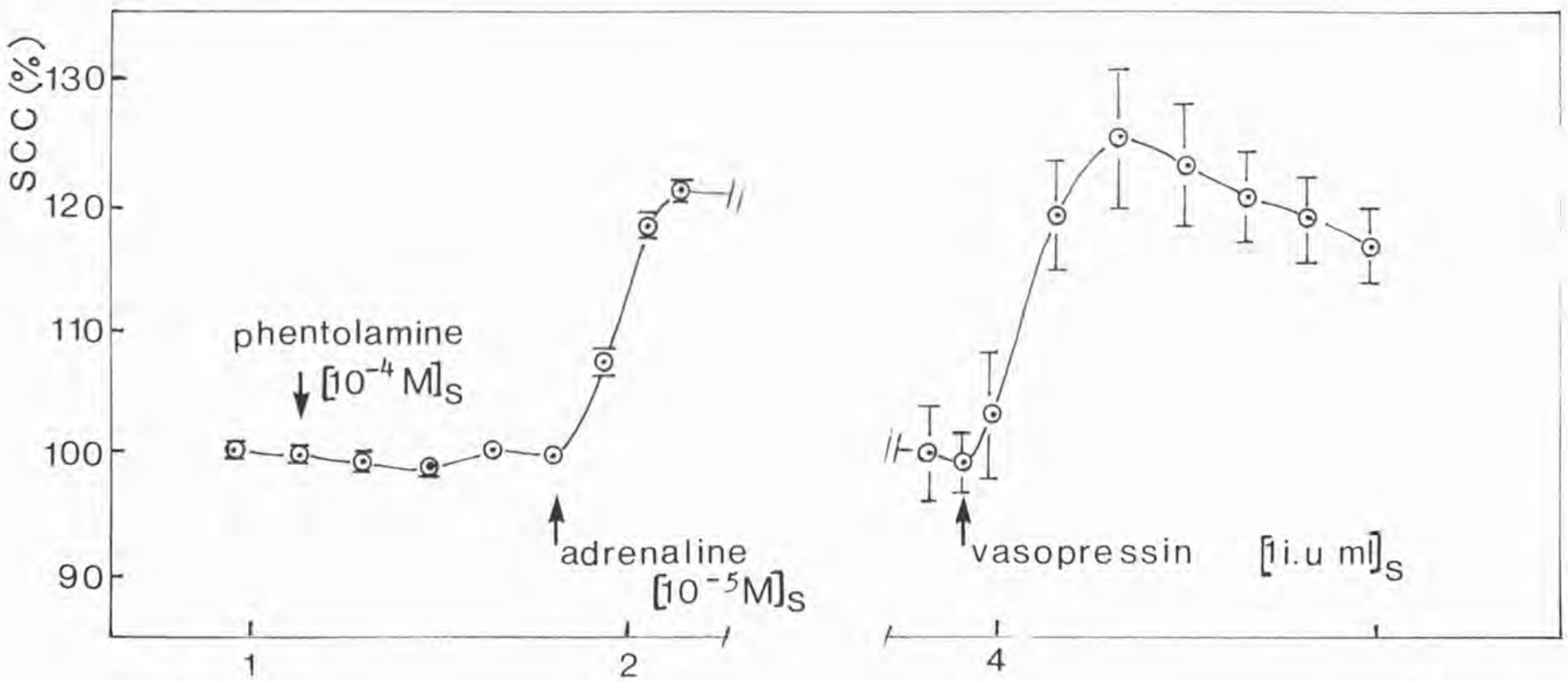


Fig.17

The effect of adrenaline [10^{-5}M] and vasopressin [1i.u./ml] on the s.c.c. across membrane preparations which were pre-treated with phentolamine [10^{-4}M]. Each point represents the mean ± s.e.m. (n = 4).
s = represents the addition of the agents to the serosal surface.

3.3.5.2. Effect of β-receptor blockers on the s.c.c. response to adrenaline and vasopressin

Propranolol was used as the β-receptor blocker of adenylate cyclase; it is highly specific for β-receptors of the cyclase and has negligible effect on α-receptors (see Chapter 4).

Application of adrenaline [10^{-5}M] to membrane preparations which had been pre-treated with propranolol [10^{-5}M] failed to increase ion transport. However the application of vasopressin [1i.u./ml] to pre-propranolol treated membrane preparations had no effect on the s.c.c. response to this hormone (Fig. 18, n = 5). Propranolol was applied to the serosal chamber. The order of hormone addition was irrelevant to the response observed.

Timolol, another β-receptor blocker was used to confirm the effect of adrenaline on β-receptors. In a series of four experiments the response to adrenaline was similarly blocked by pre-treating the gastric mucosa with timolol; it had no effect on the response to vasopressin (Fig. 19).

3.4. Effect of arachidonic acid on the s.c.c.

In a series of five experiments (Fig. 20), the application of arachidonic acid [$3x10^{-6}$M] resulted in a simultaneous, sharp increase in the s.c.c. to a maximum of 41.20 $\pm$ 9.89% (s.e.m.). This response to arachidonic acid was also reversible upon its removal from the bathing medium.

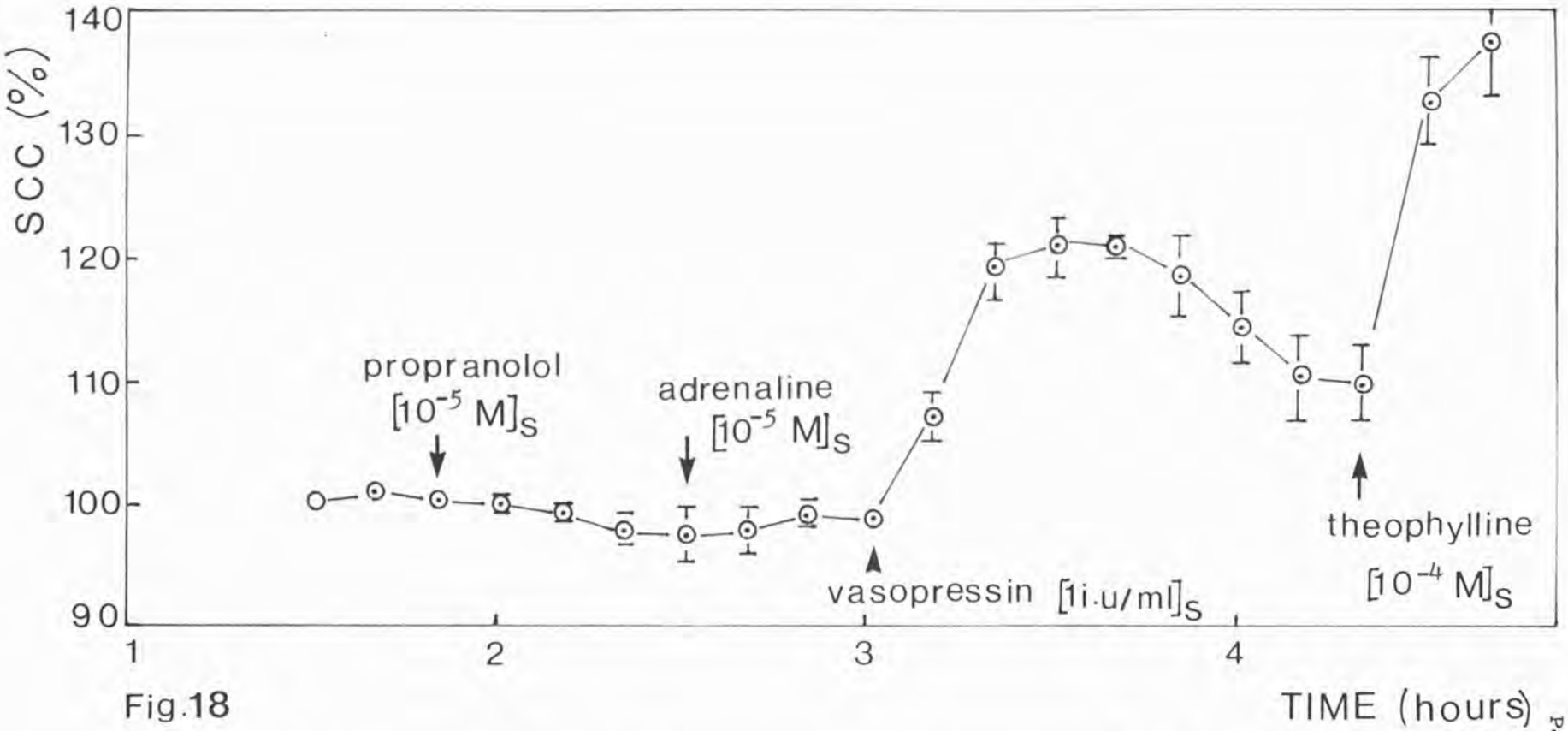


Fig.18

The effect of propranolol [10^{-5}M] on the s.c.c. response to adrenaline [10^{-5}M] and vasopressin [1i.u./ml]. Each point represents the mean ± s.e.m. (n = 5). s = represents the addition of the agents to the chamber bathing the serosal surface of the gastric mucosa. The order of hormone addition appeared to be irrelevent to the response observed.

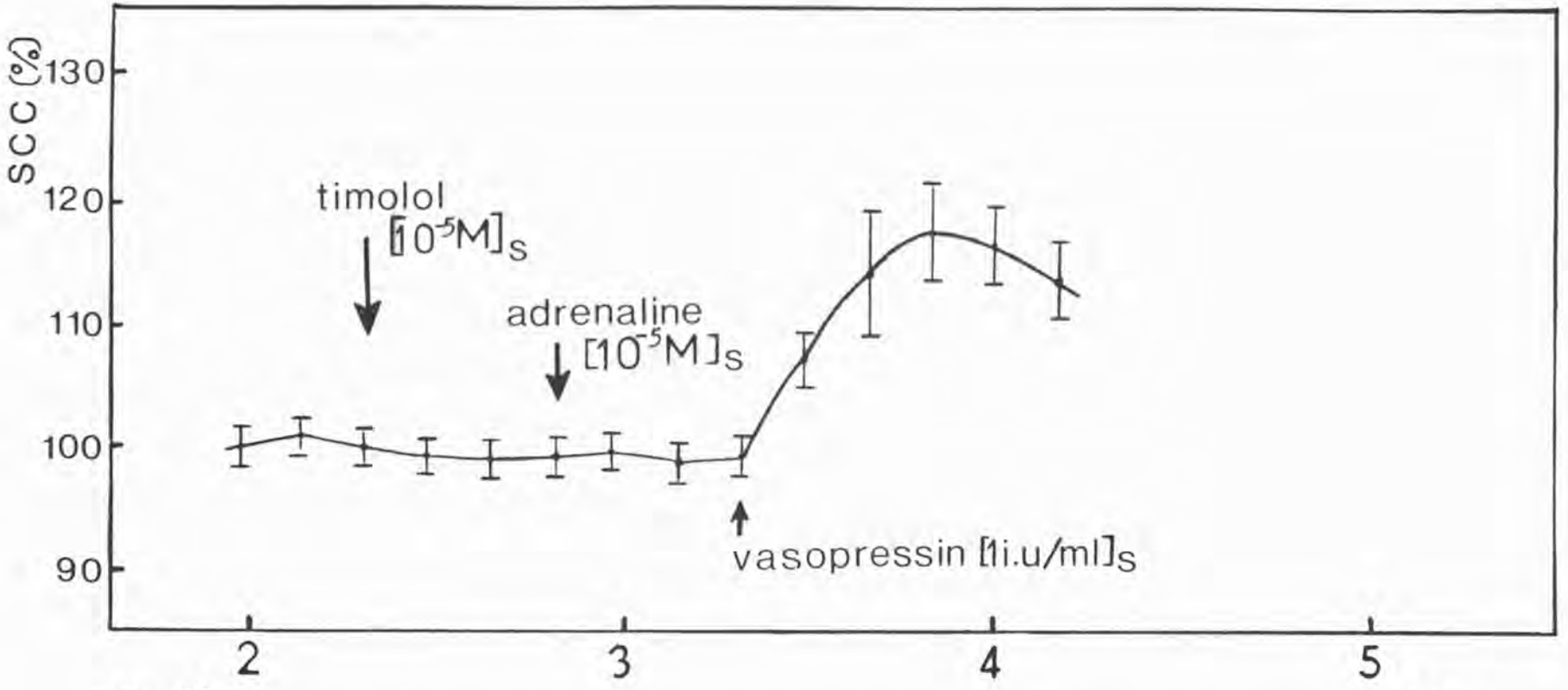


Fig. 19

The effect of timolol on the response to adrenaline and vasopressin on the s.c.c. across the gastric mucosa; n = 4. s = represents the addition of the agents to the chamber bathing serosal surface of the membrane.

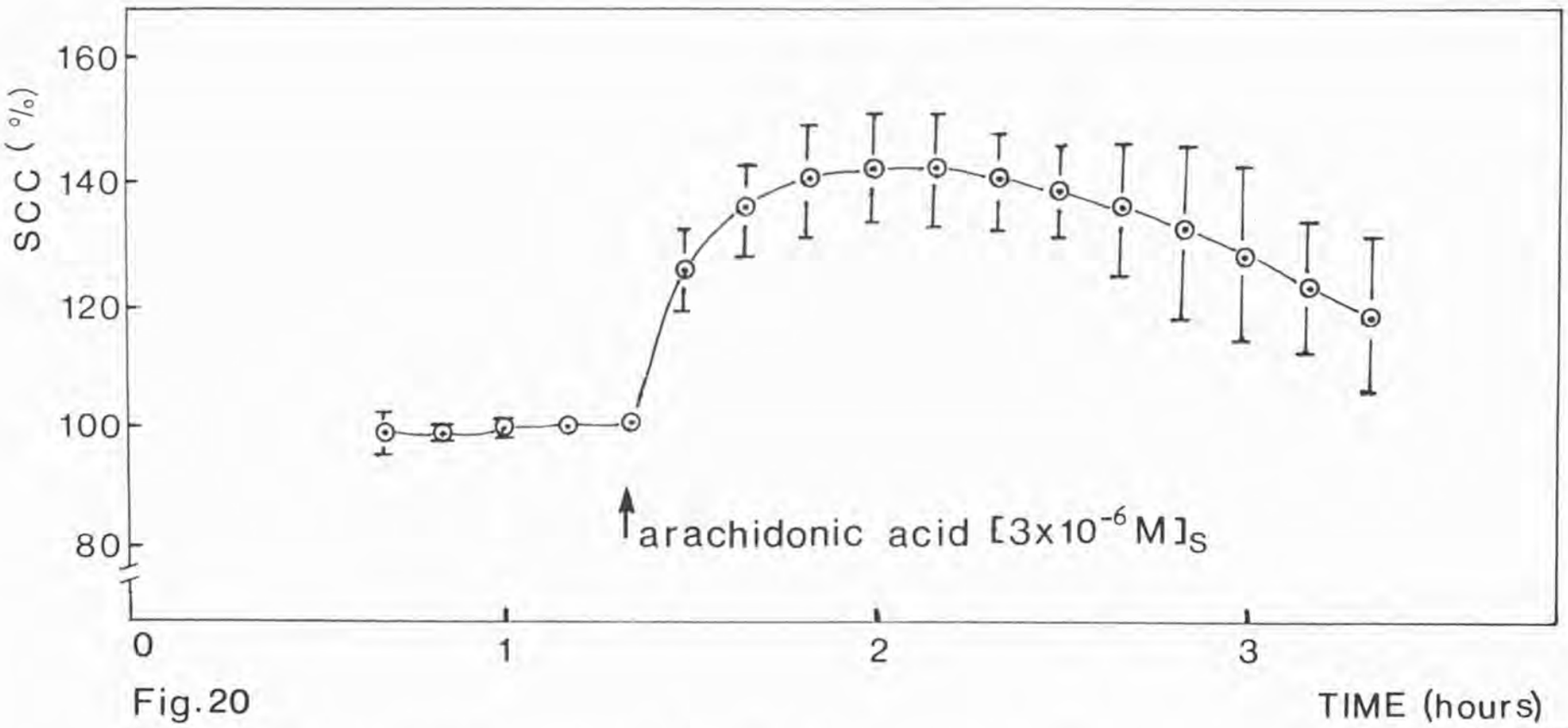


Fig.20

The effect of arachidonic acid, $3x10^{-6}$M, on the s.c.c. across the gastric mucosa of the rabbit fetus. Each point represents the mean ± s.e.m.; n = 2. s = the application of arachidonic acid to the serosal surface of the membrane.

Arachidonic acid also caused changes in the p.d. and the total conductance across the membrane but again these changes were smaller and less consistant than the changes observed in the s.c.c. (Table 9). The storage time of the membranes used are also shown in Table 9.

3.5. Effect of non-steroidal anti-inflammatory drugs on the s.c.c.

The effect of non-steroidal anti-inflammatory drugs was not always reversible particularly when aspirin and indomethacin were used at a final concentration of 10^{-4}M. However with lower concentration of these drugs, washing the membrane (several times), in a few cases prevented further decline in the s.c.c. which would then continue at a sustained level.

3.5.1. Acetyl salicylic acid

The effect of acetyl salicylic acid [10^{-4}M] is shown in Fig. 21a. The application of the drug resulted in an immediate decline in the s.c.c. across the membrane. The decline during the initial 1 hour and 20 minutes was at a rate of 0.56μA/min., followed by a much slower rate. From Table 10 it can be seen that each of the preparations used to test the effect of this agent were in a high state of activity as judged by the mean s.c.c. of 103.60 $\pm$ 9.94μA (s.e.m., n = 20) at the point of aspirin addition.

Table 9 The response of the gastric mucosa to applied arachidonic acid

The s.c.c., p.d. and the total membrane conductance at the point of arachidonic acid [3 x 10^{-6}M] addition and at maximum response.

		s.c.c. (μA /M)			p.d. (mV /M)		conductance (x 10^{-3}mho/M)	
date of experiment	storage time (hours)	at point of arachidonic addition	at maximum response	% increase in s.c.c.	at point of arachidonic addition	at maximum response	at point of arachidonic addition	at maximum response
15.07.80	24	117.00	181.50	55.13	14.00	19.50	8.30	9.31
17.07.80	72	22.00	28.00	27.27	2.50	3.00	8.80	9.33
mean $\pm$ s.e.m.		69.50 $\pm$33.69 $p > 0.10$	104.75 $\pm$54.43 $p > 0.10$	41.20 $\pm$9.89				

M = values are represented as those displayed across a total membrane area of 0.6 cm^2.

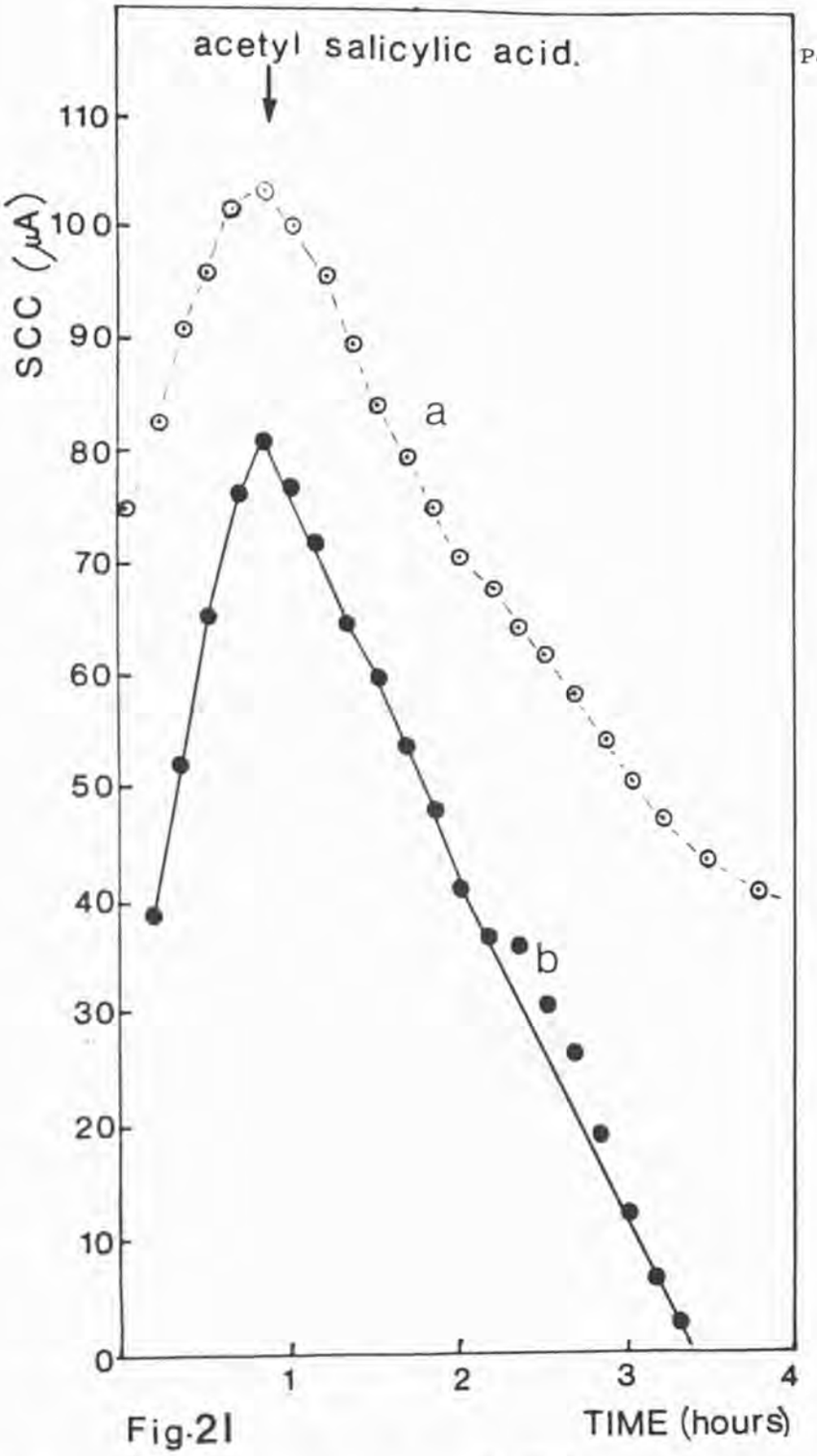


Fig.21

The effect of acetyl salicylic acid [10^{-4}M] on the s.c.c. Two different rates of decrease were observed which appeared to depend on the value of the s.c.c. at the point of aspirin addition. Curve a represents the application of the drug at a mean s.c.c. value of 103.60 $\pm$ 9.94μA (n = 20). Curve b shows the effect of aspirin on membrane preparations which displayed a mean s.c.c. value of 80.90 $\pm$ 12.80μA (n = 8).

Table 10 Effect of acetyl salicylic acid on the s.c.c. across the gastric mucosa

The effect of the addition of aspirin [10^{-4}M] on the s.c.c. across the fetal gastric mucosa. It should be noted that the membranes used to test the effect of this drug were in a relatively active state as judged by the s.c.c. at the point of anti-inflammatory drug addition (which ranged from 34μA up to 220μA).

date of experiment	storage time (hours)	s.c.c. (μA)/M at point of drug addition [10^{-4}M]
07.10.79	0	220.50
18.12.79	24	115.50
19.12.79	48	048.00
08.01.80	24	096.00
09.01.80	48	051.00
15.01.80	24	135.00
15.01.80	24	156.00
17.01.80	72	105.00
23.01.80	24	117.00
24.01.80	48	062.00
20.01.80	96	048.00
05.02.80	24	102.00
08.02.80	96	034.50
12.02.80	24	138.00
13.02.80	48	112.50
19.02.80	24	102.00
19.02.80	24	120.00
21.02.80	24	120.00
24.03.80	24	141.00
28.02.80	72	048.00
mean $\pm$ s.e.m. (n = 20)		103.60 $\pm$9.94

M = a total membrane area of 0.6cm^2

Membrane preparations which displayed low s.c.c. values with a mean of only 80.90 $\pm$ 12.80μA (s.e.m., n = 8) application of acetyl salicylic acid [10^{-4}M] resulted in a rapid sharp fall in the s.c.c., followed by complete abolition within 2 hours and 20 minutes from the point of drug addition (Fig. 21b). In this case the rate of decline during the first 1 hour and 20 minutes was 0.75μA/min., which is much faster than that observed above. The gastric mucosae used for this evaluation included membranes which had been stored for up to 96 hours.

3.5.2. Phenylbutazone

The application of phenylbutazone [10^{-4}M] was also followed by a decline in the s.c.c. (Fig. 22). The response to this anti-inflammatory drug was very similar to that observed following the addition of acetyl salicylic acid. The need to conduct further experiments with this drug therefore appeared to be unnecessary within the present work.

3.5.3. Indomethacin

The effect of indomethacin on the s.c.c. differed from those observed on the application of the above anti-inflammatory drugs. Three types of response were observed corresponding to different concentrations of the drug used.

Addition of 10^{-5}M indomethacin had a biphasic effect on the s.c.c.; it caused a small transient increase in the s.c.c. across the membrane, followed within 20 minutes, by a steady gradual decline

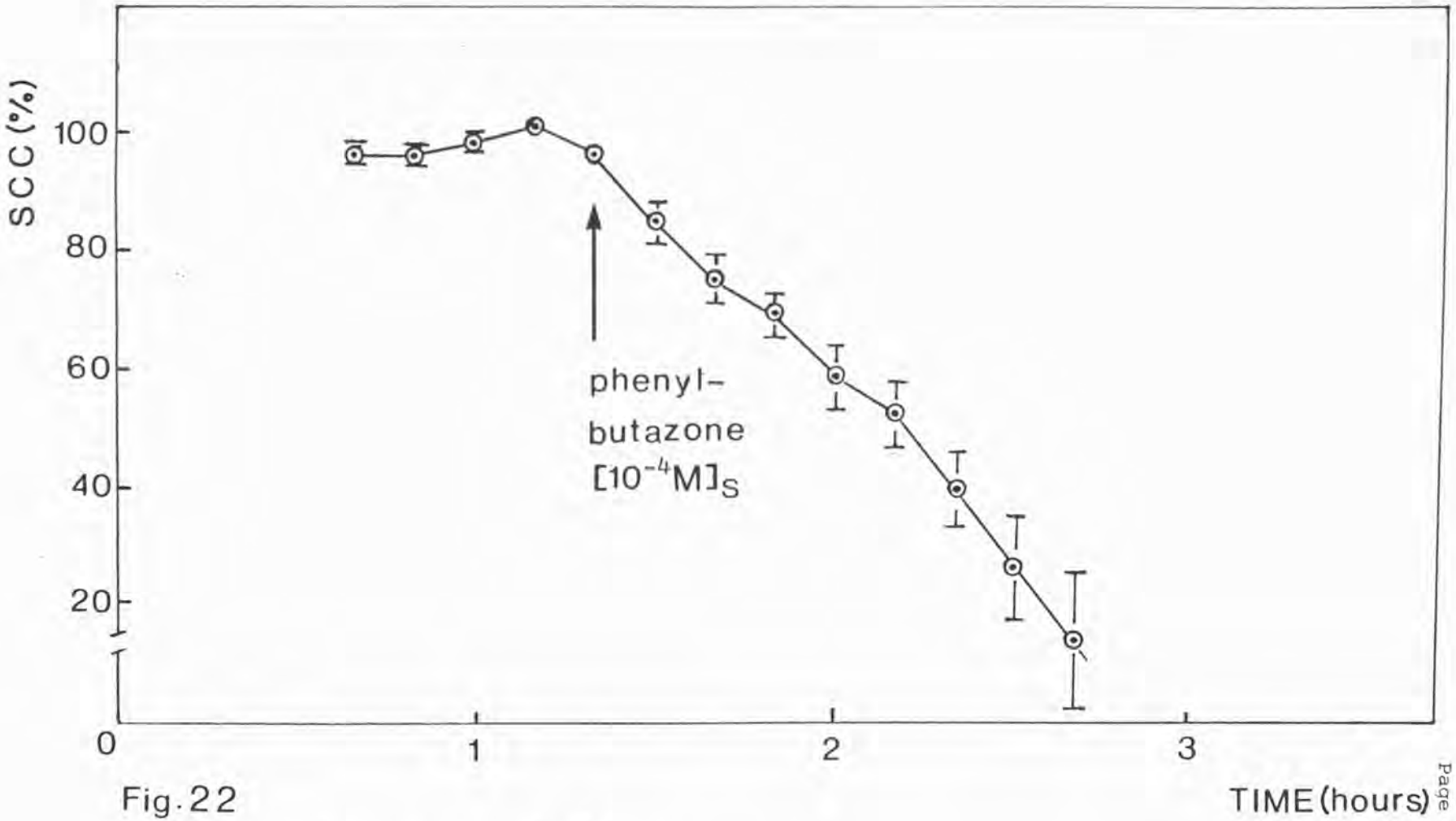


Fig. 22

The effect of phenylbutazone [10^{-4}M] on the s.c.c. response across the fetal stomach. Each point represents the mean ± s.e.m.; n = 5. s = represents the addition of the drug to the serosal chamber.

(Fig. 23). The transient increase had a mean value of 31.90 ± 6.0% (s.e.m., n = 6) of the resting s.c.c. The s.c.c. at the point of drug addition was 97.25 ± 24.24μA (s.e.m., n = 6, Table 11).

In a series of 7 experiments the application of indomethacin at a higher concentration of 10^{-4}M resulted in an immediate sharp fall in the s.c.c. (Fig. 24) omitting the initial transient increase observed when 10^{-5}M was applied. Table 12 shows the state of activity of these membranes as judged by the value of the s.c.c. at the point of drug application.

The addition of indomethacin at a lower final concentration of 10^{-7}M, resulted in an increase in the s.c.c. to a mean value of 15.28 ± 4.39%; this increase was maintained over a period of 2 hours and 50 minutes before finally declining (Fig. 25). However only two such observations were recorded involving fetal gastric mucosae which had been stored for 0 and 48 hours and which displaced a s.c.c. value of 135μA and 55μA respectively at the point of indomethacin application (Table 13).

All three prostaglandin synthetase inhibitors used also caused changes in the p.d. and total conductance (not recorded) across the membranes which followed a similar pattern to the changes observed in the s.c.c.; again these changes were smaller and less consistance in contrast to the changes observed in the s.c.c.

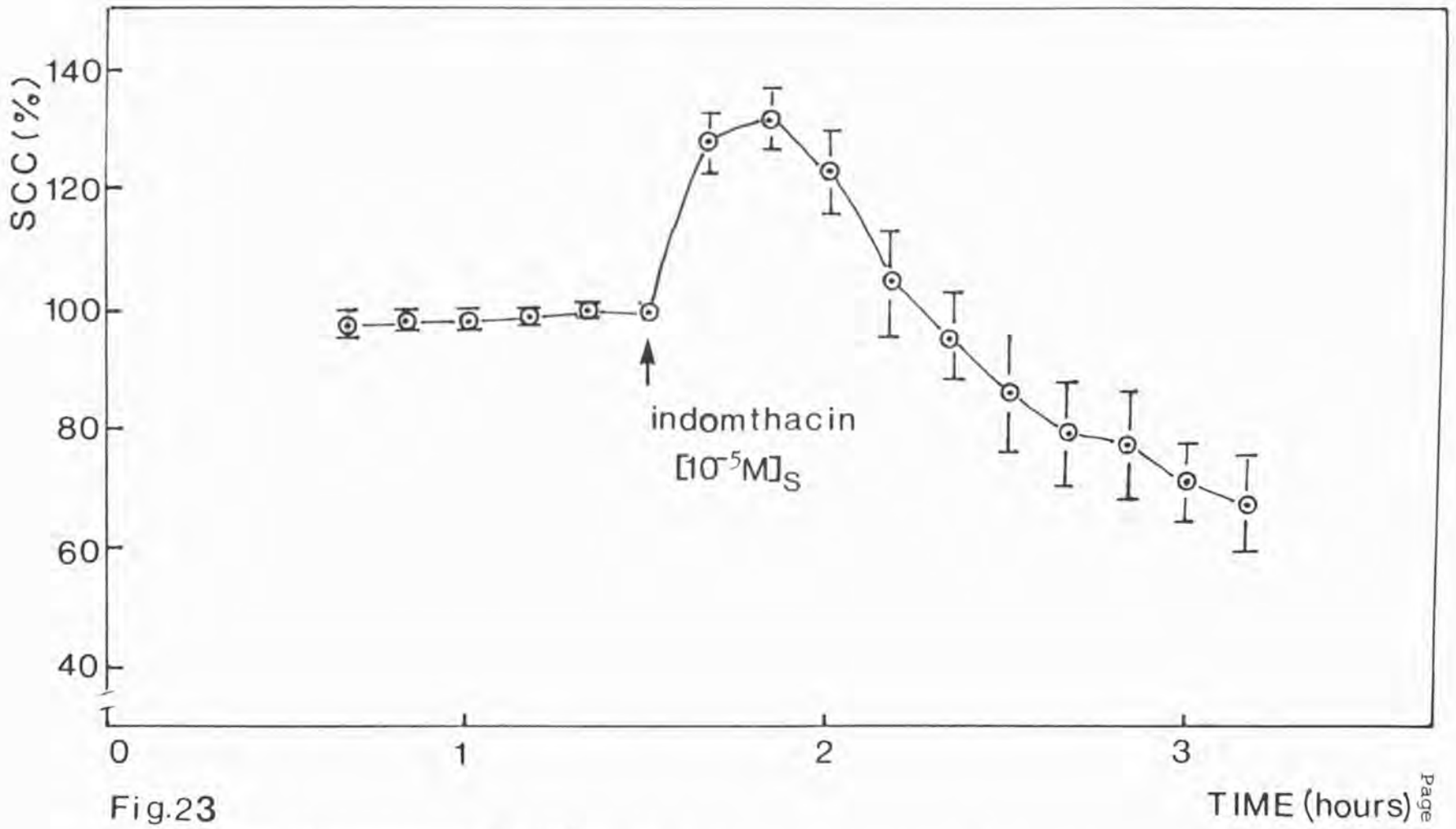


Fig.23

The effect of indomethacin [10^{-5}M] on the s.c.c. response across the gastric mucosa of the rabbit fetus. Each point represents the mean ± s.e.m.; n = 6. s = represents the application of the drug to the chamber bathing the serosal surface.

Table 11 The effect of indomethacin [10^{-5}M] on the s.c.c. response of the gastric mucosa

date of experiment	storage time (hours)	s.c.c. (μA/M) at the point of indomethacin [10^{-5} M] addition
08.04.81	48	80.00
08.04.81	48	28.00
22.04.81	24	193.50
24.04.81	72	27.00
28.04.81	0	123.00
13.05.81	24	132.00
mean $\pm$ s.e.m. (n = 6)		97.25 $\pm$41.20

M = membrane area of 0.6 cm^2.

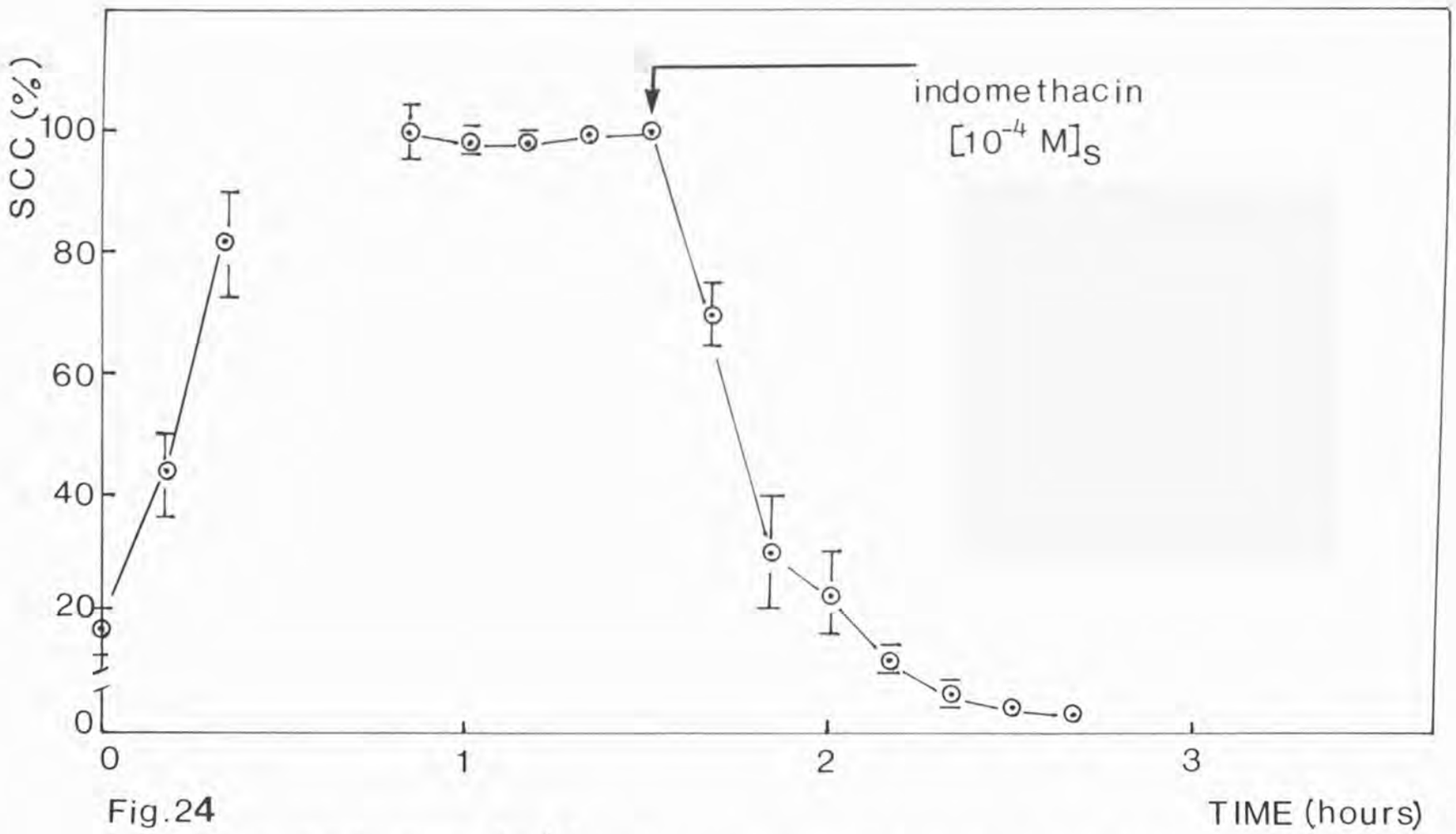


Fig.24

The effect of indomethacin [10^{-4}M] on the s.c.c. across the gastric mucosa of the rabbit fetus. Each point represents the mean ± s.e.m.; n = 7. s = represents the addition of the drug to the serosal surface.

Table 12 The effect of indomethacin [10^{-4}M] on the s.c.c. across the gastric mucosa

The s.c.c. (μA) at the point of indomethacin [10^{-4}M] addition is shown for seven preparations.

date of experiment	storage time (hours)	s.c.c. (μA/M) at point of indomethacin addition [10^{-4}M]
08.04.81	48	072.00
08.04.81	48	103.00
15.04.81	24	043.00
22.04.81	24	042.00
23.04.81	48	091.00
24.04.81	72	044.00
01.07.81	24	105.00
mean increase $\pm$ s.e.m. (n = 7)		71.40 $\pm$10.00

M = total membrane area of 0.6 cm^2.

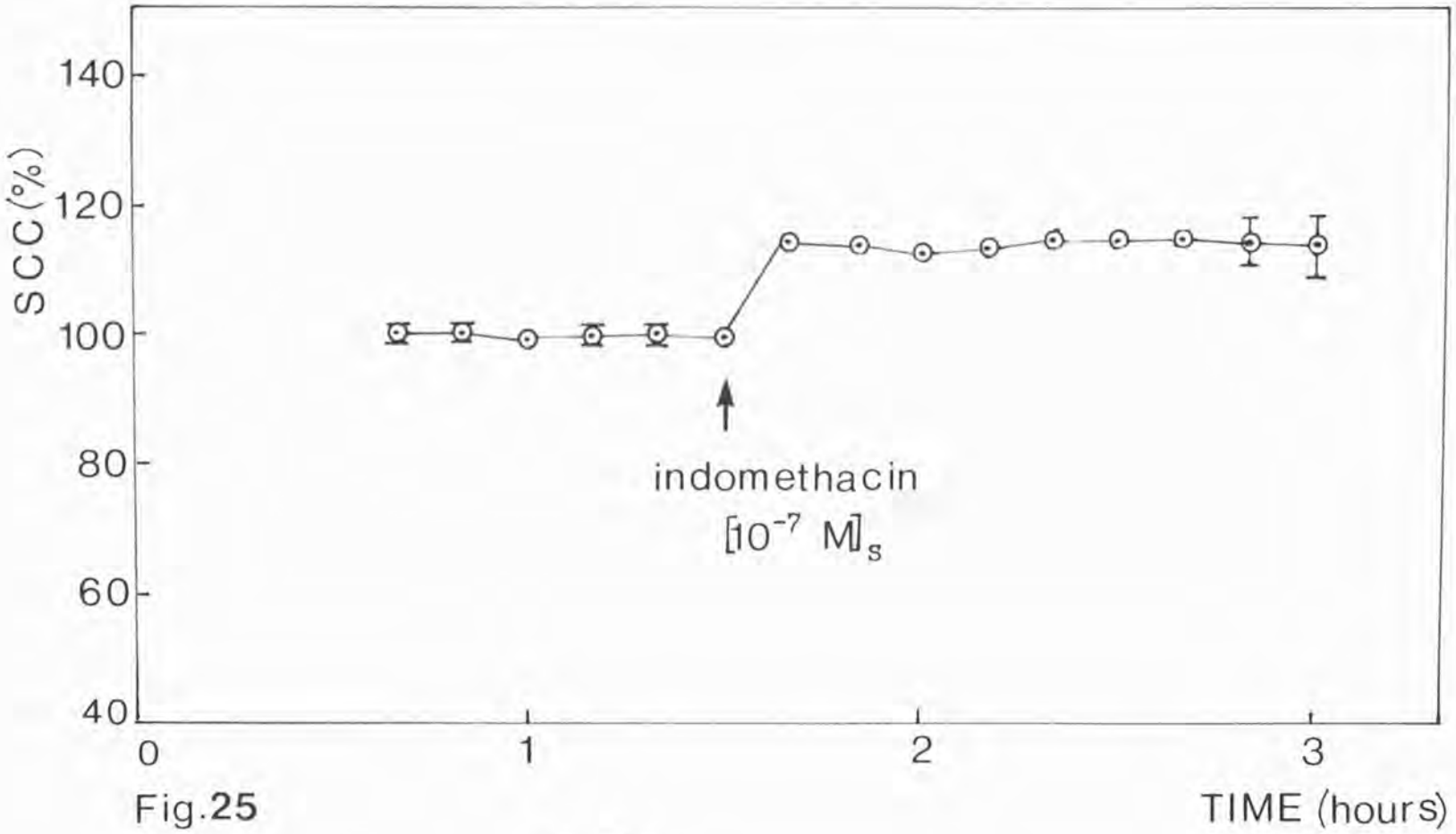


Fig.25

The effect of indomethacin [10^{-7}M] on the s.c.c. across the fetal gastric mucosa. Each point was calculated from a total of only two experiments. s = represents the addition of indomethacin to the serosal surface.

Table 13 The effect of indomethacin [10^{-7}M] on the s.c.c.

The storage time of the membrane preparations together with the actual s.c.c. (μA) values at the point of indomethacin addition and at maximum response are shown. The per cent increase in the s.c.c. is also shown.

date of experiment	storage time (hours)	s.c.c. (μA/M) at point of drug addition [10^{-7}M]	s.c.c. (μA/M) at maximum response	% increase in s.c.c.
06.04.80	0	135.00	164.00	21.48
30.04.80	48	55.00	60.00	9.09
mean ± s.e.m.		95.00 ±28.37 $p \geq 0.10$	112.00 ± 36.88 $p \geq 0.10$	15.28 ± 4.39

M = across a membrane area of 0.6^2.

3.6. Effect of theophylline on the unidirectional transport of sodium and chloride ions

Tables 14 to 17 show all the fluxes and the s.c.c. values before and after theophylline application together with the open circuit p.d. (at t_1 and t_2) and the dates at which each experiment was carried out.

All fluxes are expressed in μM $cm^{-2}hr^{-1}$. The s.c.c. or the net ion transport is expressed in the same units as the flux and was calculated in the following manner:-

$$s.c.c._{t_o \rightarrow t_1} = \frac{\int_{to}^{t_1} idt}{\int_{t_o}^{t_1} dt} \times \frac{60}{1} \times \frac{1}{M}$$

$$= \frac{\sum I_{t_1} - I_{t_o}}{\sum t_1 - t_o} \times \frac{60}{M}$$

where

$\sum I_{t_o}$ = sum of the integrator reading at time 0 (t_o) in μM

$\sum I_{t_1}$ = sum of the integrator reading at time 1 (t_1)

i = total number of ions (in μM) transported between t_1 and t_2

M = exposed area of the membrane (0.6 cm^2)

The total conductance (G) across the membrane before and after the addition of theophylline are also shown in each Table. The conductance is represented as the reciprocal of the total resistance across the membrane areas of $0.6cm^2$ and was calculated from Ohms law (which is obeyed by the gastric mucosa of the rabbit fetus, Wright, 1974).

3.6.1. Mucosal to serosal sodium flux

From Table 14 it can be seen that in a series of 8 experiments, the application of theophylline ($10^{-4}M$) to the serosal chamber resulted in an increase in the s.c.c. from a mean control value of 07.08 ± 0.87 $\mu M\ cm^{-2}hr^{-1}$ to $09.85 \pm 0.87 \mu M\ cm^{-2}hr^{-1}$, whereas this agent caused a much larger increase in the mucosal to serosal sodium flux from a control value of 13.85 ± 3.10 to 21.70 ± 5.27 $\mu M\ cm^{-2}hr^{-1}$ (n = 8). Theophylline also caused a small increase of 1.88×10^{-3}mhos (n = 8) in the total conductance across these membranes.

3.6.2. Serosal to mucosal sodium flux

The effect of theophylline on the serosal to mucosal sodium flux is shown in Table 15 from which it can be seen that the control mean s.c.c. value of 6.70 ± 0.67 increased to 9.08 ± 0.75 $\mu M\ cm^{-2}hr^{-1}$ (n = 5) following the application of this agent to the serosal surface; the serosal to mucosal sodium flux changed from 9.38 ± 1.65 to 11.25 ± 1.80 $\mu M\ cm^{-2}hr^{-1}$ (n = 5). The addition of theophylline was followed by a small increase in the total conductance across the membrane from a mean control of $13.66 \pm 1.95 \times 10^{-3}$ mhos to $14.77 \pm 1.37 \times 10^{-3}$mhos.

Table 14 The effect of theophylline on the mucosal to serosal sodium flux

Date of experiment	Na^+ influx $Jm \rightarrow s$ ($\mu Mcm^{-2} hr^{-1}$)		s.c.c. ($\mu Mcm^{-2} hr^{-1}$)		p.d. (mV/M)		G ($\times 10^{-3}$ mho/M)	
	B	A	B	A	B	A	B	A
05.12.83	1.66	3.40	8.82	11.00	16.50	19.50	9.09	9.77
06.12.83	3.16	4.54	10.70	14.07	17.00	18.00	7.94	9.75
07.12.83	15.30	18.00	10.52	10.85	19.50	20.00	8.46	9.07
04.06.85	16.51	27.00	4.67	6.58	9.00	10.50	9.33	12.71
12.06.85	17.70	27.80	5.82	8.72	16.00	17.50	5.62	7.37
12.06.85	5.97	9.60	4.03	6.75	7.00	10.00	8.14	9.75
28.06.85	27.22	44.82	5.43	8.73	15.00	17.00	16.00	19.57
28.06.85	23.25	38.42	6.68	12.08	7.50	12.50	14.53	16.16
$\bar{X}$	13.85	21.70	7.08	9.85	13.44	15.62	9.89	11.77
$\pm$ s.e.m. (n = 8)	$\pm$ 3.10	$\pm$ 5.27	$\pm$ 0.87	$\pm$ 0.87	$\pm$ 1.60	$\pm$ 1.33	$\pm$ 1.16	$\pm$ 1.37

where

$Jm \rightarrow s$ = direction of flux from mucosa to serosa

B = before theophylline [10^{-4}M] addition to the serosal surface

A = after theophylline addition

$\bar{X} \pm$ s.e.m = mean $\pm$ standard error of the mean

G = total conductance across the membrane

M = membrane area of 0.6cm^2 .

Table 15 The effect of theophylline on the serosal to mucosal sodium flux

Date of experiment	Na^+ efflux $Js \rightarrow m$ ($\mu Mcm^{-2}\ hr^{-1}$)		s.c.c. ($\mu Mcm^{-2}\ hr^{-1}$)		p.d. (mV/M)		G ($\times 10^{-3}$mho/M)	
	B	A	B	A	B	A	B	A
07.06.85	16.01	18.85	6.40	8.02	5.20	8.00	20.19	17.63
11.06.85	8.28	7.42	6.28	8.60	10.00	12.00	9.30	10.75
06.06.85	10.28	11.65	4.32	7.30	6.00	9.00	13.50	15.44
05.06.85	5.78	9.58	8.80	12.22	9.00	12.00	16.67	16.75
11.06.85	6.43	8.73	7.72	9.25	12.50	14.00	8.64	10.29
$\bar{X}$ $\pm$ s.e.m. (n = 5)	9.38 $\pm$ 1.65	11.25 $\pm$ 1.80	6.70 $\pm$ 0.67	9.08 $\pm$ 0.75	8.54 $\pm$ 1.19	11.00 $\pm$ 0.98	13.66 $\pm$ 1.95	14.77 $\pm$ 1.37

where

$Js \rightarrow m$ = direction of flux from serosa to mucosa
B = before theophylline addition
A = after theophylline [10^{-4}M] addition to the serosal chamber
$\bar{X} \pm$ s.e.m. = mean $\pm$ standard error of the mean
G = total conductance across the membrane
M = membrane area of 0.6 cm^2.

3.6.3. Serosal to mucosal chloride flux

In a series of 6 experiments the application of theophylline to the serosal solution resulted in a decrease in the serosal to mucosal chloride transport (Table 16); chloride flux fell from a control of 21.53 ± 4.13 to 16.58 ± 3.25 μM $cm^{-2}hr^{-1}$. The s.c.c. increased from a mean control value of 7.85 ± 1.33 to 9.92 ± 1.70 μM $cm^{-2}hr^{-1}$ (n = 6); similarly the total conductance across the membrane had also increased by 0.43 x 10^{-3} mhos following the addition of theophylline.

3.6.4. Mucosal to serosal chloride flux

The application of theophylline to the serosal surface was followed by an increase in mucosal to serosal chloride flux from a mean control value of 26.94 ± 2.97 μM $cm^{-2}hr^{-1}$ to 30.10 ± 2.28 μM cm^{-2} hr^{-1} (n = 4), (Table 17). The mean control s.c.c. value of 8.07 ± 1.97 increased to 11.75 ± 2.42 μM $cm^{-2}hr^{-1}$ (n = 4). The total conductance across the membrane also increased from a mean control value of 12.60 ± 0.27 x 10^{-3} to 13.83 ± 0.50 x 10^{-3} mhos (n = 4).

The effect of theophylline on the net unidirectional flux of both sodium and chloride ions are summarised in Table 18. Thus in all gastric mucosal preparations (n = 23) the application of theophylline (10^{-4}M) to the serosal surface indicated an increase in the s.c.c. and the total conductance across the membranes from a mean control value of 7.37 ± 0.60 to 10.03 ± 0.72 μM $cm^{-2}hr^{-1}$ and 11.25 ± 0.77 to 12.69 ± 0.71 x 10^{-3}mhos, respectively. In a series of 8 experiments, the addition of methyl xanthine resulted in a net increase of 7.85 ± 2.11 μM $cm^{-2}hr^{-1}$ in sodium flux in the direction

Table 16 Effect of theophylline on the serosal to mucosal chloride flux

Date of experiment	Cl^- efflux $Js \rightarrow m$ ($\mu Mcm^{-2}hr^{-1}$)		s.c.c. ($\mu Mcm^{-2}hr^{-1}$)		p.d. (mV/M)		G ($\times 10^{-3}$ mho/M)	
	B	A	B	A	B	A	B	A
18.06.85	10.84	9.12	4.05	5.12	8.00	10.00	6.75	7.10
19.06.85	19.48	10.45	12.88	13.93	14.00	17.00	12.60	12.50
19.06.85	8.30	10.73	5.90	7.98	7.00	7.00	11.36	14.10
20.06.85	32.88	16.47	8.80	10.27	7.00	10.00	14.60	13.20
20.06.85	35.33	31.85	4.62	5.72	5.00	-	12.00	-
27.06.85	22.32	20.93	10.85	16.53	12.50	19.00	12.00	13.00
$\overline{X}$ ± s.e.m. (n = 6)	21.53 ± 4.13	16.58 ± 3.25	7.85 ± 1.33	9.92 ± 1.70	8.92 ± 1.31	12.60 ± 2.05	11.55 ± 0.97	11.98 ± 1.11

where

$Js \rightarrow m$ = chloride flux from serosa to mucosa
B = before theophylline addition
A = after theophylline [10^{-4}M] additon to the serosal chamber
$\overline{X}$ ± s.e.m. = mean ± standard error of the mean
G = total conductance across the membrane
M = total membrane area of 0.6 cm^2.

Table 17 Effect of theophylline on the mucosal to serosal chloride flux

Date of experiment	Cl^- influx $Jm \rightarrow s$ ($\mu Mcm^{-2}hr^{-1}$)		s.c.c. ($\mu Mcm^{-2}hr^{-1}$)		p.d. (mV/M)		G ($\times 10^{-3}$mho/M)	
	B	A	B	A	B	A	B	A
21.06.85	36.93	31.15	3.93	4.15	-	-	-	-
25.06.85	22.83	35.47	7.77	13.38	8.00	12.00	13.12	14.25
26.06.85	22.13	23.48	14.50	17.50	17.00	19.00	12.00	12.63
27.06.85	25.87	29.80	6.10	11.97	7.50	13.00	12.67	14.61
$\bar{X}$ $\pm$ s.e.m. (n = 4)	26.94 $\pm$ 2.97	30.10 $\pm$ 2.28	8.07 $\pm$ 1.97	11.75 $\pm$ 2.42	10.83 $\pm$ 2.52	14.67 $\pm$ 1.79	12.60 $\pm$ 0.27	13.83 $\pm$ 0.50

where

$Jm \rightarrow s$ = direction of flux from the mucosa to serosa

A = after theophylline additon

B = before theophylline [10^{-4}M] addition to the serosal chamber

$\bar{X} \pm$ s.e.m. = mean $\pm$ standard error of the mean

G = total conductance across the membrane

M = total membrane area of 0.6cm^2.

Table 18 The effect of theophylline [10^{-4} M] on the unidirectional transport of both sodium and chloride ions

	$Jm \rightarrow s$ ($\mu Mcm^{-2}hr^{-1}$)	$Js \rightarrow m$ ($\mu Mcm^{-2}hr^{-1}$)	Jnet
$J_B^{Na^+}$	13.85 ± 3.10 (n = 8)	9.38 ± 1.65 (n = 5)	4.47 ± 2.64
$J_A^{Na^+}$	21.70 ±5.30 (n = 8)	11.25 ± 1.80 (n = 5)	10.45 ± 4.30
$J_{net}^{Na^+}$	7.85 ± 2.11	1.87 ± 0.70	5.98 ± 1.70 $p > 0.2$ $t = 1.19$
$J_B^{Cl^-}$	26.9= ± 2.97 (n = 6)	21.52 ± 4.13 (n = 4)	5.38 ± 3.48
$J_A^{Cl^-}$	30.10 ± 2.28 (n = 6)	16.58 ± 3.25 (n = 4)	13.52 ± 2.71
$J_{net}^{Cl^-}$	3.16 ± 3.38	-4.95 ± 2.51	8.14 ± 3.07 $p > 0.05$ $t = 1.85$

$Jm \rightarrow s$ = flux from mucosa to serosa
$Js \rightarrow m$ = flux from serosa to mucosa
J_B = flux before theophylline addition
J_A = flux after theophylline addition
J_{net} = the difference between the flux before and after theophylline addition

of mucosa to serosa whereas the net increase in the reverse direction ($J_{s \rightarrow m}$) was 1.87 $\pm$ 0.70 μM $cm^{-2}hr^{-1}$ (n = 5). In 6 experiments, the net serosal to mucosal chloride flux had decreased by 4.95 $\pm$ 2.51 μM $cm^{-2}hr^{-1}$ following the application of phosphodiesterase; however chloride flux in the reverse direction ($J_{m \rightarrow s}$) increased by 3.16 $\pm$ 3.38 μM $cm^{-2}hr^{-1}$ (n = 4).

3.7. Cellular studies

3.7.1. Microscopy and eosin exclusion test: test of viability of cells

Smears of gastric mucosal cells which had been incubated for 3 hours did not stain strongly with haematoxylin and eosin. However the faintly stained smears showed the presence of a large population of epithelial cells.

The eosin exclusion test showed that approximately 90% of the total mucosal cells did exclude eosin. However the cell count was not possible due to clumping of cells.

It has previously been reported that cell suspensions, obtained in the manner described previously (section 2.4.1.1., Chapter 2) and which had been incubated for up to 10 hours remain intact and that a large population of cells after incubation for 5 hours still possess the ability to exclude eosin and are no different from cells which had been incubated for 0 hours (Malinowska, 1974).

3.7.2. Intracellular cyclic AMP level

Additional control experiments were carried out to determine the efficiency of the extraction technique [using the cyclic AMP assay buffer] and the extent of recovery of cyclic AMP by the protein binding method.

These control recovery experiments were carried out on the ventral skin of the frog. Six pieces of the freshly removed skin of known wet weights were each dropped in 5ml of the boiling cyclic AMP assay buffer. The tubes were covered to prevent evaporation and boiled for 10 minutes. Each tissue was then homogenized in the same buffer using an electric homogenizer and centrifuged to remove the denatured protein. The supernatant was freeze dried. A known quantity of cyclic AMP (1pmol/mg wet weight) was added just before homogenization to three of the six pieces of the tissue. Cyclic AMP of the freeze dried supernatant was determined by the protein binding method described in Chapter 2.

These experiments have shown that the extraction technique for cyclic AMP provides a high degree of consistancy, i.e. cyclic AMP content of three segments of the same skin did not differ very much and known amounts of cyclic AMP added at the time of homogenization were quantitatively recovered. The efficiency of the binding protein method used in conjunction with the extraction technique for cyclic AMP was found to be in the range of $95.65 \pm 4.35\%$.

3.7.2.1. Control cyclic AMP level

Cyclic nucleotide levels are expressed in pmol/mg dry weight of mucosal cells.

Intact control membrane preparations approach maximum s.c.c. in approximately 1 hour which is maintained for a period of up to 12 hours (Kendal, 1968). Mucosal cell suspensions were therefore incubated for 2 hours to ensure maximum activity of the cells, after which time samples of cell suspensions were removed at 15 minute intervals and assayed for their cyclic AMP content.

From Fig. 26 it can be seen that the control level of cyclic AMP remained steady just below 1pmol/mg wt. of dry cells over a period of 1 hour and 30 minutes.

3.7.2.2. Effect of theophylline on intracellular cyclic AMP level

After 2 hours of incubation, theophylline [10^{-4}M] was added to the test cell suspension and incubation continued for the required length of time. A 2ml samples were withdrawn just prior to methyl xanthine addition and at 15 minute intervals there after over a period of 1 hour and 30 minutes. Simultaneous samples were also taken from control suspensions.

Fig. 27 shows the effect of theophylline on intracellular cyclic AMP level compared to the s.c.c. response of the gastric mucosa.

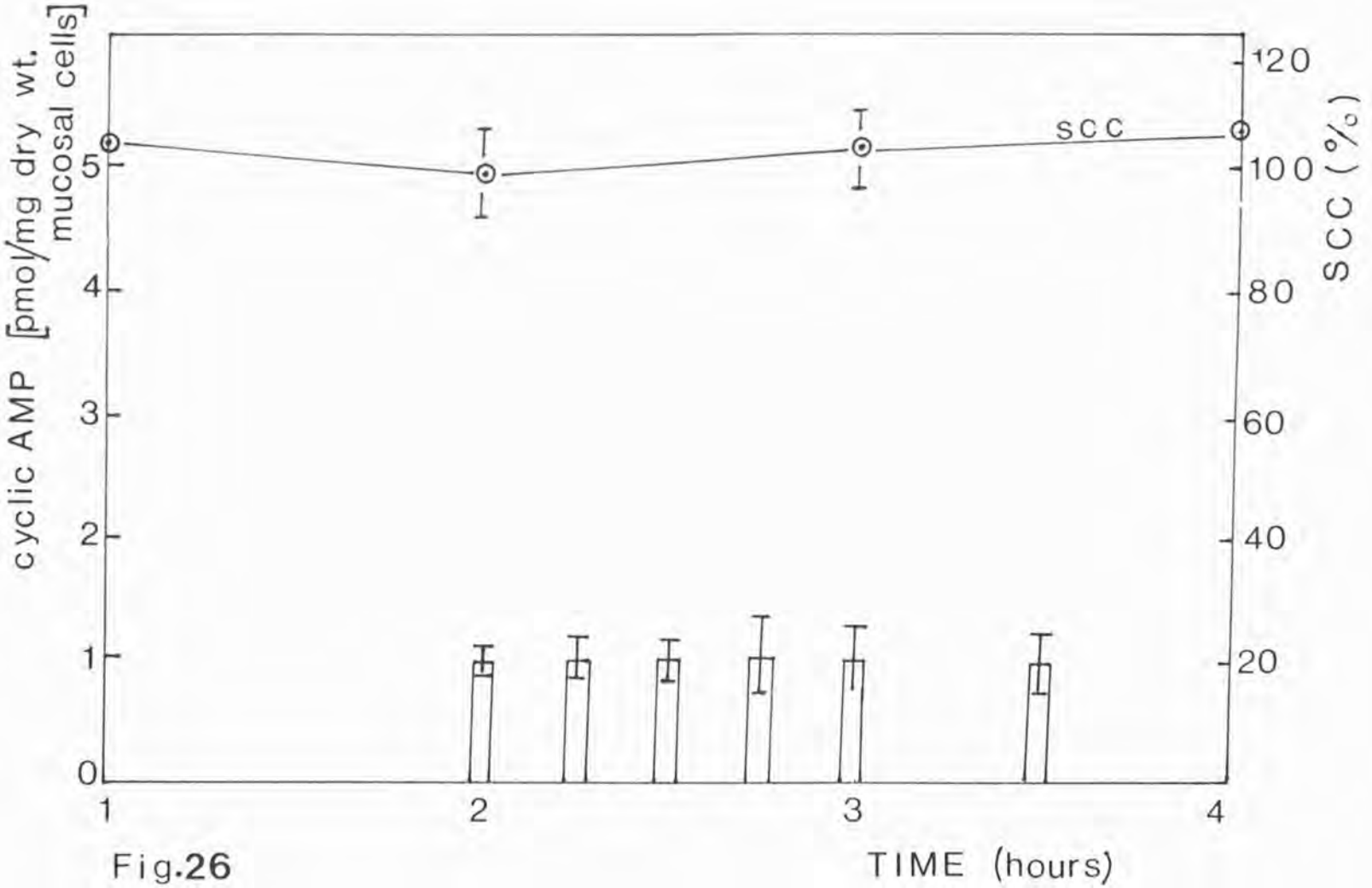
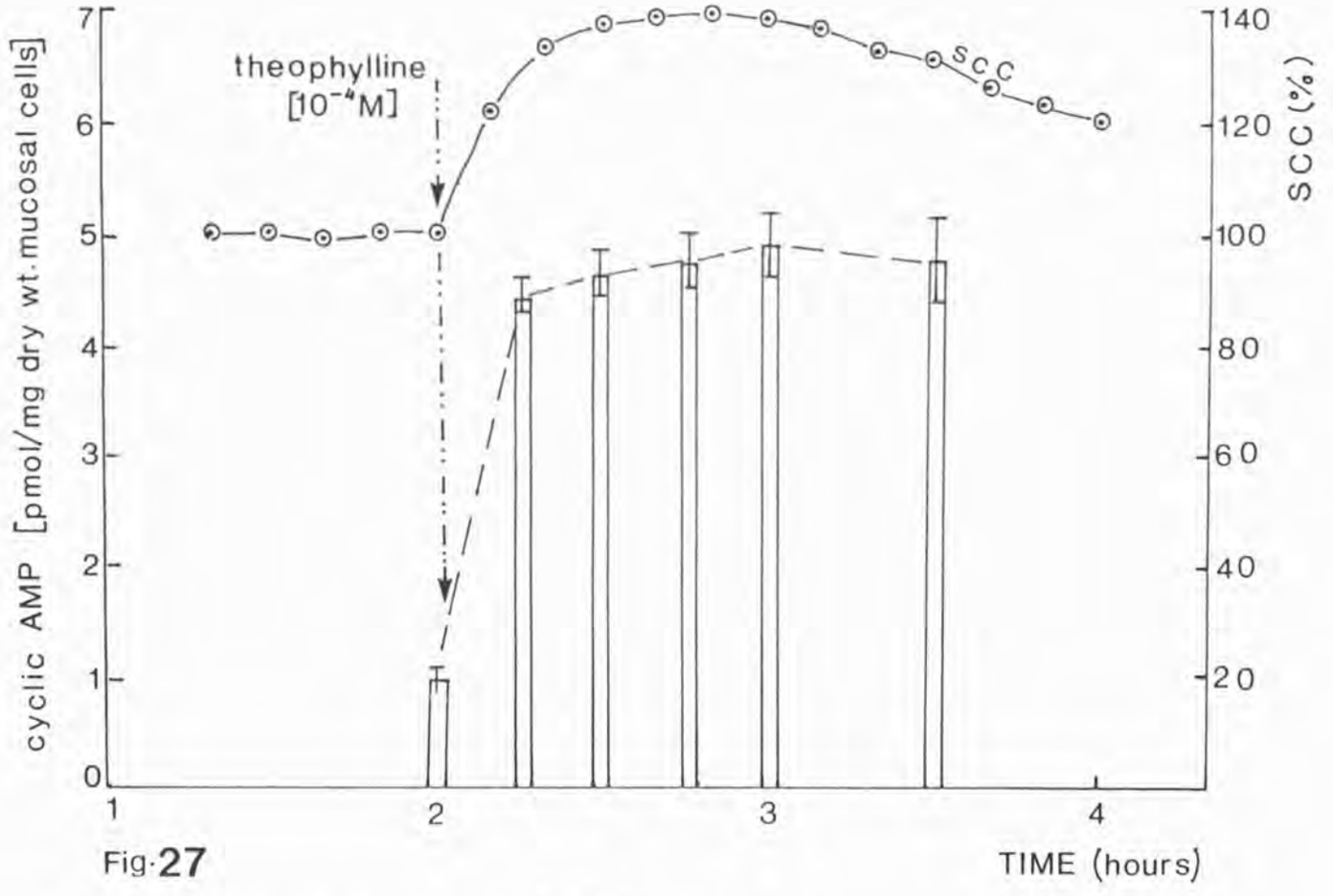


Fig.26

The control s.c.c. values across intact gastric mucosa (curve, n = 5) and the control intracellular cyclic AMP level of mucosal cells (column, n = 11).



Fig·27

The effect of theophylline [10^{-4}M] on the s.c.c. across intact gastric mucosa (curve, n = 8) and on intracellular cyclic AMP content of mucosal cells (columns, n = 13). Vertical lines represents the standard error of the mean.

Treatment of mucosal cell suspensions with theophylline resulted in an increase in cyclic AMP level from a mean control value of 0.97 ± 0.20pmol/mg dry weight of mucosal cells to 4.80 ± 0.30pmol/mg dry weight of mucosal cells (s.e.m., n = 13); an increase of 394.84 ± 38.00% was indicated.

From Fig. 27 it is seen that even when the s.c.c. response to applied methyl xanthine begins to decline after approximately 1 hour, the stimulated cyclic AMP level however did not change considerably from the maximum.

3.7.2.3. Effect of hormones on intracellular cyclic AMP level

Difficulty was encountered in measuring hormone-stimulated cyclic AMP level; in most cases no difference in the level of cyclic AMP or even a decrease was detected in cell suspensions which had been treated with adrenaline or vasopressin from those under control conditions. It was subsequently found that consistent increases in cyclic nucleotide level could be detected in cell suspensions which had been pre-treated with methyl xanthine. The inability to detect cyclic AMP level in non-theophylline treated cell suspensions was probably due to the activity of the phosphodiesterase which might be destroying cyclic nucleotide before it was assayed. Cell suspensions, the control and those under test were, therefore, pre-treated with methyl xanthine prior to hormone addition.

After 2 hours of incubation, theophylline (10^{-4}M) was added to both the control cell suspension and those under test and incubation was allowed to continue. After 40 minutes, which is the time

required for theophylline to stimulate the s.c.c. maximally (Fig. 9 in section 3.2.1. above), the respective hormone was applied to the cell suspension under test. 2ml samples of cell suspensions were taken just before hormone addition and then at 5 minute intervals there after. Concurrent samples were also taken from the control suspensions, which in this case were theophylline-treated mucosal cells.

3.7.2.3.1. Effect of adrenaline on intracellular cyclic AMP level

In section 3.3.1. above it was shown that the application of adrenaline [10^{-5}M] to intact fetal gastric mucosa, resulted in an increase in the s.c.c. by 21.02 $\pm$ 1.88% (Fig. 11 above, also superimposed as curve a in Fig. 28).

Addition of adrenaline [10^{-5}M] to pre-theophylline treated mucosal cells was followed by an increase in cyclic AMP level from a mean value of 4.82 $\pm$ 0.40pmol/mg dry weight of mucosal cells to 7.20 $\pm$ 0.21pmol/mg of dry weight of mucosal cells (s.e.m., n = 6), i.e. cyclic AMP level increased by 49.38 $\pm$ 3.00% in excess of that caused by theophylline alone (Fig. 28, columns). Curve b (Fig. 28) shows that in a series of six experiments the addition of adrenaline to the serosal surface of intact gastric mucosae which had been pre-treated with theophylline failed to further increase ion transport across the membrane. This is in contrast to an increase observed in cyclic nucleotide level even when the hormone was added to pre-theophylline treated mucosal cell suspensions over the same time course.

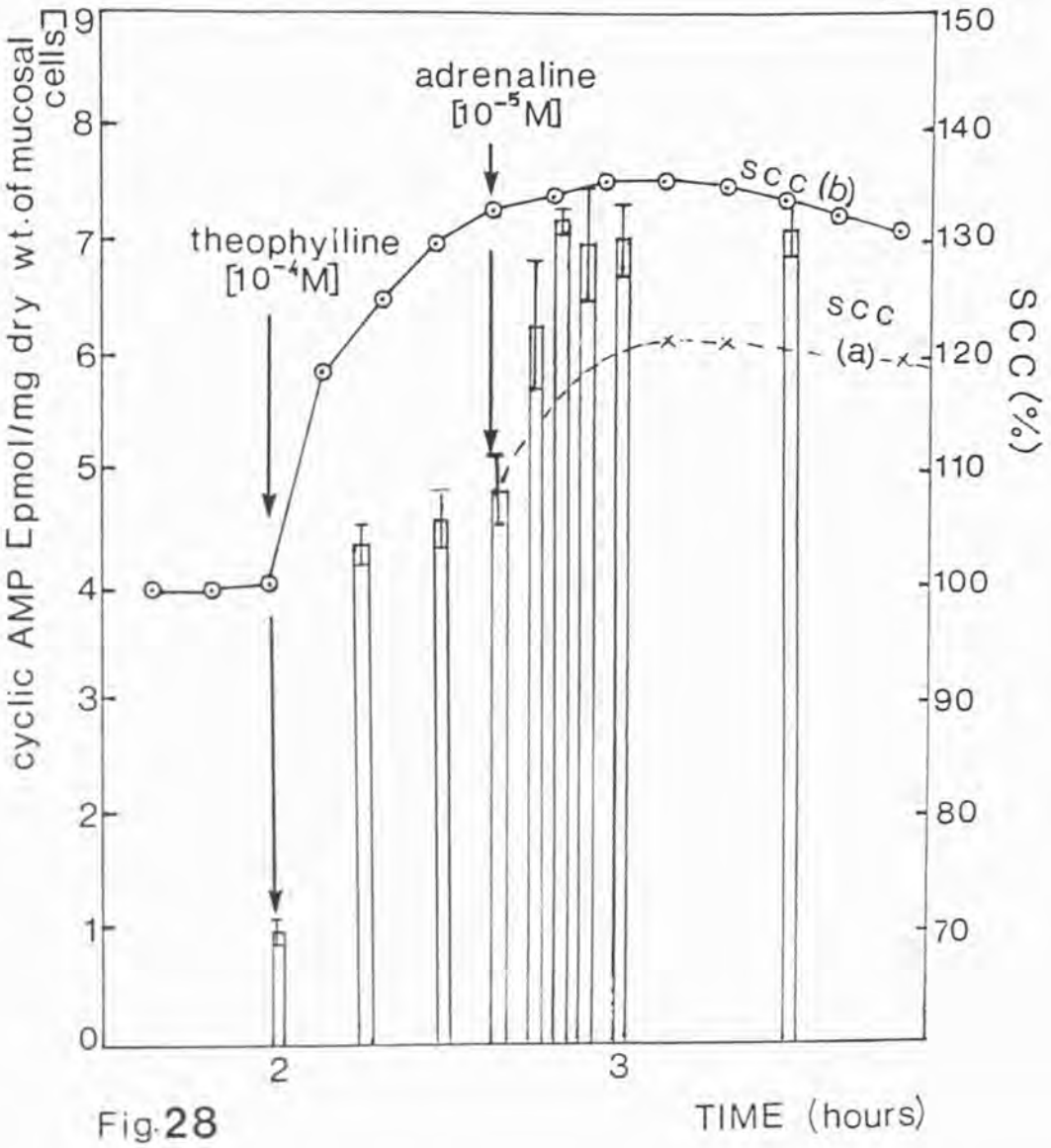


Fig.28

The effect of adrenaline [10^{-5}M] on the s.c.c. across untreated gastric mucosa (curve a, n = 15) and on pre-theophylline treated membranes (curve b, n = 6). Columns show the effect of adrenaline [10^{-5}M] on intracellular cyclic AMP content of mucosal cells which had been pre-treated with theophylline (n = 6). Vertical lines represents the mean $\pm$ s.e.m.

3.7.2.3.2. Effect of vasopressin on intracellular cyclic AMP level

It has been previously shown that the application of arginine vasopressin [1i.u./ml], to intact membrane preparations resulted in an increase in ion transport by 24.22 ± 4.41%, [Fig. 12, section 3.3.2 above also shown as curve a in Fig. 29].

Treatment of mucosal cells with arginine vasopressin [1i.u./ml] resulted in an increase in cyclic AMP level from a mean value of 4.85 ± 0.30pmol/mg to 7.65 ± 0.40pmol/mg wt. of dry cells [s.e.m., n = 5]; an increase of 57.73 ± 5.00% in excess of that caused by the addition of theophylline alone [Fig. 29, columns, n = 5].

Like adrenaline, the application of vasopressin to the serosal surface of intact gastric mucosae which had been pre-treated with theophylline, failed to further increase the s.c.c. [Fig. 29, curve b, n = 7].

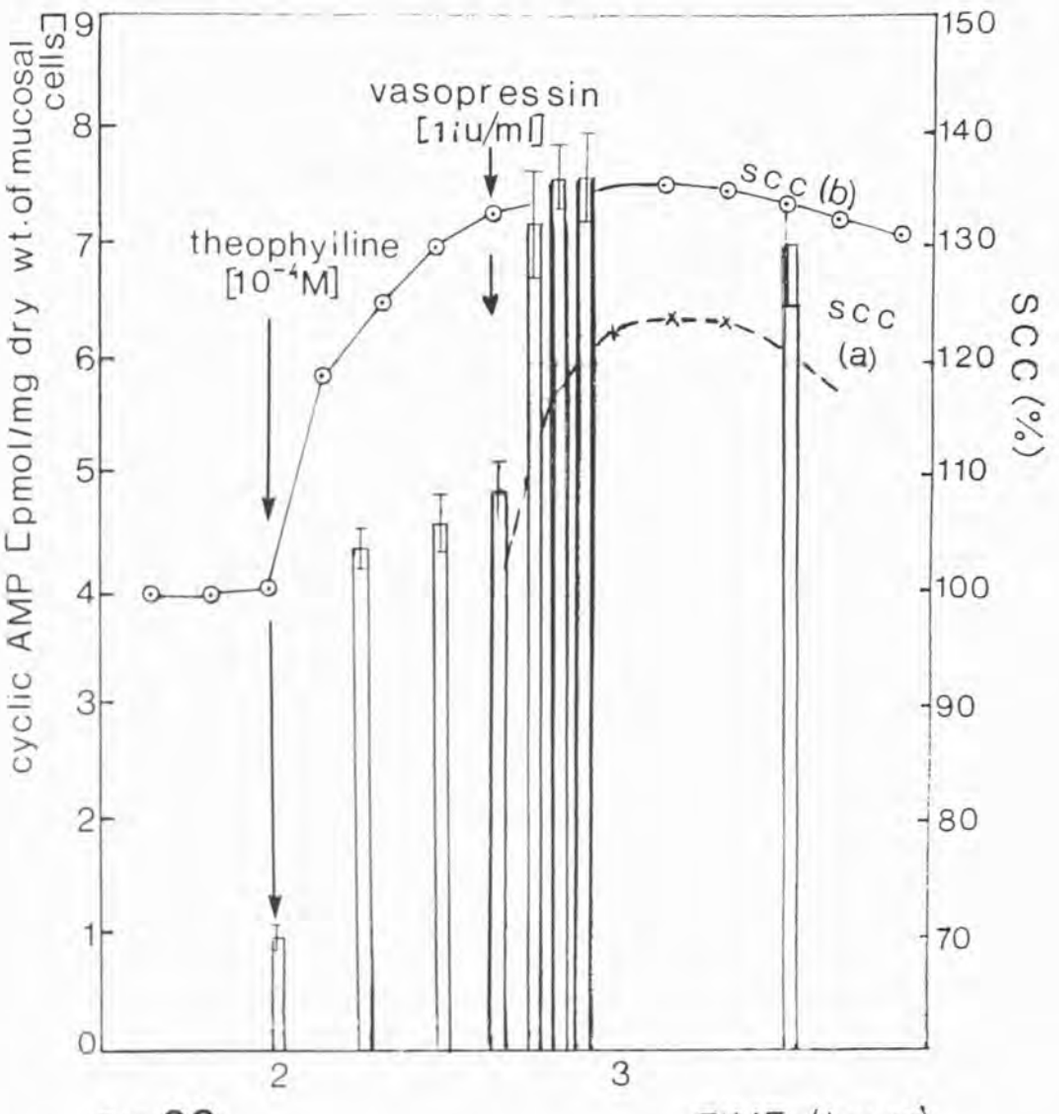


Fig.29

The effect of vasopressin [li.u./ml] on the s.c.c. response of the gastric mucosa (curve a, n = 8) and on gastric mucosa which had been pre-treated with theophylline (curve b, n = 7). Columns show the effect of vasopressin [li.u./ml] on intracellular cyclic AMP content of mucosal cells which had been pre-treated with theophylline [10^{-4}M], (n = 5). Vertical lines represents the mean $\pm$ s.e.m.

CHAPTER 4

DISCUSSION

4.1. General Introduction

The existence of a potential difference (p.d.) across the gastric mucosa of the rabbit fetus was initially reported by Wright (1961, 1962). The p.d. was shown to result from the activities of mucosal cells, the muscle coat providing no contribution (Rehm, 1946; Wright, unpublished observations). Upon reducing the electrochemical gradient and therefore the p.d. across the membrane by employing the Ussing and Zerahn (1951) technique, a s.c.c. across the membrane is observed, the magnitude of which depends upon the gestational age of the fetus (Wright, 1961, 1962).

Up to the 22nd day of gestation, the s.c.c. across the fetal stomach arises entirely from an active transport of sodium ions from the mucosal to the serosal side. In this respect, the fetal stomach resembles the frog skin and the toad urinary bladder. However, from the 23rd day to full term an additional component of the s.c.c. makes its appearance and from the work of Wright, (1961, 1962 and 1964) and Kendall and Wright (1967),this additional component of the s.c.c. was concluded to result from an active secretion of H^+ and Cl^- ions into the lumen.

The transition from the resting to an active state involves changes in the electrophysiology and the ultrastructure of the membrane. For the amphibian skin and the gastric mucosa, the concept of cyclic AMP as a second, intracellular mediator of these changes has been widely accepted. For example, methyl xanthines, which are inhibitors of cyclic nucleotide phosphodiesterase (see Chapter 1), stimulate H^+ secretion across the amphibian gastric mucosa; the

increase is associated, both in time and magnitude, with increased tissue content of cyclic nucleotide (Harris and Alonzo, 1965; Harris et al., 1969). Similarly alterations in Na^+ ion transport across the frog skin and the toad bladder are associated with changes in the intracellular cyclic AMP level (Handler et al., 1965; Hall et al., 1976; Gerencser, 1978).

Evidence is increasingly presented regarding the involvement of cyclic AMP in the secretory responses of post natal to adult mammalian gastric mucosa. For example, exogenously applied cyclic AMP and theophylline have been reported to stimulate H^+ ion secretion in the in vitro rabbit stomach (Fromm et al., 1975b). The stimulatory effect of histamine on gastric secretion is associated with increased tissue content of cyclic AMP (Ekblad et al., 1978). However, the involvement of cyclic AMP in the active transport of sodium ions across the gastric mucosa is not so well established although a limited number of reports do indicate a stimulatory effect of cyclic AMP on the sodium component of the s.c.c. across the gastric mucosa (see later).

The present studies were carried out to investigate the role of cyclic AMP in ion transport, particularly Na^+ ion transport across the gastric mucosa of the rabbit fetus. The gastric mucosa of the 28day old fetus was chosen for such studies since its ion transporting properties have been extensively studied and characterized previously (Wright, 1962, 1964; Kendall and Wright, 1967).

Experiments were carried out on intact membrane preparations. However, when determining the intracellular cyclic AMP level experiments were performed on mucosal cell suspensions obtained from such preparations. In the stomach of the rabbit fetus the mucosa is very thin, it can be easily removed by the simple mechanical means described in Chapter 2 and initially used by Malinowska (1974), to give a cell suspension. Fetal gastric mucosal cell suspensions prepared in this manner have been shown to consume O_2 (Wright, unpublished observations) which gave a preliminary indication that the cells were viable. It was therefore unnecessary to use techniques for the isolation of cells which involved the use of enzymes such as collagenase, pronase, hyaluronidase, trypsin and even the use of EDTA (Walder and Lunseth, 1963; Gatzy and Berndt, 1968; Croft and Ingelfinger, 1969 and Blum et al., 1971). Since it was possible to isolate mucosal cells by the mechanical method, the introduction of external factors which may affect the cell membrane and therefore possibly the active ion transporting systems of mucosal cells was avoided.

Mucosal cells appeared intact when examined under light microscope. The simplest test for the viability of gastric mucosal cells was the eosin exclusion test. The cells distinctly excluded eosin after 3 hours of incubation period. Furthermore, it has previously been reported that cells obtained in this manner remain viable, as judged by their ability to exclude eosin even after an incubation period of 10 hours (Malinowska, 1974).

The protein binding method of Gilman (1970) was used to measure cyclic AMP content of mucosal cells. This method is simple to perform whereas other methods currently available are either too labourous to carry out or do not have the sensitivity required by the low tissue level of the compound. The other advantages of this method are as follows:-

(i) due to the presence of the kinase inhibitor and carrying out the binding reaction at pH 4, the assay conditions become such that a binding constant approaching 10^{-9} is reached. The assay is therefore sensitive to 0.05 - 0.1 pmols of cyclic AMP: less than a milligram quantity of tissue are sufficient for the assay

(ii) tissue purification is unnecessary

(iii) destruction of cyclic AMP is not a factor

(iv) standard curves are linear since the reaction is performed at saturating concentration of ^{3}H-cyclic AMP

(v) cyclic nucleotide is assayed in deprotenized tissue extracts

(vi) recovery of cyclic AMP (see Recovery) is high

Silicon antifoam emulsion was used in all experiments, particularly when mucosal cell suspensions were used to enable continuous and thorough oxygenation. Gastric mucosal cells produce mucus which causes froth formation on gassing. In the absence of silicon antifoam emulsion this leads to a loss of the bathing medium from the chambers or experimental flasks (together with mucosal cells); it has no effect on the p.d. and therefore the s.c.c. across

the membrane (Malinowska, 1974).

The bathing medium used within the present work contained a higher concentration of K^+ ions in contrast to much lower concentration found in Krebs or Ringer solutions. This was because the gastric mucosa of the rabbit fetus is unable to survive under in vitro physiological conditions. K^+ ion concentration of about 10mM or above was found to be optimal for the stability of this membrane; below this concentration the fetal membrane is unable to retain a constant value of p.d. and therefore the s.c.c. which continues to decline (Wright, unpublished observations). Also the concentration of K^+ used within the present work is within the range found in the gastric content of the fetus on the 28th day of gestation (which varies between 13.20-19.20mM, Wright, 1964).

All intact membrane preparations studied, with the exception of those which had been treated with anti-inflammatory drugs (where p.d. measurements were not recorded) despite large increases in s.c.c., increases in the p.d. and the total conductance across the membranes were relatively small (see Tables 2-9, 14-18 in Chapter 2 and Table 19 in this Chapter). These observations are taken to indicate that the membranes did not become too "leaky" as a result of the agent addition.

It should pointed out and as indeed mentioned in Chapter 3, the gastric mucosa of the rabbit fetus obeys Ohm's law (Wright 1974). It is this obediance of the law which was used to calculate the total conductance across the membrane from the directly measured values of p.d. and the current.

4.2. Effect of the exogenous application of cyclic AMP

One test of the involvement of cyclic AMP in a particular cellular function in intact systems or membrane preparations in vitro is to administer cyclic AMP and observe its effect on the function of particular interest. In this manner, cyclic AMP for example, has been used to determine its involvement in a wide variety of cellular functions. It has been particularly used to determine and has been shown to play a second messenger role in the action of a large number of non-steroid hormones.

In the present work, the administration of cyclic AMP to intact membrane preparations has been used as an initial step towards the elucidation of its involvement in the active transport of Na^+ ions across the gastric mucosa of the rabbit fetus. However only 14 from a total of 25 preparations responded by an increase in the s.c.c. equal to or greater than 4%. Cyclic AMP molecule is somewhat hydrophylic in character attributed to it by the presence of a phosphate group at $C^{3'}$ and $C^{5'}$ of the ribose ring which renders it less permeable through the hydrophobic phospholipid bilayer of biological membranes; it is also rapidly distroyed by membrane's very active cyclic nucleotide phosphodiesterase (Butcher and Sutherland, 1962; Sutherland et al., 1962; Cheung, 1970; Weiss and Costa, 1968). It is therefore suggested that the response by only 56% of the preparations following the addition of cyclic AMP may be related to the poor permeability of cyclic AMP and its repid degration by the phosphodiesterase.

An interesting observation made from such experiments was that from the total gastric membrane preparations which did respond to the exogenous application of cyclic AMP, a relatively large number were those which had been stored for 72 hours. These observations appear to indicate that membrane permeability to cyclic AMP increases upon storage , however one can only speculate at this stage.

All membrane preparations (n = 9) to which dibutyryl cyclic AMP was applied displayed an increase in the s.c.c. $N^{6'}$-, $O^{2'}$-dibutyryl cyclic AMP, since first synthesised (Posternak et al., 1962; Falbriard et al., 1967) has been of immense value in studies of the biological effects of the naturally occuring cyclic adenosine 3', 5'-monophosphate. It is more effective then cyclic AMP when applied extracellularly to intect membrane preparations or systems. Two explanations for its unique potency have received widespread acceptance: (1) the two butyryl groups covalently bonded to $N^{6'}$ of the purine moiety and $O^{2'}$ of the sugar ring make the molecule more lypophylic permitting it to penetrate biological membranes more readily, and (2) the two butyryl groups also reduce the analogue's susceptibility to hydolysis by the catabolic cyclic nucleotide phosphodiesterase (Posternak et al. 1962; Henion et al., 1967; Monahan et al., 1969; Hepp et al., 1969). Dibutyryl cyclic AMP itself has been suggested to have an inhibitory effect on cyclic nucleotide phosphodiesterase (Klein and Berg, 1970; Heersche et al., 1971). Thus the stimulatory effect of dibutyryl cyclic AMP in all preparations used may be explained in terms of its increased ability to permeate biological membranes more readily and partly to its reduced susceptibility to membrane's phosphodiesterase.

The stimulatory effect of dibutyryl cyclic AMP on the s.c.c. following its addition and the reversibility of the response upon its removal are taken as the first indication of the vulnerability of the fetal ion transport system to alterations, or at least to increases in cyclic AMP level.

4.3. Effect of methyl xanthines

Control gastric membranes challenged with the application of theophylline or caffeine responded with increases in the s.c.c. Methyl xanthines are inhibitors of phosphodiesterase (Butcher and Sutherland, 1962); their addition to intact systems or cell suspensions should therefore result in the accumulation of intracellular cyclic AMP in cells which are involved in the production of cyclic AMP (i.e. cells which possess an adenylate cyclase system). Work on fetal mucosal cell suspensions have shown that the addition of theophylline, applied at a concentration of 10^{-4}M, was followed by an increase in the intracellular cyclic AMP level. The initial increase in cyclic AMP level was followed but not led, by an increase in the s.c.c. (Fig. 30). These results further indicate the involvement of intracellular cyclic AMP in the regulation of active ion transport across the gastric mucosa of the rabbit fetus.

If the increases in cyclic AMP level are examined further, particularly in terms of per cent increases, it is seen that about 90% of the increase in cyclic nucleotide level occurred during the first 15 minutes following the addition of theophylline whereas 90% of the increase in the s.c.c. response was reached in approximately

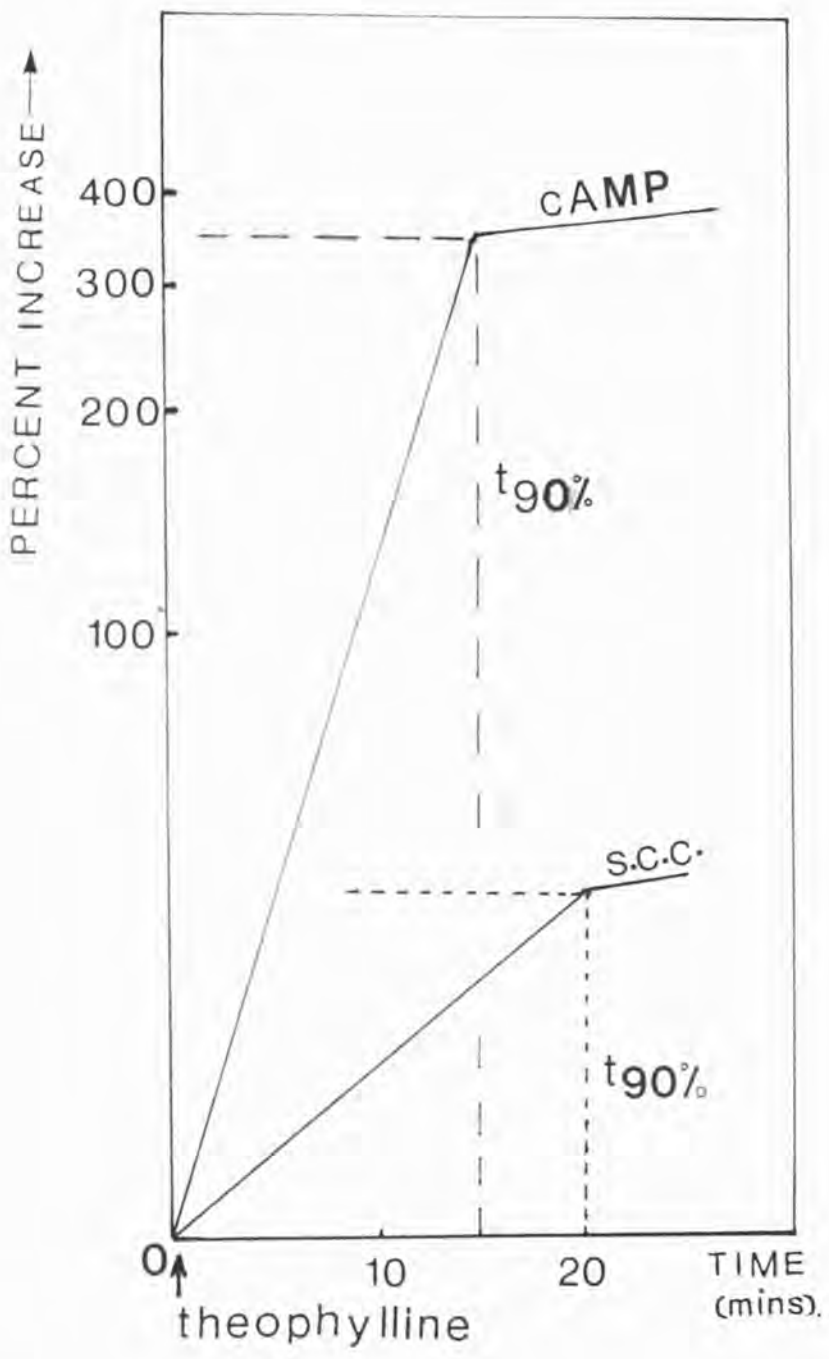


Fig.30

A comparison between the per cent increase in the s.c.c. and cyclic AMP level during the initial twenty minutes following the application of theophylline. $t_{90\%}$ represents the time required to reach an increase of 90%.

20 minutes. From these results it appears that there exists a 'lag' period of about 5 minutes between an increase in cyclic AMP level and the increase in the s.c.c. However, this lag period of 5 minutes is only an approximate measure extrapolated from the measurement of cyclic AMP level taken at 15 minute intervals. These results nevertheless indicate that the increase in the cyclic nucleotide level occurs prior to the observed increase in ion transport across the fetal stomach and in this respect are comparable with those reported for the frog skin (Hall et al., 1976), the piglet gastric mucosa (Ekblad et al., 1978; Machen et al., 1982) and rabbit gastric glands (Chew et al., 1980). In each case, an increase in cyclic AMP level was followed by a lag period when no observable changes occurred; the increase in ion transport occurring after the lag period. It is suggested that the lag period between an increase in cyclic nucleotide level and the increase in the electrical parameters may result from the involvement of other factors distent to the production of cyclic AMP such as the activation of protein kinases and the consecutive phosphorylation of substrates etc. which may be involved in increasing the permeability of the membrane.

As mentioned above, theophylline is an inhibitor of cyclic nucleotide phosphodiesterase and as such its application to the gastric membrane of the fetus should result in the accumulation of cyclic AMP but only in cells which contain an adenylate cyclase system, i.e. cells which have the ability to synthesise and are already in a process of producing cyclic AMP at a control rate. Theophylline has been reported to stimulate cyclic AMP accumulation and the concurrent increase in gastric secretion by parietal cells

(see for example Soll and Wollin, 1977; Sonnenberg et al., 1978; Wollin et al., 1979). Adenylate cyclase system has been reported to be present in parietal cells (Ekblad et al., 1978; Rutten and Machen, 1981).

However flux measurements carried out within the present work have shown that the administration of theophylline had no significant effect on the active transport of Cl^- ions (in the direction serosa to mucosa) across the gastric mucosa (see section 4.7 of this chapter) whilst it did significantly increased the active absorption of Na^+ ions. As far as the author is aware, there are no reports in the literature which indicate or even associate the active transport of Na^+ ions to the functions of parietal cells. Hence from the results obtained on intact membrane preparations and on mucosal cell suspensions and taking into consideration the presence of a large number of non-differentiated epithelial cells and relatively few parietal cells in the gastric mucosa of the 28 day old fetus (Menzies, 1958) it is possible to conclude that theophylline stimulates the active transport of Na^+ ions through the accumulation of cyclic AMP in non-parietal cells. If active transport of sodium is indeed a function of non-differentiated epithelial cells as suggested by Wright (1961, 1962) then these results can be taken to further indicate the presence of a cyclase system in such cells; however further work is required on intact gastric membranes prior to the 22nd day of gestation (when no parietal cells are present) and directly on non-differentiated epithelial cells to precisely relate the active transport of sodium to the function of such cells.

The application of theophylline was followed by an increase in the s.c.c., the magnitude of which was very similar to that observed following the addition of dibutyryl cyclic AMP (38.15 ± 1.67% and 39.40 ± 6.08% respectively). These observations appear to indicate that both of these agents might be stimulating the same ion transport system to its maximum. This suggestion would then be in consistent with the results presented in Chapter 3, section 3.7.2.3., where it was found that whilst both adrenaline and vasopressin are capable of stimulating the s.c.c., but when applied to the gastric mucosae which had been pre-treated with theophylline failed to further stimulate ion transport and yet each of the two hormones further stimulated cyclic AMP production in mucosal cell suspensions which had been pre-treated with theophylline.

During stimulation with theophylline, tissue cyclic AMP content increased to 394.84 ± 38.0% whereas the maximum increase in the s.c.c. only reached a value of 38.15 ± 1.67%. This non-correlation between the increase in cyclic AMP level and the increase in electrical parameters across the membrane, once the tissue had been maximally stimulated has also been reported by others (Chew et al., 1980; Machen el al., 1982). It appears that once the tissue is maximally stimulated the active ion transport processes no longer depends upon the concentration of intracellular cyclic AMP but upon the existence of some factor or factors other than cyclic AMP. The comparison between cyclic AMP level to the magnitude of the s.c.c. is not feasible as the addition of theophylline would not only result in the accumulation of cyclic AMP in those systems which might be involved in ion transport but in all other systems which also

utililize this compound.

4.4. Effect of non-steroid hormones

All four non-steroid hormones used within the present work stimulated the s.c.c. across the gastric mucosa of the rabbit fetus (Chapter 3, section 3.3.). Work on mucosal cell suspensions have shown that the addition of adrenaline or vasopressin was followed by an increase in the intracellular cyclic AMP level (Figs. 28 and 29, Chapter 3). From Fig. 31 it can be seen that the increase in cyclic nucleotide level was followed, but not led, by an increase in the s.c.c. These results indicate and further support the results obtained with theophylline that intracellular cyclic AMP is involved in the regulation of ion transport across the fetal stomach. These results also indicate the presence of an adenylate cyclase system in the fetal membrane which is responsive to all four hormones used.

The increased production of cyclic AMP following the addition of adrenaline or vasopressin could have been predicted from the well established fact that each of the four hormones used within the present work elicit their respective responses through the stimulation of adenylate cyclase activity (Birnbaumer et al., 1970; Robison et al., 1971a). Nevertheless an important implication of these results is that the adenylate cyclase system is an integral part of the fetal membrane the receptor site of which, or at least those responsive to adrenaline, vasopressin, glucagon and pentagastrin, are located on the serosal surface as indicated by the responsiveness of the tissue only when each hormone was applied to the serosal surface of the gastric mucosa.

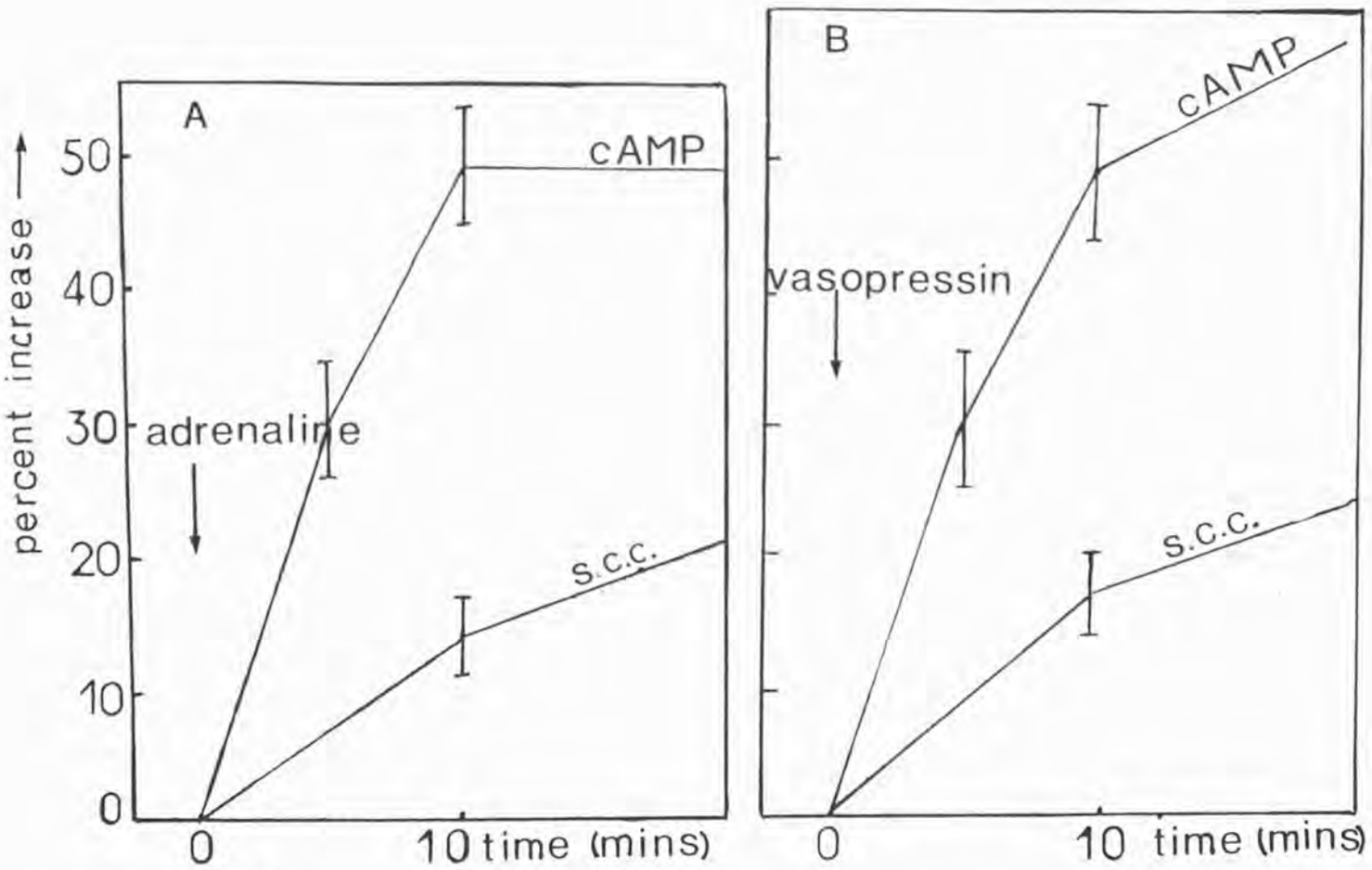


Fig.31

A comparison between the per cent increase in the s.c.c. and cyclic AMP level during the initial twenty minutes following the addition of adrenaline (A) and vasopressin (B).

Fetal stomach displayed a response to both arginine and lysine vasopressin. These results indicate the inability of the fetal adenylate cyclase system to distinguish between the two analogues of the hormone (which differ only in their terminal peptide). Arginine vasopressin is the naturally occurring hormone found in the adult rabbit and other mammalian membranes (Frieden, 1976; Barrington, 1979) although lysine vasopressin has been isolated from domestic pigs (Frieden, 1976). The responsiveness of the fetal stomach to both analogues of vasopressin, at this stage, does not, however, indicate the presence of both forms in the fetus.

The maximum increases in the s.c.c. following the application of adrenaline or vasopressin were of a smaller magnitude (21.02 $\pm$ 1.88% and 24.22 $\pm$ 4.41% respectively) compared with the much larger increases observed from the addition of dibutyryl cyclic AMP (39.40 $\pm$ 6.08%) or theophylline (38.15 $\pm$ 1.67%). One explanation for this relatively low magnitude of stimulation of the s.c.c. may be that the production of cyclic AMP by the cyclase system is continuously offset by the very active phosphodiesterase (Butcher and Sutherland, 1962; Sutherland et al., 1962; Weiss and Costa, 1968; Cheung, 1970). Thus in the absence of a phosphodiesterase inhibitor, the cyclic nucleotide level at any particular point is dependent upon the relative activities of the two enzymes. Whereas methyl xanthines, by inhibiting phosphodiesterase activity, and dibutyryl cyclic AMP's high permeability, together with its inhibitory action on the membrane's phosphodiesterase, may raise the intracellular cyclic nucleotide level well above the physiological level (10^{-6}-10^{-9}M), thereby maximally stimulating the ion transport system across the

gastric mucosa.

The application of pentagastrin was followed by a relatively small increase in the s.c.c. (11.13 ± 1.20%) in contrast to the increases observed from the addition of adrenaline, vasopressin or glucagon. This may be due to the stimulatory action of gastrin on the Na^+-independent component of the s.c.c. only. Pentagastric have been reported to stimulate H^+ ion secretion across other gastric mucosae (Enoch and Johnson, 1977; Kuo and Shanbour, 1979).

Further work was carried out to determine whether both adrenaline and vasopressin increase ion transport across the fetal gastric mucosa by stimulating a single adenylate cyclase system or whether the fetal membrane possesses separate adenylate cyclases each being responsive to only one hormone. It was argued that if each of these hormones stimulate a separate adenylate cyclase system then the application of one hormone during the maximum response to the first applied hormone, should result in a further increase in the s.c.c., i.e. the response to the application of the second hormone, through the stimulation of a second adenylate cyclase system, should produce an additive effect on the s.c.c. but only when both hormones are applied at their maximum effective concentrations. Results presented in Chapter 3, section 3.3.5., have shown that no further increase in the s.c.c. was observed when the second hormone was applied during the maximum response to the first applied hormone. However the addition of a second hormone, at a point when the response to the first hormone just begins to decline from the maximum, was followed by a small increase in ion transport; the second increase was additive but only up to the previous maximum. The order of hormone

addition appeared to be irrelevant to the response observed. The inability of the second hormone to elicit a further increase in ion transport when applied during the maximum response to the first hormone could not have been due to maximum saturation of membrane's capacity to transport ions or its inability to respond to a further increase in the intracellular cyclic AMP level since the addition of theophylline, in each case, was followed by a further increase in the s.c.c. These results therefore indicate that in the gastric mucosa of the rabbit fetus, both adrenaline and vasopressin increase ion transport by stimulating a single adenylate cyclase system which is responsive to both hormones. The small increase in ion transport observed upon the addition of the second hormone at a point when the response to the first hormone just begins to decline from its maximum can be explained in the following mannor: the application of the first hormone maximally stimulates the adenylate cyclase system and the decline from maximum in the s.c.c. represents the dessociation between the receptor sites of the cyclase system and the first hormone molecules. At this point the application of the second hormone then immediately occupies the now vacant binding sites or sites which are specific for the second hormone and thereby maximally stimulating the cyclase system once again.

These observations further led to the question of whether both adrenaline and vasopressin stimulate the cyclase system by interacting with a common set of receptor sites or whether the cyclase system possesses seperate sites which are specific for each hormone.

Two sets of receptor sites of adenylate cyclase have been largely studied since their identification by Ahlquist (1948) and later confirmation by Powell and Slater (1958). These receptors are generally referred to as the alpha (α) and the beta (β) receptors. If both adrenaline and vasopressin stimulate the adenylate cyclase system by interacting with a common receptor site, then the selective blockage of that site should inhibit the action of both hormones. Adrenaline is generally known to stimulate adenylate cyclase by interacting with the β-receptors (Robison et al., 1968). If the same situation applies to the fetal cyclase system than by selectively blocking the β-receptors only, it should be possible to inhibit the stimulatory action of adrenaline on the s.c.c. and observe the effect, if any, of vasopressin.

Propranolol or timolol was used as the β-receptor blockers; they have high affinity for β-receptors with almost negligible action on α- receptor sites (Meyers et al., 1980). Phentolamine was used to confirm that α-receptors are not involved. This agent selectively blocks α-receptors while it has negligible effect on β-receptors (Meyers et al., 1980).

Results presented in Chapter 3, section 3.3.5.1 and 3.3.5.2., have shown that pre-treatment of the gastric mucosa with phentolamine (10^{-4}M) applied to the serosal surface, had no observable effect on the action of either adrenaline or vasopressin on ion transport whereas pre-treatment with propranalol or timolol (10^{-5}M), applied to the serosal chamber, prevented the stimulatory effect of adrenaline on the s.c.c. but not that of vasopressin. These results indicate that in the gastric mucosa of the rabbit fetus adrenaline also

stimulates the adenylate cyclase system by interacting with its β-receptors whereas vasopressin, although stimulates the same cyclase system, acts upon some other site of the enzyme and that these are not α- or β-receptors.

One argument against the inhibitory effect of propranolol on the action of adrenaline on ion transport could be that this effect of propranolol may have resulted not from its β-receptor blocking properties but from its anaesthetic effect on the membrane (Lucchesi et al., 1967; Parmley and Braunwald, 1967) i.e. the addition of propranolol may have temporarily anaesthetized the gastric mucosa which then prevented the stimulation of ion transport by adrenaline. However two observations against this argument are (1) the order of hormone addition did not affect the response observed: if vasopressin, instead of adrenaline was applied as the first hormone, an increase in the s.c.c. was observed; the addition of adrenaline (at the point when the response to vasopressin had declined back to the control level) was still unable to stimulate the s.c.c. and (2) similar observations were made when timolol was used in place of propranolol.

From these results it is possible to conclude that the gastric mucosa of the rabbit fetus (i) contains adenylate cyclase systems which are responsive to all four hormones used within the present work; (ii) both adrenaline and vasopressin increase the s.c.c. by stimulating a single adenylate cyclase system which is responsive to both hormones whereas adrenaline interacts with the β-receptors of this enzyme, while vasopressin acts upon some other sites but which are not α- or β-receptors of the cyclase system.

In this respect these results are comparable with those reported for the rat adipose tissue where a single adenylate cyclase is also responsive to more than one hormone (Butcher et al., 1968; Bar and Hechter, 1969; Rodbell et al., 1970) and where the different hormones do not interact with a common receptor site but on sites which are specific for each of the active hormone. Single adenylate cyclase systems which respond to more than one hormone, with each hormone interacting with a specific site have also been reported to be present in other tissues such as the cat liver (Makman and Sutherland, 1964), rat and cat heart (Murad and Vaughan, 1969; Levey and Epstein, 1969).

The addition of theophylline was followed by an increase in the active transport of Na^+ ions while it had no significant effect on the active secretion of Cl^- ions (see section 4.7 below). In the present work it is assumed that both adrenaline and vasopressin stimulated the s.c.c. through their effect on the active transport of Na^+ ions since these two hormones, like theophylline, elicit their respective responses through increases in the intracellular cyclic AMP level (confirmed in chapter 3, section 3.7.2.3.) although by different means. Vasopressin has been reported to inhibit gastric acid secretion (Ivey, 1975; Carter, 1976) but without stimulating cyclic AMP production in parietal cells (Batzi and Gardner, 1978). These reports are in contrast to the results obtained within the present work where an increase in cyclic nucleotide level was observed following the addition of vasopressin to mucosal cell suspensions of the rabbit fetus. As mentioned above the active transport of Na^+ ions is not a function of parietal cells these

results may therefore be taken to indicate the presence of an adenylate cyclase system in membranes of those cells which carry out the active transport of Na^+ ions but which are not parietal cells.

4.5. Effect of non-steroidal anti-inflammatory drugs

Non-steroidal anti-inflammatory drugs inhibit prostaglandin biosynthesis by interfering with the activity of prostaglandin synthetase (Vane et al., 1971; Ferreira et al., 1971; Smith and Willis, 1971: Flower et al., 1972). This enzyme is involved in the final insertion of two oxygen atoms into the fatty acid, arachidonic acid followed by its cyclization to prostaglandins (Von Dorp et al., 1964; Bergstrom et al., 1964). In the present work anti-inflammatory drugs were used to determine indirectly the possibility of the involvement of prostaglandins in the regulation of the active transport of ions across the gastric mucosa of the 28 day old rabbit fetus.

Although anti-inflammatory drugs were used at a concentration which has been reported to inhibit synthetase activity in other tissues (Vane et al., 1971; Ferreira et al., 1971; Smith and Willis, 1971; Flower et al., 1972; Hall et al., 1976), as prostaglandin output was not measured the decline in the s.c.c. (and p.d. [not given]) may have resulted from one of the three or a combination of the three following possibilities:

(a) due to a decrease in prostaglandin biosynthesis;

(b) concurrent with (a) above, due to the removal of the cytoprotective effect of prostaglandins on the gastric membrane;

(c) directly as a result of the destructive action (other than the removal of the protective barrier) of anti-inflammatory drugs on the gastric membrane.

On the mucosal surface of the stomach, there is present a mucus layer, called the gastric mucosal barrier, which consists of a matrix of glycoproteins. Its function is to act as a barrier between the acidic contents of the lumen and the mucosal cells and thereby prevent the mucosa from autodigestion (Davenport et al., 1964a, 1964b, 1967). Prostaglandins have been reported to maintain this barrier by stimulating the secretion of mucus (Fung et al., 1974; Bicket and Kaufmann, 1981; Lamont et al., 1983) and by preventing its destruction by agents such as aspirin and indomethacin (Cohen, 1975; Colton et al, 1979; Bommelar and Guth, 1979).

Upon the 28th day of gestation the gastric mucosa of the rabbit fetus secretes hydrochloric acid but at a very low rate, in the range of approximately 0.4-0.5μEq. $cm^{-2}hr^{-1}$ (Wright, 1962, 1964). It is therefore unlikely that the decline in the s.c.c. which led to complete abolition of ion transport as seen in Figs. 21b, 22 and 24, had resulted from the removal of the protective barrier as a result of the inhibition of prostaglandin biosynthesis with concurrent autodigestion of the gastric mucosa by the gastric acid thereby ruling out possibility (b).

Non-steroidal anti-flammatory drugs have been reported to directly damage the gastric mucosa (for a review see Kimberg, 1974) through their accumulation, in the non-ionized form, in the mucosal and sub-mucosal layers of the stomach membrane (Garner, 1978). This

results in the loss of mucosal cells. As mucosal cells have a high turnover rate, under in vivo conditions the damaged cells are thereby rapidly replaced. However in the in vivo gastric mucosa such replacement may not be possible. It is therefore likely that the sharp decline in the s.c.c., specially that seen in Figs. 21b, 22 and 24 following the addition of acetyl salicyclic acid, phenylbutazone or indomethacin, respectively (at their final concentration of 10^{-4}M) resulted from such a direct action of aspirin-like drugs on the gastric mucosa. In a number of preliminary experiments (not reported in the text), the application of cyclic AMP to membrane preparations which had been pre-treated with aspirin for about 15-20 minutes, failed to prevent the s.c.c. from a further decline. These observations are consistent with the direct destructive effect of synthetase inhibitors.

Observations where the direct addition of acetyl salicyclic acid (n = 20, Fig. 21a) or indomethacin (at a final concentration of 10^{-5}M, Fig. 23, n = 8)) was followed by a sustained decrease in the s.c.c. which was maintained for several hours (2-3 hours) before reaching zero values are similar to those reported for the frog skin where the decline in the s.c.c. was further reported to be accompanied by the reduced release of prostaglandin-like material from the skin (Hall et al., 1976).

Taking into account the similarity of the present results with those reported for the frog skin, it is suggested that the sustained decrease in ion transport as seen in Figs. 21a and 23 may have been due to the inhibition of prostaglandin biosynthesis which would than further indicate the involvement of prostaglandins in the regulation

of the active transport of ions across the gastric mucosa. However the direct measurement of prostaglandin output is required to confirm this suggestion.

Unlike the other prostaglandin synthetase inhibitors used, indomethacin had a biphasic effect on the s.c.c. (Fig. 23). The second phase of the s.c.c. response to this drug, i.e. the decrease, can be attributed to the inhibition of prostaglandin biosynthesis whereas the first phase, the initial increase, may be due to the inhibitory effect of indomethacin on the activity of membrane's phosphodiesterase (Flores and Sharp, 1972; Ciosek et al., 1974), thereby facilitating cyclic AMP accumulation. A similar effect of indomethacin has been reported for the frog skin, where the initial increase in the s.c.c. was further shown to be associated with increase in cyclic nucleotide level and the second phase with its decrease (Hall et al., 1976).

Application of indomethacin at a final lower concentration of 10^{-7}M was followed by a sustained increase in the s.c.c. across the fetal gastric mucosa. Indomethacin, at this concentration range had been reported to stimulate ion fluxes in some smooth muscles (Northover, 1972). However the direct measurements of intracellular cyclic AMP level may indicate whether a similar action of indomethacin is also responsible for the observed effect across the gastric mucosa of the rabbit fetus.

In conclusion from the results obtained with anti-inflammatory drugs, particularly taking into account the sustained decreases in the s.c.c. following the addition of acetyl salicylic acid and

indomethacin (at a final concentration of 10^{-5}M), it is possible to conclude that prostaglandins are involved in the regulation of the active transport of ions across the fetal stomach. However it must be emphasised that the direct measurement of prostaglandin output is necessary to positively establish this whereas the present results only provide an indirect indication of the existance of such a possibility across the gastric mucosa of rabbit fetus.

4.6. Effect of arachidonic Acid

Results presented in Chapter 3, section 3.4. indicate that arachidonic acid applied directly to the gastric mucosa was followed by an increase in the s.c.c. Arachidonic acid is an immediate precursor of prostaglandins (Bergstrom et al., 1964; Von Dorp et al., 1964): oxidization and the consecutive cyclization of which leads to the formation of prostaglandins. Administration of arachidonic acid to the frog skin have been reported to be associated with the increased release of prostaglandin-like material from the skin (Hall et al., 1976). Increases in prostaglandin output following the exogenous application of arachidonic acid was similarly reported from mammalian gastric mucosae (see for example Konturek et al., 1979). The stimulatory effect of arachidonic acid on the s.c.c. across the gastric mucosa of the rabbit fetus is therefore probably due, at least in part, to the enzymatic transformation of arachidonic acid to prostaglandin. These results further indicate the involvement of prostaglandins in the regulation of the active ion transport across the fetal stomach.

Prostaglandins are widely distributed in various tissues including the gastrointestinal tract. High concentration of prostaglandins have been found to be present in the gastric mucosa and they are secreted into the gastric juice following vagal and hormonal stimulation (Bennett et al., 1967, 1968 and 1973; Zozois and Thompson, 1974). Enzymes responsible for their biosynthesis and degradation have been identified in the epithelial wall of the stomach (Bebiak et al., 1979; Spenny and Barton, 1981).

Although prostaglandins have been reported to simulate Cl^- ion secretion and decrease Na^+ ion absorption across epithelial membranes such as the treachea (Al-Bazzaz et al., 1981) and even the small intestine (Al-Awqati and Greennough, 1972; Bukhave and Rash-Madsen, 1980), their role in gastric ion transport is uncertain. Naturally occuring prostaglandins and their analogues are known to be potent inhibitors of gastric secretion both under basal conditions and in response to various exogenous and endogenous stimulants in man as well as in laboratory animals (Nezamis et al., 1971; Wilson et al., 1971; Robert, 1974; Robert et al., 1976; Konturek et al., 1976; Mihas et al., 1976).

Work on cell suspensions rather than on intact membrane preparations have shown that prostaglandins decrease cyclic AMP level in parietal cells and actually increase its level in non-parietal cells or in suspensions containing low parietal cell content. For example the addition of prostaglandins or arachidonic acid to mucosal cell suspensions stimulate cyclic AMP production in cell fractions containing fewer parietal cells but has only a slight effect on cyclic AMP level of enrich parietal cell population (Soll and Wollin,

1977; Sonnenberg et al., 1978; Batzi and Gardner, 1978; Wollin et al., 1979; Thompson et al., 1981).

That the active secretion of H^+ and Cl^- ions is a function of parietal cells is well established (see for example Forte et al., 1977; Ito and Schofield, 1978; Chew et al., 1980; Rutten and Machen, 1981). It has been suggested that prostaglandins, by decreasing intracellular cyclic AMP level of parietal cells, act as local controller of gastric secretion rather than initiating acid secretion (Robert et al., 1974 and 1976; Konturek et al., 1979).

Although prostaglandins have been reported to stimulate the production of cyclic AMP in non-parietal cells, no mention, as far as the auther is aware, regarding its function in non-parietal cells have been made so far. Prostaglandins have been reported to stimulate the production of mucus (see section 4.5 of this Chapter) but whether this is associated with increased production of cyclic AMP in such cells is not known. However it is unlikely that prostaglandins would stimulate cyclic AMP production in mucus-produceing cells, if indeed the increase production of cyclic nucleotide is associated with the secretion of mucus, at a stage when the gastric mucosa is not actually secreting acid or at a very low rate.

Because the gastric mucosa of the rabbit fetus carries out an active transport of Na^+ ions even before the appearance of parietal cells, it was therefore concluded that the active transport of sodium ions is a function of non-differentiated epithelial cells (Wright, 1962, 1964).

Taking into consideration that in the gastric mucosa of the 28 day old rabbit fetus (i) a large proportion of the total s.c.c. (approximately 70%) arises as a result of the active transport of sodium ions from the mucosa to the serosa (Wright, 1962 and 1964; Kendall and Wright, 1967); (ii) it contains a large number of non-differentiated epithelial cell whereas parietal cells are relatively few (Menzies, 1958); (iii) the accumulation of cyclic AMP, as a result of the presence of theophylline, stimulated the active transport of sodium ions while it had no significant effect on the active secretion of chloride ions (see section 4.7 below); (iv) prostaglandins stimulate the production of cyclic AMP in non-parietal cells and actually decrease its production by parietal cells with a concurrent decrease in gastric secretion and (v) arachidonic acid is a precursor of prostaglandins, its addition to the fetal gastric mucosa was followed by an increase in the s.c.c. it is thus possible to conclude that the stimulatory effect of prostaglandins, on the production of cyclic AMP in non-parietal cells is probably associated with its effect on the active transport of sodium ions. Hence the addition of arachidonic acid and its conversion to prostaglandins by the synthetase present in the fetal stomach would then stimulate the production of cyclic AMP by the membrane's adenylate cyclase which in turn stimulates the active tranport of sodium ions across the membranes of non-parietal cells. However further work is required on intact membrane preparations, preferably before the 22nd day of gestation, to positively establish and relate the active transport process of sodium ions to the functions of non-differentiated epithelial cells.

4.7. Effect of theophylline on the Unidirectional fluxes of both sodium and chloride ions

Theophylline was chosen because, being an inhibitor of phosphodiesterase, its effect on ion transport would result from the accummulation of intracellular cyclic AMP produced by those cells which contain an adenylate cyclase system. Theophylline, therefore, is an ideal agent for such studies since it forms a link, as it were, between the effect of exogenously applied cyclic AMP and that resulting from the addition of a number of non-steroid hormones.

The Student t test was used to analyse the statistical significance of the mean ($\bar{x}$) of each set of results (Table 19). Where required the t test for related samples (paired test) was also used to compare the significance of the difference between the two means; p values (for a two tailed test) less than 0.05 were regarded as significant.

Before analysing the results using the Student t test, if the results summarised in Table 18 (Chapter 3) are examined taking into account all the mean influx and efflux values, whilst disregarding the p values for the present, it then appears that the application of theophylline to the serosal surface was followed by an increase in the net active transport of Na^+ ions by 5.98 ± 1.70 $\mu M\ cm^{-2}hr^{-1}$, in the direction of mucosa to serosa whereas the s.c.c. increased by a value of only 2.66 ± 0.34 $\mu Mcm^{-2}hr^{-1}$. Hence the net increase in sodium influx was about 2 times greater than the increase in the s.c.c. which, at this point, suggests that some other ion species was also stimulated by increases in intracellular cyclic AMP level.

If chloride fluxes are now examined it appears that the addition of theophylline was actually followed by a considerable decrease in chloride efflux (serosa to mucosa) by a value of 4.95 ± 2.50 $\mu Mcm^{-2} hr^{-1}$, whereas its influx in the direction of the passive transport, increased by 8.14 ± 3.07 $\mu Mcm^{-2} hr^{-1}$. These results appear to indicate that the difference between the increase in net sodium influx and the increases in the s.c.c. may have resulted from an increase in chloride influx as if pulled along by the positively charged Na^+ ions moving in the same direction (against an electrochemical gradient). This co-transport of Cl^- ions, in the direction of mucosa to serosa, was similarly reported for the dog gastric mucosa following the application of cyclic AMP, where a decline in the active transport of Cl^- ions (efflux), was also reported (Kuo and Shanbour, 1980).

The net increase in Cl^- influx appears to be well within the standard error of the net increase in the active transport of Na^+ ions from mucosa to serosa which may be taken to indicate that the increase in the s.c.c. may have resulted from an increase in some ion species, other than from an increase in Na^+ or Cl^- ion fluxes. However if this was the case and equal quantities of both sodium and chloride ions were moving in the direction of mucosa to serosa per unit area per unit time then these results would indicate that the increase in the s.c.c. may not have actually resulted from an increase in either sodium or chloride ions but from an increase in some other ion species. The results nevertheless indicate the stimulatory effect of theophylline on the active transport of sodium ions.

The increase in the unidirectional transport of Na^+ ions from the mucosa to the serosa could have resulted either from the effect of cyclic AMP or theophylline on the mucosal membrane to increase its permeability to Na^+ ions or from its effect on the rate of Na^+ pump or both. As theophylline also increased the unidirectional transport of Cl^- ions from the mucosa to the serosa this suggests that an increase in the permeability of the mucosal membrane is a possibility. However an increase in the rate of the Na^+ pump cannot be excluded.

If these results are now analysed with the stringent application of the t test (see Table 19) the following points emerge:-

(i) in all experiments electrical measurements indicated that the addition of theophylline resulted in a net statistically significant increase in the s.c.c. by $2.66 \pm 0.34 \mu Mcm^{-2} hr^{-1}$ ($p < 0.01$, $n = 23$);

(ii) experiments in which the unidirectional sodium transport was measured (using Na^+ isotope) have shown that the addition of theophylline was followed by an increase of $7.85 \pm 2.11 \mu Mcm^{-2} hr^{-1}$ in sodium influx (in the direction of active transport of this cation); this increase was found to be statistically significant ($p < 0.01$, $n = 8$);

(iii) theophylline also caused a small increase in sodium efflux, in the direction of passive transport of this cation. However the Student t test showed this increase of $1.87 \pm 0.70 \mu Mcm^{-2} hr^{-1}$ to be statistically insignificant ($p > 0.05$, $n = 5$);

Table 19 Statistical analysis of the unidirectional transport of sodium and chloride ions following the addition of $[10^{-4}M$ theophylline

	Na^+ flux ($\mu Mcm^{-2}hr^{-1}$)		Cl^- flux ($\mu Mcm^{-2}hr^{-1}$)		s.c.c. ($\mu Mcm^{-2}hr^{-1}$)	G ($\times 10^{-3}$ mho/M)
	$Jm \rightarrow s$	$Js \rightarrow m$	$Js \rightarrow m$	$Jm \rightarrow s$		
$\bar{X}_B \pm$ s.e.m.	13.85 ±3.10 (n = 85 $p < 0.005$	9.38 ± 1.65 (n = 5) $p < 0.01$	21.53 ± 4.13 (n = 6) $p < 0.01$	26.94 ± 2.97 (n = 4) $p < 0.01$	7.37 ± 0.60 (n = 23) $p < 0.001$	11.55 ± 0.77 (n = 21) $p < 0.001$
$\bar{X}'_A \pm$ s.e.m.	21.70 ± 5.27 (n = 8) $p < 0.005$	11.25 ± 1.80 (n = 5) $p < 0.01$	16.58 ± 3.25 (n = 6) $p < 0.01$	30.10 ± 2.28 (n = 4) $p < 0.01$	10.03 ± 0.72 (n = 23) $p < 0.001$	12.69 ± 0.71 (n = 21) $p < 0.001$
$\bar{X} - \bar{X}' \pm$ s.e.m.	7.85 ± 2.11 $p < 0.01$ t = 3.48	1.87 ± 0.70 $p > 0.05$ t = 2.37	-4.95 ± 2.51 $0.2 > p > 0.1$ t = 1.8	3.16 ± 3.38 $p > 0.4$ t = 0.81	2.66 ± 0.34 $p < 0.01$	1.14 ± 0.30 $p < 0.01$

where

$Jm \rightarrow s$ = flux in the direction of mucosa to serosa
$Js \rightarrow m$ = flux in the direction of serosa to mucosa
$X \pm$ s.e.m. = mean ± standard error of the mean
B = before theophylline addition
A = after theophylline addition
G = total conductance across the membrane
M = membrane area of 0.6 cm^2.

The increase in the net sodium transport of $5.98 \pm 1.70\ \mu Mcm^{-2}hr^{-1}$ was found to be statistically insignificant with $p > 0.2$.

(iv) the application of theophylline was followed by a decrease in chloride efflux (active transport of this ion); the decrease of $4.95 \pm 2.51 \mu Mcm^{-2}hr^{-1}$ was statistically insignificant ($P > 0.1$, $n = 6$) as determined by the t test;

(v) chloride influx increased by $3.16 \pm 3.38 \mu Mcm^{-2}hr^{-1}$ ($n = 4$) upon the addition of methyl xanthine, but again this increase was found to be statistically insignificant with $p > 0.4$;

The decrease in the net chloride transport of $8.14 \pm 3.07 \mu Mcm^{-2}hr^{-1}$ was also found to be statistically insignificant with $p > 0.05$.

(vi) in all experiments theophylline application was followed a small increase in the total conductance across the membranes from a mean value of $11.55 \pm 0.77 \times 10^{-3}$mho ($p < 0.001$, $n = 23$) to $12.69 \pm 0.71 \times 10^{-3}$mho/$0.6cm^2$ ($p < 0.001$, $n = 23$); the difference between the two means of $1.14 \pm 0.30 \times 10^{-3}$mho was statistically significant ($p < 0.01$).

From analysis of the results using the Student t test, it appears that the addition of theophylline was followed by a statistically significant increase in the active transport of sodium ions from the mucosa to the serosa ($7.85 \pm 2.11 \mu Mcm^{-2}hr^{-1}$) whereas its transport in the reverse direction (passive flux) was statistically insignificant. As other ions beside sodium also contribute to the

s.c.c., it is necessary to postulate, at this stage, that the difference of approximately 3-fold between the increase in Na^+ influx and that in the s.c.c. may have resulted from the stimulation of some other ion species which may be either a negatively charged ion(s) moving in the same direction as Na^+ ions or a positively charged ion(s) or even an exchange between Na^+ moving in the direction of serosa with a negativelly charged ion moving in the direction of mucosa. Since the increase and the decrease in Cl^- influx and efflux were not statistically significant it follows that some ion(s), other than Cl^- ions was also stimulated by methyl xanthine. The stimulation of H^+ ions and even perhaps HCO_3^- ion secretion are a distinct possibility. Stimulation of H^+ ions, independent of chloride ion secretion, has been reported from the gastric mucosa of the frog (Shoemaker et al., 1974), necturus (Spenney et al., 1972), rat (Brennan et al., 1975) and even from the new born rabbit (Fromm et al., 1975b).

Although these results indicate that the active transport of sodium ions was stimulated by theophylline, and this increase was statistically significant, further work is nevertheless required to ascertain that the effect of cyclic AMP on sodium efflux, chloride efflux and influx is indeed statistically insignificant and did not infact result from the experiments being performed under non-parallel conditions i.e. both the influx and efflux measurements, within the present work, were carried out in different set of preparations

From the results presented in this Thesis it is possible to conclude that intracellular cyclic AMP is involved in the regulation of the active transport of ions across the gastric mucosa of the 28 day old rabbit fetus. The application of theophlline, a phosphodiesterase inhibitor, resulted in a significant increase in the active transport of sodium ions which further indicates the involvement of cyclic AMP in the regulation of sodium transport in this tissue.

Experiments with arachidonic acid and anti-inflammatory drugs indicate that intracellular prostaglandins also affect active transport across the fetal stomach.

Finally the author would like to point that from the work presented in this Thesis it is possible to conclude that all four criteria put forward by Sutherland et al. (1968) for cyclic AMP as an intracellular second messenger in the stimulation of ion transport across the gastric mucosa by adrenaline and vasopressin have been satisfied. Thus (a) the ability of cyclic AMP to stimulate Na^+ transport across the fetal gastric mucosa satifies the 3rd criterion of the second messenger concept which states that "it should be possible to mimic hormonal effects with the exogenous application of cyclic AMP or its dibutyryl derivative; (b) the stimulation of Na^+ ion transport by theophylline satisfies the 4th criterion which states that "it should be possible to potentiat adenylate cyclase activity by substances which inhibit cyclic nucleotide phosphodiesterase activity"; (c) the increase in cyclic nucleotide level following the addition of adrenalline or vasopressin was followed but not led by an increase in ion transport. These

observations thus satisfy the 2nd criterion which is that "the changes in cyclic nucleotide level should preceed but not follow the physiological response" and (d) the stimulation of cyclic AMP production by adrenaline and vasopressin, in mucosal cell suspensions satifies the 1st criterion of this concept which is that "adenylate cyclase in cell suspensions should respond to the same hormone which is effective in the intact tissue.

SUMMARY

The results presented in this thesis indicate the following:-

1. the direct application of cyclic AMP or its dibutyryl derivative was followed by an increase in the s.c.c. thus indicating the effect of cyclic AMP on the active transport of ions across the gastric mucosa of the rabbit fetus;

2. (a) the addition of methyl xanthines and non-steroid hormones was also followed by an increase in the s.c.c. which indicates the involvement of intracellular cyclic AMP in the regulation of active ion transport across the gastric mucosa;

   (b) measurement of intracellular cyclic AMP content have shown that the addition of theophylline, adrenaline or vasopressin was followed by an increase in cyclic nucleotide level which preceeds the increases in ion transport. These observations further confirm the involvement of intracellular cyclic AMP in the regulation of the active transport of ions across this membrane;

   (c) measurements of the unidirectional flux of sodium and chloride ions have shown that the increase in the s.c.c., following the addition of theophylline, results from an increase in the active transport of sodium ions in the direction of mucosa to serosa. These results also indicate the stimulation of some other ion species. However the stimulation of the active transport of chloride ions appeared

to be unaffected.

From these observations it is possible to conclude that theophylline by stimulating cyclic AMP accummulation, stimulates the active transport of sodium. As the active transport of sodium ions is not a function of parietal cells, these results therefore may be taken to further indicate the effect of methyl xanthine or cyclic AMP in non-parietal cells (possibly on non-differential epithelial cells) but cells which carry out the active transport of sodium ions;

3. the administration of arachidonic acid, a prostaglandin precursor, was followed by an increase in the s.c.c. These observation indicate the involvement of prostaglandins in the regulation of active transport across the gastric mucosa of the rabbit fetus. Prostaglandins elicit their respective response by stimulating or inhibiting cyclic AMP production; they have been reported to inhibit gastric acid secretion with associated decrease in cyclic AMP level of parietal cells and increase its production in non-parietal cells.

It is suggested that the stimulatory effect of arachidonic acid on the s.c.c. across the fetal gastric mucosa is associated with the increased production of cyclic AMP in non-parietal cells or cells which carry out the active transport of sodium ions. This suggestion is consistent with the stimulatory effect of prostaglandins on the active transport of sodium ions across the frog skin (Hall et al., 1976).

4. the addition of non-steroidal anti-inflammatory drugs was followed by a sustained decrease in the s.c.c. which further indicates the involvement of prostaglandins in the control of ion transport across this membrane. However further work is required to eliminate the possibility of a destructive action of such drugs whic may have also caused a decline in the s.c.c.

5. Each of the non-steroidal hormones used stimulated the s.c.c. indicating that the gastric mucosa of the rabbit fetus contain an adenylate cyclase (or cyclases) which is responsive to all the hormones used within the present work.

6. Experiments involving the consecutive applications of adrenaline or vasopressine, or vice versa, have shown that both hormones enhance ion transport by stimulating a single common adenylate cyclase system. From the work with α- and β-receptor blockers it is concluded that each of the two hormones act at a different site of the same cyclase system; adrenaline interacts with the β-receptors whereas vasopressin acts upon some other site but which are not α- or β-receptor sites.

OUTLINE OF FUTURE WORK

Although sodium influx was found to be statistically significant further ion flux measurements should nevertheless be carried out to confirm that sodium efflux, chloride efflux and influx are indeed statistically insignificant and did not infact resulted from experiments being performed under non-parallel conditions and on different membrane preparations. Sodium influx and efflux should, therefore, be measured simultaneously across the same preparations; $^{22}Na^{+}$ /$^{24}Na^{+}$ or $^{22}Na^{+}$/$^{36}Cl^{-}$ should be used. If the stimulation of some other ion species is still indicated then the ion species involved should be investigated.

Prostaglandin output should be measured both under control conditions and following the addition of arachidonic acid and anti-inflammatory drugs. The method of Vane, 1971 and Ferriera et al., 1971 may be used. Such experiments should directly indicate the involvement of prostaglandins in the regulation of sodium transport across the fetal gastric mucosa.

Fetal stomach prior to 22nd day of gestation would be ideal for such experiments, when no parietal cells are present and when the total s.c.c. is entirely dependent upon the active transport of sodium ions.

The chambers, used within the present work, to clamp the gastric mucosa should be redesigned to hold fetal membranes of smaller sizes

(20 to 22 day old fetal stomachs). Experiments with such stomachs would directly indicate the effect of various agents on the active transport of sodium ions; they would also through some light on the relationship, if any, between non-differented epithelial cells and the active transport of sodium ions.

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APPENDIX

Mechanism of action of cyclic AMP

A large amount of work has been carried out in the hope of elucidating the mechanism by which the low molecular weight cyclic nucleotides, such as cyclic AMP and cyclic GMP might bring about such a diversity of effects ranging from the mediation of hormone actions in the vertebrates (Sutherland et al., 1968; Robison et al., 1968, 1971a and 1971b; Sutherland and Robison, 1969; Greengard and Costa, 1970; Pastan and Perlman, 1971; Greengard et al., 1972; Bitensky and Gorman, 1972 and Greengard and Robison, 1973) to the stimulation of enzyme synthesis in bacteria (Pastan and Perlman, 1970, 1971). Control of the enzymes mediating glycogenolysis in skeletal muscles is one of the most thoroughly studied examples of hormonal regulation via cyclic AMP. In this system adrenaline increases tissue concentration of cyclic AMP which then activates a class of enzymes, called the cyclic AMP-dependent protein kinaes (Huijing and Larner, 1966; Walsh, et al., 1968; soderling et al., 1970; Villar-Palasi and Schendler, 1970 and Reimann et al., 1971). Protein kinases, in turn stimulate the phosphorylation of the inactive phosphorylase b kinase into its active "a" form which then carries out the break down of glycogen into glucose; hence the reaction between protein kinase and phosphorylase kinase forms a link between increase in cyclic AMP production by the hormone and the stimulation of glucogenolysis. Adrenaline also causes inactivation of glycogen synthetase (enzyme involved in the synthesis of glycogen) by converting the

nonphosphorylated but active form to its phosphorylated inactive form. This reaction is also mediated by the same cyclic AMP dependent protein kinase which activates phosphorylase b kinase (Soderling et al., 1970 and Villar-Palasi and Schendler, 1970).

Similar cyclic AMP dependent protein kinases and recently cyclic GMP dependent protein kinases have since been identified in a large number of mammalian tissues and also in a number of species of the invertebrate phyla (Kuo and Greengard, 1969a; Greengard and Robison, 1973). In all cases studied the cyclic AMP dependent protein kinases have a requirement for a divalent cation such as Mg^{2+}, Co^{2+} or Mn^{2+} for their activity which is inhibited by Ca^{2+} ions (Kuo et al., 1970 and Miyamato et al., 1969). These enzymes, in general can catalyse the phosphorylation of the substrate in the presence of cyclic AMP and ATP, however the physiological significance of phosphorylation of these substrates is unknown at present. It has been suggested that in brain, at least, the molecular basis of memory lies in the activation of such cyclic nucleotide-dependent protein kinases with the consequent phosphorylation of macromolecules: following synaptic activation increased levels of cyclic AMP would cause the activation of protein kinases in the synaptic region, the stimulated protein kinases would then bring about phosphorylation of a protein constituent of the plasma membrane which would result in permeability changes and thereby modify synaptic transmission, either by facilitation or inhibition (Greengard and Kuo, 1970).

Even though the physiological substrates for protein kinases have not yet been identified in many cases, it is nevertheless presumed that many of the effects of cyclic AMP are brought about by the activation of such kinases. Kuo and Greenard [1969a and b] have even postulated that all of the biochemical and physiological effects elicited by cyclic AMP are mediated by protein kinases which using ATP as substrate, phosphorylate endogenous substrate within the target cells. There is also evidence of tissue cross reactivity of such kinases, i.e. a protein kinase from one tissue may stimulate an enzyme from another tissue. For example, the protein kinase from rabbit skeletal muscle can activate the hormone-sensitive lipase in fat cells [Corbin et al., 1970; Huttunen et al., 1970a and 1970b]. If tissue reactivity is a generalized occurrence then the tissue specificity of protein kinases may depend upon different cells having different substrates for the enzymes or in the intracellular location of the kinases.

Considerable progress has been made towards understanding the mechanism by which cyclic AMP increeses protein kinase activity and the same molecular event has been shown to apply in each tissue studied [Greengard and Robison, 1973 and Robison et al., 1971b]. Cyclic AMP dependent-protein kinases are composed of two subunits, a cyclic AMP-binding protein or the regulatory subunit and a catalytic subunit which when released becomes the active form of the kinase. In the absence of cyclic AMP the enzyme exists as a single inactive unit but when present, cyclic AMP binds specifically [and reversibly] to the regulatory subunit causing conformational changes of the kinase molecule which results in dissociation of cyclic AMP-bound

regulatory subunit and thereby releases the catalytic subunit which is now the active form of the enzyme. In this manner cyclic AMP acts as an allosteric modifier of protein kinases.

It should be pointed out that certain effects of cyclic AMP on cell function may not involve a protein kinase activation step. Cyclic AMP has been shown to be involved in the synthesis of β-galactosidase, tryptophanase, and other enzymes in E. Coli by acting either at the transcriptional or translational level (Pastan and Perlman, 1969, 1970 and 1971; DeCrombrugghe et al., 1969, 1971; Emmer et al., 1970 and Nissley et al., 1971). These effects require the presence of a cyclic AMP binding protein which in the case of β-galactosidase acts in the operator region of the lac gene and permits transcription in the presence of cyclic AMP. However this cyclic AMP-binding protein does not appear to be a protein kinase (Pastan and Perlman, 1970, 1971; Emmer et al., 1970; Nissley et al., 1971 and DeCrombrugghe et al., 1971).