GASTRIC ACID SECRETION: SUBSTRATE-DEPENDENCY AND INTRACELLULAR MECHANISMS OF ACTION OF SECRETAGOGUES AND INHIBITORS.

by

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degree of
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SUMMARY

The principal objectives of this study were: a) to establish which substrates, or combination of substrates, could provide by their metabolism, energy to support acid secretion by the rat parietal cell; b) to establish the putative involvement of protein kinase C in the acid-secretory process, and to determine the principal protein substrates for this enzyme within the parietal cell; c) to investigate the mechanism of action of a probable physiological inhibitor of gastric acid secretion, epidermal growth factor. Acid secretion was estimated by direct measurement in vivo, and by the accumulation of the weak base, aminopyrine, in vitro.

The effects of metabolic substrates were investigated using a preparation of isolated parietal cells maximally stimulated with secretagogues. Concentrations of glucose, oleate, lactate, D-3-hydroxybutyrate, L-isoleucine, acetoacetate, and L-valine similar to those found in plasma could enhance acid secretion above the level found in the absence of exogenous substrates. Supraphysiological concentrations of acetate, butyrate and L-leucine, but not L-glutamine also supported acid secretion. Supraphysiological concentrations of lactate and L-isoleucine produced a greater secretory activity than 5mM-glucose. Addition of combinations of certain substrates at physiological concentrations to 5mM-glucose, increased acid-secretory activity. Thus, maximal rates of acid secretion by isolated cells require a combination of substrates in addition to glucose.

12-O-tetradecanoylphorbol-13-acetate, exerted a specific inhibitory action on acid secretion in vivo, most likely by activation of protein kinase C in the parietal cell. An enzyme exhibiting many of the characteristics of protein kinase C phosphorylated an 89Kda protein present in a 100,000g cytosolic fraction of an homogenate derived from a preparation highly enriched with parietal cells.

Epidermal growth factor inhibited acid secretion stimulated by histamine, but had no effect on basal secretion or on that stimulated by the other secretagogues tested. The inhibitory action of epidermal growth factor on acid secretion by rat parietal cells probably did not involve prostaglandin production or activation of protein kinase C. A site of action close to the generation or hydrolysis of cAMP is suggested.

Key words: Acid secretion. Parietal cell. Substrate metabolism. Protein kinase C. Epidermal growth factor.

For my Family

ACKNOWLEDGEMENTS.

I would like to express my sincere gratitude to my supervisor Dr P.J. Hanson for his continual assistance and advice during the course of this work. I would also like to thank everyone involved in research in the Department of Molecular Sciences with whom I have enjoyed both working and socializing. Finally, I would like to thank Hazel Wright for cheerfully typing this thesis.

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ABBREVIATIONS.

The following, non-standard, abbreviations were used throughout this work.

cAMP	cyclic AMP
cGMP	cyclic GMP
dbcAMP	dibutyryl cyclic AMP
IBMX	3-isobutyl-1-methylxanthine
i.d.	internal diameter
o.d.	outside diameter
Pi	inorganic phosphate
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
TPA	12-O-tetradecanoylphorbol-13-acetate
4αPDD	4α-phorbol-12,13-didecanoate

CHAPTER 1.

INTRODUCTION.

1. INTRODUCTION.

This study investigates three aspects of the process of gastric acid secretion. Firstly, using an isolated parietal cell preparation *in vitro*, work was carried out in an attempt to establish the nature of the substrate-dependency of acid secretion. Thus, the primary objective of the first part of this study was to determine which substrates, or combinations of substrates, could provide by their metabolism energy to support acid secretion.

The second part of this work is concerned with an investigation of one aspect of the intracellular mechanism of acid secretion in parietal cells. Using an enriched parietal cell preparation *in vitro* this work aimed to establish the involvement of a calcium-sensitive, phospholipid-dependent protein kinase (protein kinase C), in the acid-secretory process. Since acid secretion cannot be measured directly using this model, an intact stomach preparation *in vivo* was used to determine whether the observed effect of 12-0-tetradecanoylphorbol-13-acetate (TPA) *in vitro* genuinely represented a reduction in acid secretion. The putative role of protein kinase C in this process was further investigated using a cell-free homogenate derived from a preparation highly-enriched with parietal cells. In this section, experiments were performed to establish the principle protein substrates for protein kinase C in parietal cells.

Finally, again using an isolated parietal cell preparation, experiments were performed in an attempt to establish the mechanism by which epidermal growth factor (EGF) inhibits acid secretion.

1.1 BASIC PHYSIOLOGY OF THE RAT GASTRIC MUCOSA.

1.1.1 Cell-types in the gastric mucosa.

The stomach forms the expanded region of the alimentary tract between the oesophagus and the duodenum, where it serves both as a reservoir and a digestive organ. The rat stomach is divided into distinct regions. The uppermost non-glandular region , above the entrance of the oesophagus , takes no part in the secretion of acid . Acid-secreting parietal cells are present in the fundic glandular region , and the G-cells which secrete gastrin are present in the antral glandular region . Mucus is secreted by every region of the stomach and serves an important protective function .

The surface of the gastric mucosa consists of mucous-secreting columnar cells. The surface is punctuated with tiny pits (approx. 100/mm^2), at the bottom of which are the gastric glands. More than one gland may be associated with a single pit. These gastric pits serve to increase the surface area of the gastric mucosa 20-fold. A typical fundic gastric gland is shown in figure 1.1. The acid-secreting parietal cells are scattered throughout the gland but predominate in the area of the isthmus. The neck of the gland is composed mainly of mucous neck cells, while the base of the gland is populated predominantly by chief cells.

Several other, less common, cell types have been identified in the rat gastric mucosa, and these may play an important role in the acid-secretory process. In particular, the endocrine-like cells located in the rat fundic gastric mucosa have been shown to contain histamine,

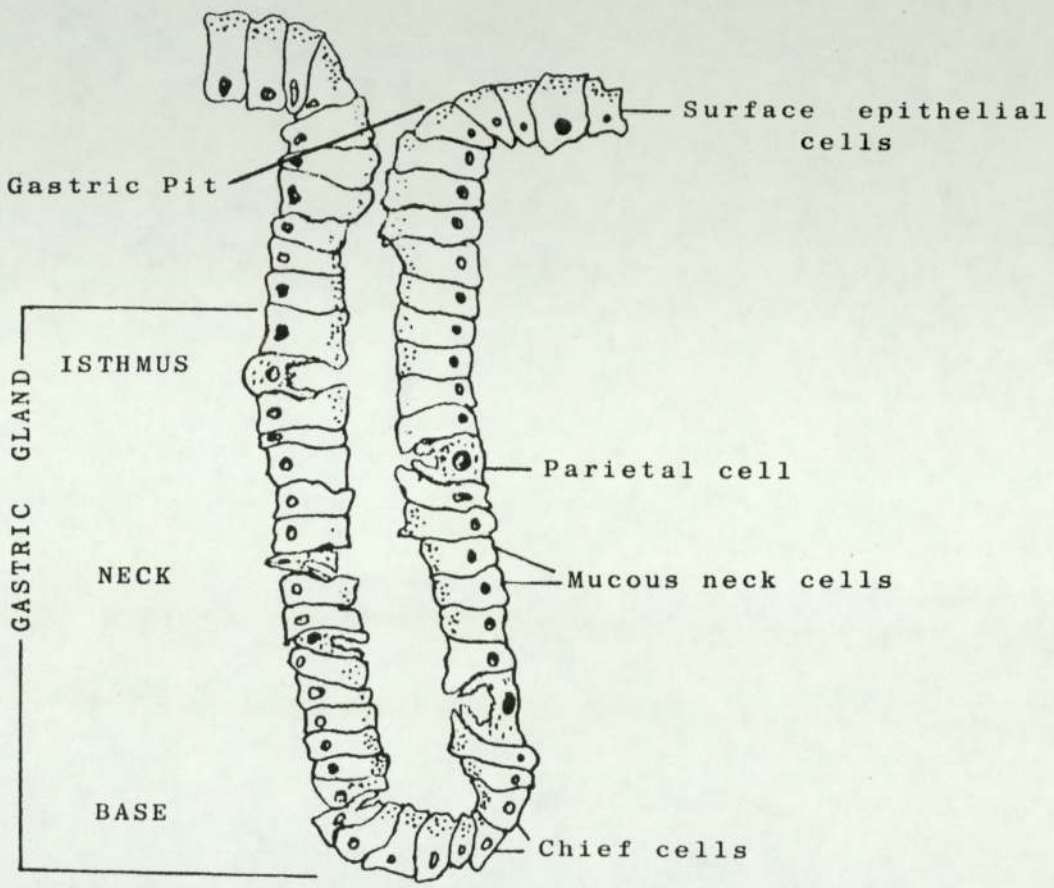


Figure 1.1

Illustration of a gastric gland showing some of the cell-types present in the stomach.

which stimulates acid secretion by interacting with a receptor on the parietal cell surface. These endocrine-like cells have been shown to be distinct from the rat peritoneal mast cells (Soll et al., 1981). The D-cells of the rat gastric glands secrete somatostatin from long cytoplasmic processes which terminate on the parietal cell. The release of somatostatin from these extensions may cause the inhibition of acid secretion by a direct effect on the parietal cell (Gespach et al., 1980).

1.1.2 Parietal cell morphology.

The mammalian parietal cell is characteristically conical in shape, and may have a width of up to 25µm at its widest point. These cells are generally located peripherally in the gastric gland so that their basolateral membrane appears to extend into the lamina propria, and their apical membrane appears not to reach the gland lumen. Under the electron microscope the parietal cell is easily identified (plate 1.1), since in addition to its large size, it possesses a large centrally-located nucleus, and numerous mitochondria which may account for up to 34% of the cell volume (Helander and Hirschowitz, 1972). A small golgi apparatus may also be present and free ribosomes may be visible. An unusual feature of the acid-secreting parietal cell is the presence of an intracellular canaliculus (plate 1.1). This is a network of branching canals into which protrude numerous finger-like projections or microvilli. This secretory canaliculus occupies a large proportion of the cytoplasmic volume of the active parietal cell (Berglindh et al., 1980a). In the resting, i.e. non-secreting parietal cell, the intracellular canaliculus is absent and instead the cytoplasm is occupied by large numbers of tubulovesicles.

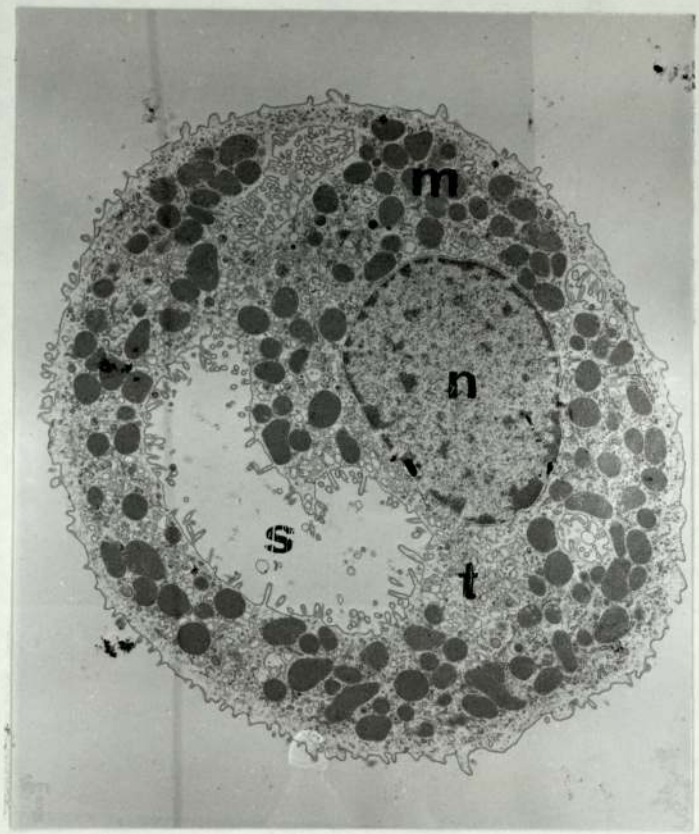


Plate 1.1

Ultrastructural appearance of a typical acid-secreting parietal cell showing; nucleus (N), secretory canaliculus (S), mitochondria (M), and relatively few tublovesicles (t).

Magnification 5,600 x.

1.2 REGULATION OF ACID SECRETION.

1.2.1 Stimulation of acid secretion.

Parietal cell activity is stimulated by three chemical messengers which act either directly or indirectly to increase acid secretion. These are gastrin, acetylcholine and histamine, and each of these chemical messengers uses one of the three possible modes of delivery to stimulate acid secretion. An example of endocrine delivery is the release of the hormone gastrin from the G-cells of the antral mucosa and the first part of the duodenum, into the blood from were it can activate the parietal cell directly (dog), (Grossman, 1981), or induce the release of histamine from another cell-type (enterochromaffin-like cells, rat; Håkansan, 1974). An example of neurocrine delivery is the release of acetylcholine by post-ganglionic nerve endings at or near the parietal cell. Acetylcholine then interacts with a muscarinic cholinergic (probably M_2) receptor on the parietal cell surface. An example of paracrine delivery is the release of histamine from mast-like cells or enterochromaffin-like cells in the rat, (Soll et al., 1981), adjacent to the parietal cell, into the extracellular fluid from were it can activate the parietal cell directly via the H_2-receptors on its surface.

A considerable amount of work has been carried out to investigate the existence of histamine, acetylcholine and gastrin receptors on the parietal cell surface. Using oxygen consumption by a gastric mucosal cell preparation as an index of acid secretion, and specific histamine (H_2), and acetylcholine receptor antagonists, Soll (1978a) suggested that gastrin, acetylcholine and histamine have a direct action on the parietal cell. This observation has subsequently been confirmed by the use of biologically

active radiolabelled ligands, and the gastrin (Magous and Bali, 1982; Soll et al., 1984), histamine (Batzri et al., 1982) and acetylcholine (Magous et al., 1985) receptors on the parietal cell surface, have been more fully characterized. Potentiation between these three parietal cell secretagogues is known to occur in vivo, i.e. when two secretagogues act simultaneously, the response is greater than the sum of the individual responses (Grossman, 1967). More recently, Soll (1978b) demonstrated a two-way potentiation in vitro between histamine and carbachol and histamine and gastrin, and a three-way potentiation between histamine, carbachol and gastrin using isolated canine parietal cells. This potentiation suggests that these secretagogues exert their effects on acid secretion via distinct second messenger systems (fig 1.2), and may explain the apparent non-specificity of receptor antagonists in vivo. Stimulation of acid secretion can be divided into three closely linked phases as follows:

1.2.1.1 Cephalic phase.

The cephalic phase of gastric acid secretion contributes approximately 10% of the gastric secretion associated with eating a meal, and is initiated by the smell, thought and taste of food. The cephalic phase is mediated solely by vagus nerves and is abolished by truncal vagotomy. Vagal stimulation of the gastrin-secreting G-cells of the antral mucosa in addition to the direct vagal stimulation of the parietal cell contributes to the overall secretory response during the cephalic phase (Nilsson et al., 1972).

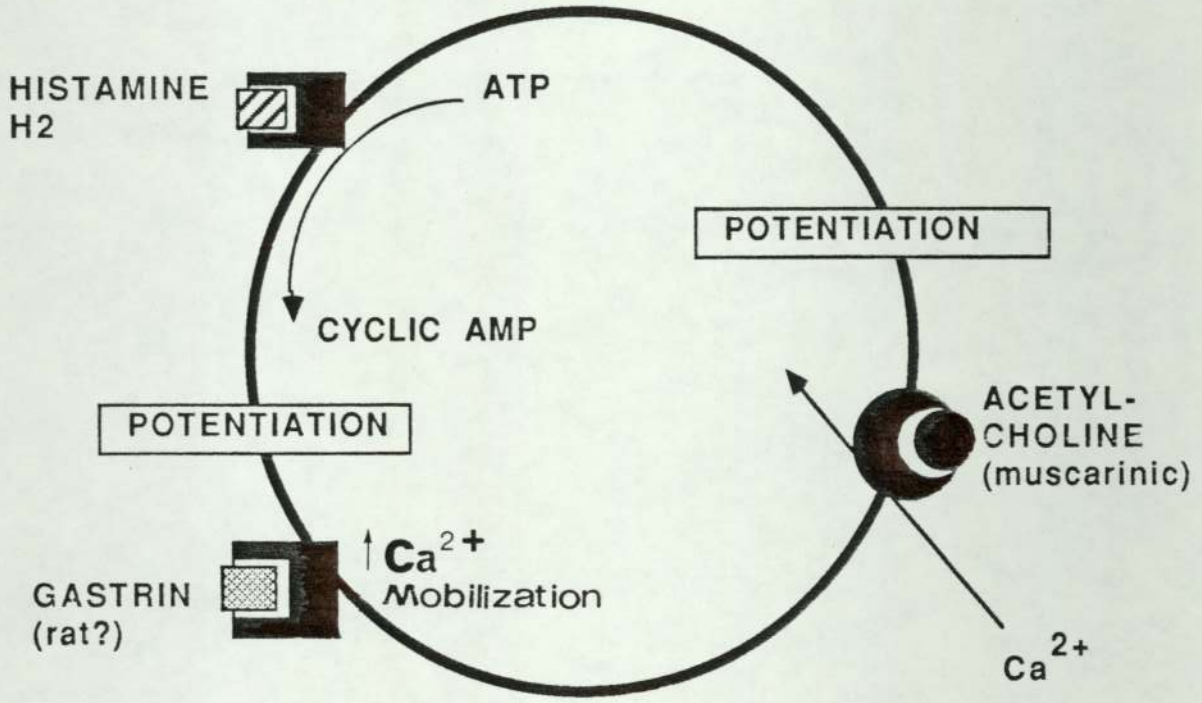


Figure 1.2

Diagrammatic representation of the secretagogue-receptor interactions involved in the stimulation of acid secretion.

1.2.1.2 Gastric phase.

The gastric phase accounts for up to 66% of the total acid secretion associated with eating a meal, and is initiated by gastric distension and the chemical constituents of food. Distension of the stomach stimulates gastric acid secretion primarily by activating vagovagal and intramural reflexes (Grossman, 1981), while chemical agents such as calcium and the digestion products of proteins may stimulate secretion by a direct action on the antral G-cells, thereby increasing serum gastrin levels (Feldman and Grossman, 1980).

1.2.1.3 Intestinal phase.

The presence of the digestion products of proteins in the duodenum, which can be absorbed into the blood, and duodenal distension are responsible for the intestinal phase of gastric acid secretion. In addition, small amounts of gastrin (enteric gastrin) are secreted by the duodenal mucosa in response to these stimuli, and may contribute to the increase in acid secretion during this phase.

1.2.1.4 Morphological transformation associated with stimulation of acid secretion.

The parietal cell undergoes a rapid morphological transformation upon stimulation, characterized by a reduction in the number of cytoplasmic tubulovesicles and a 5-10 fold increase in the surface area of the apical

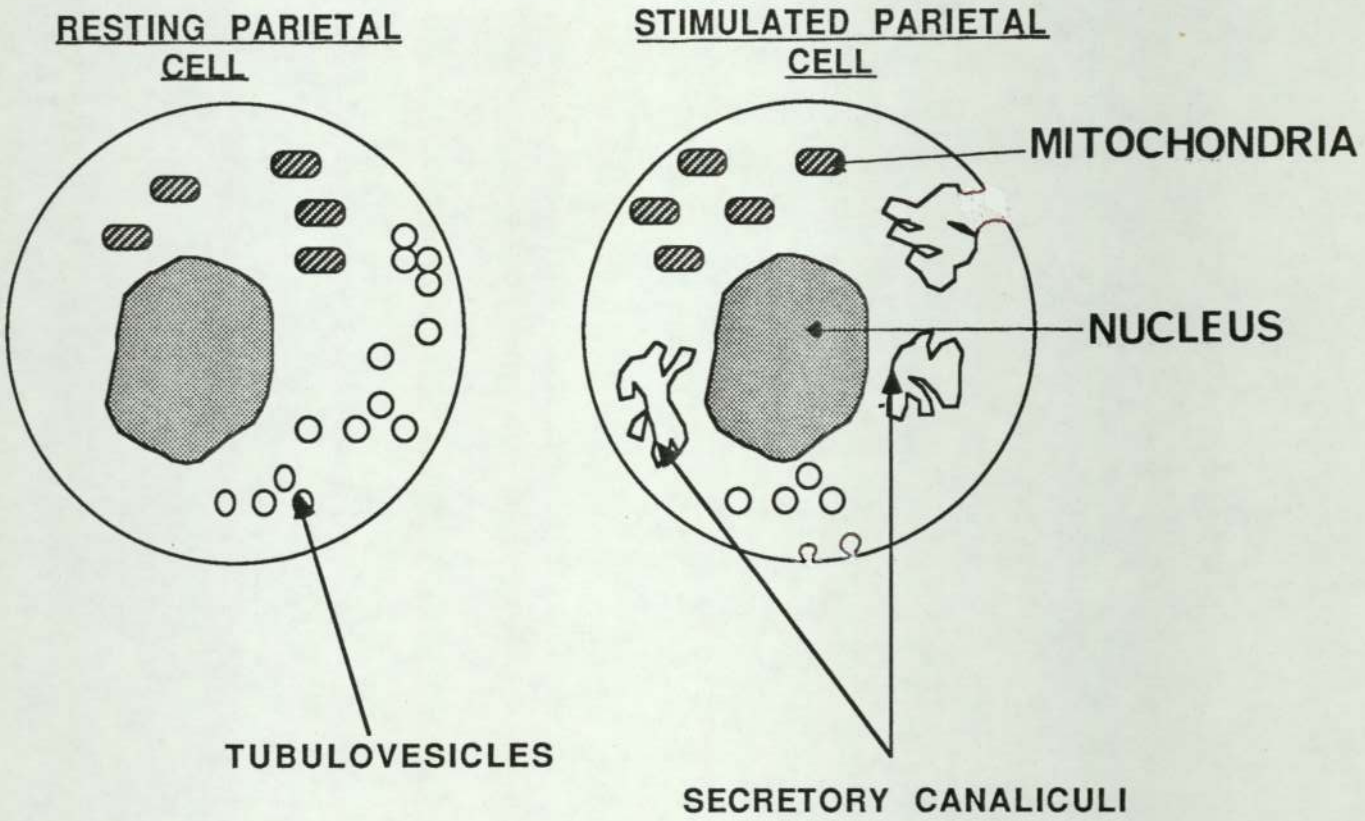


Figure 1.3

Diagrammatic representation of the transition between the resting state and the acid-secreting state in parietal cells.

membrane, (Ito and Schofield, 1974), (fig 1.3). This expansion of the apical membrane forms the intracellular secretory canaliculus, from the surface of which project numerous microvilli. Two alternative explanations of the mechanism by which this membrane expansion phenomena occurs have been proposed:

Firstly, the osmotic expansion theory of Berglindh et al., (1980a), suggests that the secretory canaliculus exists in a collapsed form in the resting parietal cell, but with stimulation the canaliculus expands due to an influx of H^+ and Cl^- from the cytoplasm followed by an influx of water. Secondly, the membrane recycling hypothesis of Forte et al., (1977), suggests that the cytoplasmic tubulovesicles migrate to the secretory surface upon stimulation, fuse, and are incorporated into it, thereby increasing the surface area. Evidence in favour of this latter hypothesis has come from a number of studies including morphometric observations and freeze-fracture analysis of parietal cells. Using the former technique, Helander and Hirschowitz (1972) clearly demonstrated a close correlation between the increase in the surface area of the apical membrane of the acid-secreting parietal cell and the decrease in the surface area of the cytoplasmic tubulovesicles. Freeze-fracture data demonstrated distinct differences in the substructure of the apical membrane and the cytoplasmic tubulovesicles of the piglet non-secreting parietal cell. However, upon stimulation, the substructure of the apical membrane changes to a form similar to that of the tubulovesicles (Black et al., 1980). An observation consistent with tubulovesicle integration into the apical membrane with the onset of secretion.

1.2.2 Inhibition of acid secretion.

A number of signals are known to inhibit acid secretion, these include:

(i) High levels of $[H^+]$ in the pyloric antrum or the proximal duodenum.

(ii) The presence of fat in the duodenum.

(iii) The presence of hyperosmolar concentrations in the duodenum.

When the pH of the pyloric antrum falls below 1.5, gastric acid secretion is inhibited as gastrin release is prevented. The inhibitory effect of acid in the duodenum is mediated by a putative hormone termed enterogastrone. The nature of this inhibitory agent is unknown, although it was originally thought to be either cholecystokinin or secretin which are both released from intestinal cells and both are known inhibitors of acid secretion. However, recent work (Schepp et al., 1983a), has indicated that these agents are unlikely to be enterogastrone since they have no effect on acid secretion at physiological concentrations. The presence of fat, in an absorbable form, in the small intestine also inhibits acid secretion by a humoral mechanism. The hormone responsible was thought to be gastric inhibitory polypeptide (GIP), since GIP release is stimulated by the presence of fat in the small intestine (Brown et al., 1975), and the hormone is known to inhibit acid secretion (Pederson and Brown, 1972), possibly by promoting the release of somatostatin (Ipp et al., 1977). However, GIP has been shown to have only a minimal effect on pentagastrin-stimulated gastric acid secretion in man (Maxwell et al., 1979), and the

concentration of GIP required to inhibit acid secretion, from rat parietal cells is unlikely to be found under physiological circumstances (Kuzio et al., 1974). This evidence therefore casts considerable doubt on GIP as a physiological modulator of gastric acid secretion. The presence of solutions which are hyperosmolar with respect to blood plasma in the small intestine, also inhibit acid secretion by a mechanism which is at least partly humoral (**Grossman,1981**).

Agents which are probably of physiological significance as inhibitors of acid secretion are somatostatin, prostaglandins, adenosine and EGF. Inhibition of acid secretion effected by somatostatin has been shown to occur by a direct action on the parietal cell (Chew, 1983), possibly by inhibition of the parietal cell adenylate cyclase (Schepp et al., 1983b). Prostaglandins may also inhibit histamine-stimulated acid secretion in a similar manner (Major and Scholes, 1978), as indeed may adenosine, (Gerber et al., 1985). At present, the mechanism of the anti-secretory action of EGF is uncertain and this is the subject of part of this investigation (Chapter 6).

1.3 SECOND MESSENGER SYSTEMS FOR SECRETAGOGUES.

Histamine stimulates acid secretion from parietal cells by interacting with a plasma membrane H_2-class receptor, thereby activating the receptor-associated adenylate cyclase and increasing intracellular cAMP levels (Soll and Wollin, 1979). The mechanism by which these elevated intracellular cAMP levels induce acid secretion most likely involves activation of the type I cAMP-dependent protein kinase (Chew, 1985).

Cholinergic stimulation of the parietal cell enhances the permeability of the plasma membrane to calcium, thereby effecting an increase in the intracellular calcium concentration (Muallem and Sachs, 1985). Gastrin stimulates acid secretion, at least in part, by a direct action on the parietal cell (Chew and Hersey, 1982), which may also involve an increase in the intracellular calcium concentration (Muallem and Sachs, 1985). Although the parietal cell response to both carbachol and gastrin involves an increase in the intracellular calcium levels, and is independent of cAMP concentration, several differences exist. The gastrin effect is insensitive to atropine and calcium-channel blocking agents (Muallem and Sachs, 1985), and may therefore be mediated by the mobilization of calcium from intracellular stores (fig 1.2). The increase in the intracellular calcium concentration which occurs after parietal cell stimulation by carbachol or gastrin, may activate specific calcium-dependent protein kinases, (Shaltz et al., 1981). These enzymes may phosphorylate cytosolic or particulate proteins, thereby linking the hormone-receptor interaction with the physiological response, i.e. the secretion of acid from the parietal cell. During this work, an investigation of the action of a novel protein kinase, protein kinase C, was undertaken in an attempt to establish the involvement of this enzyme in the stimulus-secretion coupling mechanism of the acid-secreting parietal cell (chapter 4).

As histamine-stimulated acid secretion is mediated by cAMP, and gastrin and carbachol-stimulated acid secretion is mediated by calcium, it should be possible to explain the mechanism of potentiation between histamine and gastrin, and histamine and carbachol in terms of the combined effects of the two second messenger systems (fig 1.2). It is clear that histamine has no effect on intracellular calcium levels, and that changes in the intracellular calcium concentration are not involved in the

mechanism of histamine-stimulated acid secretion from isolated parietal cells (Muallem and Sachs, 1984). Similarly, both cholinergic agonists and gastrin failed to increase the intracellular cAMP concentration of isolated canine parietal cells (Soll and Wollin, 1979).

1.4 MECHANISM OF ACID SECRETION.

The observation that $H^+ : K^+$ exchange in gastric vesicles was accompanied by the hydrolysis of ATP (Lee *et al.*, 1974), provided strong evidence that a $H^+ + K^+$ ATPase had a central role in the mechanism of gastric acid secretion. The use of immunochemical techniques enabled the localization of this $H^+ + K^+$ ATPase to the secretory canalicular membrane of the acid-secreting parietal cell (Saccomani *et al.*, 1979). More recent work using monoclonal antibodies to the $H^+ + K^+$ ATPase suggested that this enzyme may be translocated from the tubulovesicles of the resting parietal cell to the secretory surface upon stimulation (Smolka *et al.*, 1983). This evidence is consistent with a central role of this enzyme in the acid-secretory process.

The observation by Berglindh *et al.*, (1980b) that the secretory capacity of shock-permeablized rabbit gastric glands, incubated in medium containing a high concentration of K^+ (108mM), was restored by exogenous ATP, strongly suggested that ATP may serve as the primary source of energy for the acid-secretory process. This is not unlikely given the role suggested above for the H^+/K^+ ATPase. Although the role of ATP as the energy source for acid secretion seems certain, the substrates which, by their metabolism produce the ATP have not been clearly identified, and this is the subject of the first part of this investigation (chapter 3).

Much of the recent work on the mechanism of gastric acid secretion has employed gastric vesicles as a tool to study this process. Two functionally and physically distinct types of vesicles have been isolated from homogenates of the oxyntic mucosa, depending on the secretory state of the tissue (Wolosin and Forte, 1981). Stimulation-associated vesicles are derived from the apical membrane of the acid-secreting parietal cell, and may accumulate H^+ in the presence of ATP. This type of vesicle is highly permeable to K^+ and Cl^- (Wolosin and Forte, 1981). The second vesicle type most likely originates from the cytoplasmic tubulovesicles of the resting parietal cell. Consequently, these vesicles are relatively impermeable to K^+ and Cl^-, and ATP-generated H^+ accumulation by these vesicles requires the presence of specific ionophores (Wolosin and Forte, 1984). The K^+ and Cl^- conductances of these vesicles can also be improved by agents which promote S-S or tyrosine-tyrosine cross-linking, such as Cu^{2+}-o-phenanthroline (Takeguchi et al., 1985). Work with vesicles also supports the involvement of the H^+/K^+ ATPase in the secretory process, and suggests that its activity is switched on by the provision of K^+ at the external face through the opening or insertion of a K^+ or Cl^- conductance in the membrane. Work with gastric vesicles has enabled a model for the mechanism of gastric acid secretion from parietal cells to be proposed and this is shown in figure 1.4, but can be more fully explained as follows:

K^+ and Cl^- transport into the lumen of the secretory canaliculus occurs via a single physical unit in the apical membrane of the parietal cell, even though both conductances are distinct. HCl is formed within the lumen of the intracellular canaliculus when K^+ is recycled in exchange for H^+ by the H^+/K^+ ATPase with the consumption of ATP. Water is drawn into the canaliculus as a result of this accumulation of HCl.

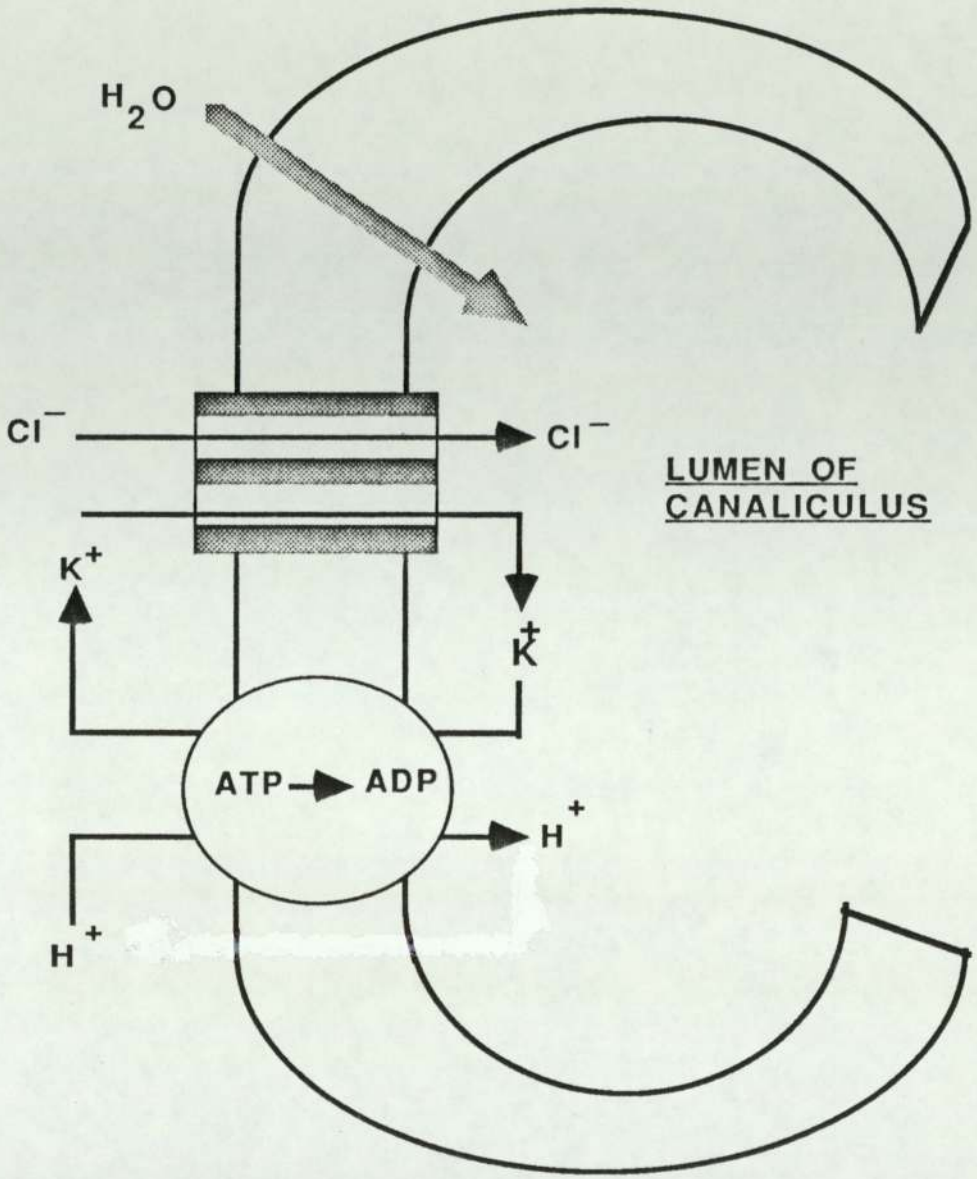


Figure 1.4

The current model for the mechanism of acid secretion:

The $H^+ + K^+$ ATPase located on the secretory membrane of the active parietal cell transports H^+ into the intracellular canaliculus in exchange for K^+. K^+ and Cl^- move into the lumen of the canaliculus via separate conductances.

In order for the parietal cell to function efficiently, it is necessary for a constant intracellular pH to be maintained. This is achieved by the Na^+ : H^+ and the Cl^- : HCO_3^- exchange mechanisms located on the basolateral membrane of the parietal cell (Muallem et al., 1985). With the onset of H^+ secretion from the parietal cell, alkalinization of the cell interior occurs (Paradiso and Machen, 1984), and in order to maintain a constant intracellular pH it is necessary to remove base from the cell. This is achieved largely by electroneutral Cl^- and HCO_3^- exchange (Muallem and Sachs, 1985). Under resting, i.e. non-secreting conditions, when the intracellular H^+ concentration may increase, the Na^+ : H^+ exchange mechanism is responsible for maintaining the intracellular pH (Muallem et al., 1985).

This investigation aims to extend current knowledge in three major areas of gastric research.

(i) The relationship between the control of acid secretion and metabolism.

(ii) The intracellular mechanisms involved in stimulus-secretion coupling.

(iii) The molecular mechanism of action of inhibitors of acid secretion and their physiological function.

CHAPTER 2.

ISOLATION AND PURIFICATION OF PARIETAL CELLS FROM RAT STOMACH FOR USE IN AMINOPYRINE TESTS.

2.1 INTRODUCTION.

2.1.1 Advantages and disadvantages of the use of isolated parietal cells.

A rat isolated parietal cell preparation was used throughout this study to investigate the effect of substrates (Chapter 3), and EGF (Chapter 6) on gastric acid secretion. This *in vitro* preparation was also used in conjunction with an *in vivo* system to investigate the putative role of protein kinase C in the regulation of gastric acid secretion (Chapter 4). Isolated cells were used routinely in preference to a stomach preparation *in vivo*, and intact mucosa *in vitro*, since certain fundamental problems are associated with the use of both these systems to investigate the mechanisms of regulation of acid secretion.

The site of action of the agent to be investigated is frequently difficult to determine using stomach preparations *in vivo* or intact mucosa *in vitro*, since the agent may either act directly on the parietal cell to, for example, inhibit acid secretion or it may act on another cell type to release an inhibitor of acid secretion. In both situations the same effect, i.e. inhibition of acid secretion would be observed, although the site of action of the agent of interest could not be determined with certainty. In addition, problems associated with agents gaining access to the parietal cell surface may be encountered, either because of metabolism of agents in the blood *in vivo*, or because of diffusion barriers in intact mucosal preparations *in vitro*. Also, oxygenation may present a problem with the use of the intact mucosa *in vitro*, but not so with isolated parietal cells. It is also difficult to design complex experiments using *in vivo*

systems, without the use of large numbers of experimental animals at a considerable cost in time and money. By contrast, it is possible to design relatively complex experiments using a single batch of isolated parietal cells.

However, there are some disadvantages in using isolated cells *in vitro*, e.g. the loss of intercellular connections and cellular polarity which occur upon isolation may reduce cellular responsiveness. This is true for isolated single acinar cells which are substantially less responsive to secretagogues than dispersed acini (Peikin *et al.*, 1978). In addition, the proteolytic enzymes often used in cell isolation procedures may disrupt receptors on the parietal cell plasma membrane. In fact, exposure of isolated rat adipocytes to trypsin for only fifteen seconds reduces the binding capacity of these cells for insulin by eighty percent (Kono and Barham, 1971). In contrast to these effects of the isolation procedure on pancreatic acini (Peikin *et al.*, 1978) and adipocytes (Kono and Barham, 1971), isolated parietal cells from rabbit seem to respond to secretagogues at least as well as gastric glands (Chew, 1983).

2.1.2 Techniques used for isolating parietal cells.

A wide range of techniques have been employed for isolating parietal cells from frog (Blum *et al.*, 1971), dog (Soll, 1978a), rabbit (Glick, 1974), and guinea-pig (Batzri and Gardner, 1978) gastric mucosa, and most have several stages in common. The initial stage usually involves the separation of the gastric mucosa from the submucosa and muscle. This is usually achieved by careful blunt dissection (Soll, 1978a), and is essential for adequate cell dispersion at low enzyme concentration. Alternatively, the proteolytic enzyme can be injected into

an everted sac prepared from the fundic mucosa (Lewin et al., 1974).

The second stage of the isolation procedure most commonly involves mucosal digestion by a proteolytic enzyme (pronase or crude collagenase) followed by a calcium-chelation step (incubation in medium containing 1-2mM EDTA). The use of separated mucosa and the calcium-chelation stage facilitate digestion of the intercellular matrix by collagenase, permitting the use of enzyme preparations with lower proteolytic activity (Soll, 1978a). The final stage of the isolation procedure usually incorporates mechanical dispersion followed by filtration of the cell suspension through a nylon filter.

2.1.3 Techniques used for parietal cell enrichment.

A heterocellular gastric preparation can be used to measure acid secretion from parietal cells by using aminopyrine accumulation as an indirect index of acid secretion. This is possible because the ionized form of the weak base, aminopyrine, becomes trapped only within the acidic spaces of stimulated parietal cells (2.1.4.3). However, for studies on the intracellular mechanisms of acid secretion, enrichment in the proportion of parietal cells may be required. Since the parietal cell is the largest and one of the least dense [due to its high proportion of membranes, Soll (1981a)], of the cell types present in the gastric mucosa, it may be separated from the remaining cell-types using both velocity and density-gradient separation techniques.

Velocity separation is achieved due to differences in cell size, large cells sedimenting faster, and relies on the principle of Stokes's law (equation 2.1).

Equation 2.1: $$SV = (\frac{2}{9})r^2 \ (\rho p - \rho m) \ \frac{g}{\eta}$$

SV = Sedimentation velocity
ρp = density, parietal cell
ρm = density, medium
r = cell radius
η = viscosity of medium
g = gravitational field

Separation on the basis of cell size may be achieved by unit gravity sedimentation. Romrell et al., (1975), obtained a highly-enriched fraction of mouse parietal cells using this technique although it does suffer from the disadvantage of providing a low yield, and requires long periods in the cold room at 4-5^{0}C. Parietal cell enrichment on the basis of cell size may also be achieved by successive 100g x 45s centrifugation steps (Soumarmon et al., 1977), which gives an improved yield, and does not require specialized instrumentation such as the elutriator rotor. The elutriator rotor has been used by Soll (1980 a,b) to produce an enriched fraction of canine parietal cells. This system relies on the principle of counter-flow centrifugation. The separation vessel is constantly perfused in a direction opposing the centrifugal force, and the cells equilibrate within the vessel as a function of their sedimentation velocity. The smaller, slower sedimenting, cells near the centre and the larger, faster, sedimenting cells towards the outside. As the flow-rate is increased progressively larger cells are eluted. (Soll, 1981a).

Density separation is achieved on the basis of variations in cell density. Upon centrifugation, all cells migrate through the medium until their sedimentation velocity reaches zero when the densities of the medium and of the cell are equal. All cells reach this equilibrium position providing that the speed of the centrifuge and the duration of centrifugation are sufficient. During the approach to equilibrium, separation will depend on both cell-size and density. This may be an advantage when enriching parietal cells since this cell type is the largest and one of the least dense of the cells present in the gastric mucosa.

Density separations have been performed using a wide range of media. Soll et al., (1979), employed a density-gradient of bovine serum albumin to produce an enriched mast cell fraction from canine fundic mucosa, and Glick (1974) used Ficoll (Pharmacia, Piscataway, N.J) to prepare an enriched parietal cell fraction from rabbit mucosa. Although a linear gradient of sucrose has been employed to enrich rat parietal cells (Lewin et al., 1974), this medium is considered unsuitable due to problems associated with hyperosmolality in the high-density region of the gradient. Problems associated with the use of some density separation media such as elevated viscosity and osmolality may be overcome by the use of Percoll (Pertoft et al., 1977). Percoll is composed of colloidal silica particles coated with polyvinylprrolidone (PVP). These particles do not penetrate cell membranes, and since solutions of high density have only a slightly increased osmolality over media without Percoll, water is not drawn from the cells present in the high density region of the gradient (c.f. sucrose) (Pertoft et al., 1977). Percoll does not adversely affect cell viability (Pertoft et al., 1977) and has therefore been used as a density separation medium for numerous cell-types. The isolation of mouse leydig cells

from testicular tissue has been achieved by the use of a linear continuous gradient of Percoll (Schumacher et al., 1978), with complete retention of morphological and biochemical integrity. Murine polymorphonuclear neutrophils have also been enriched by centrifugation on a self-generated continuous density gradient of Percoll (Watt et al., 1979). Enrichment procedures using Percoll have also been applied to the purification of parietal cells from gastric mucosal cell preparations. Sonnenberg et al., (1979), achieved an enriched parietal cell fraction by centrifugation in an iso-osmotic Percoll solution of density 1.03g/ml. After centrifugation, the parietal cells remained in suspension, and the more dense non-parietal cells were concentrated in the pellet. Ecknauer et al., (1981), utilized a discontinuous Percoll gradient consisting of layers of 10%, 20% and 30% Percoll. The gastric cell preparation was layered on top of the discontinuous gradient and after centrifugation an enriched parietal cell fraction banded at the 10%/20% Percoll interface.

2.1.4 Assessment of the viability of isolated parietal cells.

2.1.4.1 Dye exclusion.

The dye exclusion viability test was performed rountinely throughout this study and its methodology is simple. Microscopic examination reveals the ability of cells to exclude the dye, trypan blue (mwt. 961da), thereby giving an indication of the integrity of the cell membrane. It should be noted that the dye exclusion test only gives an indication of the structural integrity of the cell membrane and several factors such as inadequate cell dispersion in the dye, or insufficient duration of cell-dye

contact may result in artificially elevated viability readings (Elliot, 1979). Alternatively, such high viability readings may be obtained in the presence of bovine serum albumin which can bind trypan blue (Seglen, 1976). However, the concentration of bovine serum albumin routinely used in the incubation medium in this work was only 0.1%.

2.1.4.2 Cellular respiration.

Since acid secretion and respiration are closely linked (Soll, 1978a), it is not surprising that oxygen consumption and/or carbon dioxide production can be used to assess the metabolic integrity of the acid-secreting parietal cell. The increase in oxygen consumption by an enriched isolated parietal cell preparation stimulated with secretagogues has been interpreted as evidence of good cellular viability, and the presence of functional receptors on the cell surface (Soll, 1978a). This increase in cellular respiration can also be used as an index of cell responsiveness. Care must be taken when using changes in respiration as the sole index of responsiveness to secretagogues, since when using unenriched cell preparations it is not possible to assign the changes in oxygen consumption or carbon dioxide production to a single cell-type.

2.1.4.3 Aminopyrine accumulation.

The accumulation of aminopyrine within the acidic spaces inside the parietal cell, serves as a simple and reliable index of their response to secretagogues and can therefore be used to assess cellular responsiveness as well

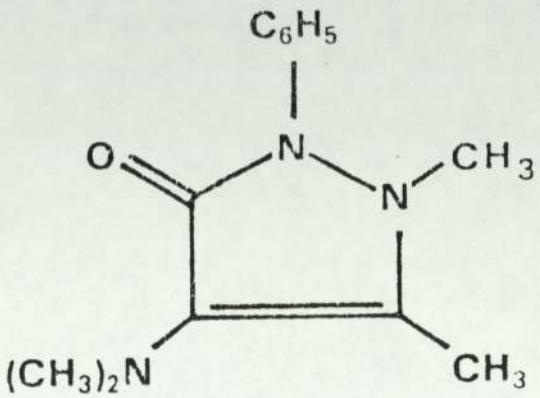
as the metabolic integrity of the cell. Berglindh et al., (1976), demonstrated that viable rabbit gastric glands accumulated [^{14}C] aminopyrine and subsequent work (Berglindh et al., 1980b), indicated that the radio-labelled aminopyrine was actually accummulated within the parietal cell.

Aminopyrine is a weak hetrocyclic base of pKa = 5.0, which exists almost entirely in an unionized form at physiological pH (approx. 7.4). It is able to diffuse across the parietal cell plasma membrane into the acidic spaces of the tubulovesicles and secretory canaliculi where it becomes ionized and trapped (fig 2.1). Aminopyrine accumulation is not a measure of the quantity of acid secreted but rather an indicator of the average intra-cellular pH (fig 2.1) which will be determined by the amount of acid sequestered within the parietal cell. As a result, aminopyrine accumulation data serves as an indirect index of acid secretion and may not entirely correspond with other more direct indices of acid secretion, such as measurements of oxygen consumption. Soll (1980a), indicated that aminopyrine accumulation may be a more sensitive index of acid secretion than oxygen consumption.

Aminopyrine accumulation may be expressed in a variety of ways, although probably the most universal is the aminopyrine accumulation ratio used by Soll (1981b). The aminopyrine accumulation ratio is the concentration of intracellular aminopyrine divided by the concentration of aminopyrine in the medium, and this is related to the mean intracellular pH (fig 2.1). Alternatively, aminopyrine accumulation data may be expressed as aminopyrine accumulation per 10^6 cells (Ecknauer et al., 1981), although this method is inferior because it depends on the concentration of aminopyrine in the medium.

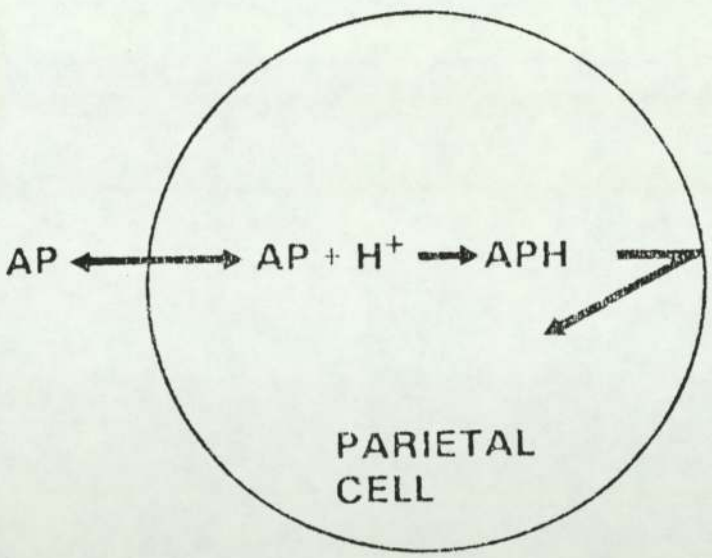
Fig 2.1 Intracellular aminopyrine (AP) accumulation as an indirect index of acid secretion by parietal cells.

Aminopyrine



pKa = 5

WEAK BASE



PROTONATED FORM OF AMINOPYRINE IS TRAPPED INSIDE THE CELL

$$\text{AMINOPYRINE RATIO} = \frac{[AP]\ \text{cell}}{[AP]\ \text{medium}} = \frac{1+10^{(pKa-\ pH\ \text{cell})}}{1+10^{(pKa-pH\ \text{med})}}$$

2.1.4.4 Morphological transformation.

Upon stimulation by secretagogues the parietal cell undergoes characteristic ultrastructural changes (chapter 1), which can be used to assess the structural integrity and responsiveness of the parietal cell. Berglindh et al., (1976), studied parietal cell morphology by electron microscopy to assess the secretory activity of their isolated rabbit gastric gland preparation. In addition, Soll et al., (1976), utilized the morphological transformations associated with stimulation of parietal cell secretion as an index of secretory activity. However, this technique is not suitable for routine use as an assessment of secretory activity since equivalent results can be obtained by simpler and less time-consuming procedures.

2.2 METHODOLOGY.

2.2.1 Routine preparation of an everted stomach sac.

Rats were anaesthetized with an intraperitoneal injection of sodium pentabarbitone (Sagatal; 60mg/Kg body weight). A mid-line incision was made to expose the stomach and the oesophagus was ligated. The stomach was removed by cutting above this ligature and by transecting the duodenum. Everted stomach sacs were prepared using the method of Dikstein and Sulman (1965), with some modifications as follows:

The stomach was rinsed in ice-cold saline (NaCl, 9g/l) and a ligature was tied between the antrum and the fundus. This boundary was not easily discernable but was judged to be one-third of the distance between the pylorus and the pale non-glandular region. The antral region and the proximal part of the duodenum were then removed. An incision was made in the non-glandular region opposite the ligature and a glass rod was pushed through from the antral end thereby everting the stomach. The interior of the fundic wall (now exteriorized) was rinsed in ice-cold saline, and another ligature was tied across the open end of the stomach. The resulting sac was injected with medium A (table 2.1) containing pronase (1000 PUK units/ml) until it was well-inflated.

Table2.1 Additions to Eagle's minimum essential medium

Medium	Additions
A	EDTA(2mM) + Soybean trypsin inhibitor(0.1mg/ml) + Dextran(30mg/ml)
B	Bovine serum albumin, fraction V (30mg/ml)
B´	Bovine serum albumin, fraction V (1mg/ml)
C	EGTA (3.85mM) + Dithiothreitol (0.5mM) + Bovine serum albumin, fraction V (1mg/ml)

2.2.2 Preparation of isolated parietal cells.

Parietal cells were isolated using a method similar to that of Trotman and Greenwell, (1979) which is a modification of the method of Lewin *et al.*, (1974). The everted stomach sac was incubated for 30 minutes in 40ml of medium A (table 2.1) at 37^{0}C with constant shaking (84 cycles/min), and continuous gassing (95% O_2 : 5% CO_2). After digestion, the sacs were removed from medium A, blotted, and added to 20ml of medium B (table 2.1) in a covered plastic beaker. The space above the medium was gassed continuously, and the sacs were stirred using a magnetic stirrer at room temperature for 30 minutes to remove the cells. The sacs were then returned to 40ml of fresh medium A and the cycle repeated two more times. The cells released into medium B were harvested by filtering the medium through nylon mesh (195µm pore size, Sericol Group Limited, London, UK), into plastic centrifuge tubes and centrifuging for 10 minutes at 100g at 15^{0}C. The pellet containing the parietal cells was resuspended in fresh medium B, and stored at 37^{0}C with constant gassing above the medium, and shaking (as above). After the completion of three cycles, the pooled parietal cell suspension was centrifuged at 100g at 15^{0}C for 10 minutes and the cells resuspended in the required volume of experimental medium [Krebs-Ringer bicarbonate medium containing dialysed fatty acid-free bovine serum albumin (3g/100ml, appendix A2.1) for the work on the substrate-dependency of acid secretion, but medium B′ (table 2.1) for all other work]. For the substrate-dependency work an additional wash in Krebs-Ringer bicarbonate medium containing bovine serum albumin (0.1g/100ml) essentially free of fatty acids was incorporated into the isolation procedure before the final resuspension.

The protocol used routinely in this study differs from the method of Trotman and Greenwell, (1979) in the use of Eagle's minimum essential medium (1.8mM-Ca^{++}, appendix A2.2) rather than minimum essential medium for suspension cultures (Ca^{++}-free) as the basis for the medium in which the stomach is incubated, and in the addition of soybean trypsin inhibitor to this medium. Trotman and Greenwell, (1979) also appeared to incubate the stomach sacs continuously for 90 minutes in a Ca^{++}-free medium which was changed every 30 minutes. In this study each 30 minute incubation in medium A (Ca^{++} -free) is separated by a 30 minute period in medium B (1.8mM-Ca^{++}).

2.2.3 Routine preparation of an enriched parietal cell suspension.

The parietal cells were purified by isopycnic centrifugation in Percoll as described by Sonnenberg et al., (1979). A stock Percoll solution was prepared by adding 4.5ml of Percoll to 0.5ml of 10xconcentrated-Eagle's minimum essential medium. Sodium bicarbonate was added to this stock solution to produce a final concentration of 26mM. The solution was gassed for 5 minutes with 95% O_2 : 5% CO_2 at room temperature and the pH adjusted to 7.4 with 3M-HCl, before 3ml was added to 7.0ml of the crude parietal cell suspension in medium B′ (table 2.1) to provide a cell suspension in 30% Percoll. This mixture was centrifuged at 2000g for 1 minute and the supernatant cell suspension added to 20ml of medium B′ . This diluted supernatant was centrifuged at 100g for 5 minutes to sediment all the cells present and the pellet resuspended in 5ml of medium B′ .The suspension was then centrifuged at 50g for 5 minutes and the pellet was resuspended in 5ml of medium B′ . This final step was repeated, with the final resuspension in 1.5ml of medium B′ being an enriched parietal cell preparation.

The Percoll centrifugation stage also yielded a pellet which was used as a parietal cell-depleted fraction in experiments on the cellular distribution of glutaminase (EC 3.5.1.2) (Section 3.2.4).

2.2.4 Preparation of a highly-enriched parietal cell fraction for the identification of endogenous protein kinase C substrates.

This novel method was developed to improve the yield and purity of the parietal cell fraction over that obtained with the method described above. A gastric cell suspension (2.2.2) was centrifuged at 100g for 10 minutes and the particulate fraction containing the parietal cells was resuspended in 4ml of oxygenated medium C (table 2.1) to give a final cell concentration of 5×10^6 cells/ml. This cell suspension was added to 0.5ml of medium C and 3ml of oxygenated iso-osmotic percoll (9 parts Percoll : 1 part 10 x tissue culture medium), and the mixture centrifuged at 30,000g for 13 minutes at 4^0C. The less dense parietal cells formed a layer in the top 1.5ml of the centrifuge tube, corresponding to a density of approximately 1.03g/ml. This layer was pipetted off into another centrifuge tube and the total volume made up to 10ml with medium B′ (table 2.1). The cell suspension was centrifuged at 100g for 5 minutes and the pellet resuspended in 3ml of medium B′ . This suspension was split between two 1.5ml microfuge tubes and centrifuged at 10,000g for 30 seconds (Beckman microfuge). The final cell pellet contained on average $1.8 \times 10^7 \pm 0.3$ (5) cells of which $92.8 \pm 1.6\%$ (5) were parietal. The pellets were resuspended in the required volume of homogenization buffer [50mM-MES pH 7.0, 1mM-$MgCl_2$, 0.1mM-Dithiothreitol, 10mM-Benzamidine, 10mM-EGTA, leupeptin (1µg/ml), and pepstatin (1µg/ml)].

2.2.5 Aminopyrine accumulation.

1.2 - 1.5ml of an isolated parietal cell preparation (usually 2 - 5 x 10^6 cells/ml) in experimental medium [Krebs -Ringer bicarbonate medium containing dialysed fatty acid-free bovine serum albumin (3g/100ml, appendix A2.1) for the substrate-dependency work and medium B´ (table 2.1) for all other work], was added to siliconized 20ml glass scintillation vials (appendix A3) containing [^{14}C] aminopyrine, (0.1μCi/ml ; 0.9μM), [^{3}H] polyethyleneglycol (0.4μCi/ml) and where appropriate, secretagogues, substrates and inhibitors. The total volume in each vial was the same, since in control vials where test agents were not added, an equivalent volume of the appropriate solvent was added to compensate. Each vial was gassed with 95% O_2: 5% CO_2, capped and incubated for 30 minutes at 37^{0}C with continual shaking (120 cycles/min). Aminopyrine accumulation by stimulated parietal cells reached a plateau after 30 minutes of incubation (work of N. G. Anderson in Shaw *et al.*, 1985), and therefore the aminopyrine accumulation ratio was determined at this time.

After incubation, two 0.5ml aliquots were taken from each vial and centrifuged at 10,000g for 30 seconds (Beckman microfuge) in 1.5ml plastic tubes [L.I.P (Equipment and Services) Limited, Shipley, W. Yorks, England], to separate the cells from the media. From each aliquot, 50μl of the supernatant was added to a single scintillation vial, and each pellet was washed once without resuspension with medium B´ . The tip of each microfuge tube was then cut off and the pellet added to a separate scintillation vial. The contents of all scintillation vials were then dissolved in 1ml of protosol at 37^{0}C overnight. Econofluor (NEN, 10ml) was then added to each scintillation vial and the radioactivity of the samples determined by liquid scintillation counting (appendix A4).

Calculation of the aminopyrine accumulation ratio was determined using equation 2.2:

$$\text{Equation 2.2: Aminopyrine accumulation ratio} = \frac{A - (B \times \frac{C}{D})}{B} \times \frac{1}{E} .$$

where:

A = ^{14}C dpm in pellet fraction
B = ^{14}C dpm in 1µl of supernatant fraction
C = ^{3}H dpm in pellet fraction
D = ^{3}H dpm in 1µl of supernatant fraction
E = volume of intracellular fluid (µl).

The total ^{14}C dpm in the pellet fraction may be divided into actual intracellular aminopyrine and also that trapped in the extracellular fluid (ECF) which is associated with the pellet. The aminopyrine trapped in the ECF was corrected for by using [^{3}H] polyethyleneglycol (approx. mwt 4,000da), to determine the volume of the ECF since due to its high molecular weight it cannot enter the cells.

The intracellular fluid (ICF) volume can be calculated since it has been shown (Berglindh et al., 1976), that 2µl of ICF was equivalent to 1mg dry weight. The dry weight of the pellet was determined in duplicate by centrifuging known volumes of cell suspension (10,000g, Beckman microfuge), in 1.5ml plastic tubes [L.I.P. (Equipment and Services) Limited, Shipley, W. Yorks, England], cutting off the tip of the tubes containing the cell pellets and drying in an oven overnight at 90^{0}C. The pellets were weighed accurately using a microbalance (Mettler) and the measured weight corrected for the contribution of salts and albumin in the ECF associated with the cell pellet since medium B′ (table 2.1) contains 17.3mg of dissolved salts and albumin/ml.

2.2.6 Dye exclusion test and identification of parietal cells

An aliquot of the final cell suspension was well-mixed with an equal volume of trypan blue (4mg/ml) in saline (9g/l NaCl) and counted using a haemocytometer (E. Leitz, Wetzlar) by light microscopy. At least 150 cells were counted to ensure the validity of the technique, and only cells showing no dye uptake were considered intact and viable.

Parietal cells were identified by size and morphology (plate 1.1). Parietal cells usually have a diameter in excess of 12µm and are therefore the largest cell-type present in the preparation. They possess a large, centrally-located nucleus and the cytoplasm has a granulated appearance due to the large proportion of mitochondria. Indeed, mitochondria may occupy up to 40% of the cytoplasm in rat parietal cells (Helander, 1969).

2.3 RESULTS AND DISCUSSION.

2.3.1 Cell yield and dye exclusion.

2.3.1.1 Whole gastric mucosal cell preparation.

The mean yield of cells isolated per stomach by the method described in section 2.2.2 was 18.8 ± 1.5mg dry weight (10). The average total number of cells isolated per stomach was $7.5 \pm 0.9 \times 10^7$ (10), and the mean proportion of parietal cells was 16.9 ± 0.9% (10).

Using an isolation procedure similar to that employed in this study, Schepp et al., (1983a,b), isolated an equivalent number of cells from the rat stomach (8×10^7 cells/ stomach). However, a considerably reduced yield is achieved if the rat gastric glandular mucosa is incubated in medium containing 0.75% pronase (2.5×10^7 cells/ stomach; Dial et al., 1981). A lower yield of isolated cells than that achieved in this study was also obtained by collagenase digestion of the guinea pig fundic mucosa (3.3×10^7 cells/ stomach; Batzri and Dyer, 1981). Although collagenase digestion did produce a higher yield of isolated cells from the dog stomach (7×10^8 cells/ stomach; Soll 1978b), this improved yield must be balanced against the greater cost of purchasing and maintaining dogs.

The viability, as determined by trypan blue exclusion, of the whole cell preparation obtained in this study was on average 98.9 ± 0.2% (10). This is better than the viability of cells isolated by any of the procedures mentioned previously. The viability of the parietal cell fraction was found to be 97.3 ± 1.2% (10).

In general, it appears that sufficient, apparently viable cells can be isolated from the rat stomach by the method described in section 2.2.2 to enable investigation of the mechanisms of action of secretagogues and inhibitors. Cells isolated in this way may also provide suitable starting material for parietal cell enrichment.

2.3.1.2 Enriched and highly-enriched parietal cell preparations.

On average the enriched parietal cell fraction contained $2.6 \pm 0.3 \times 10^6$ cells/stomach (8) of which $74 \pm 2\%$ (8) were parietal cells and $92.6 \pm 1.2\%$ (8) excluded trypan blue. The recovery of parietal cells from the whole gastric mucosal cell preparation was $14.8 \pm 0.8\%$ (8). This parietal cell purity was improved upon by using the method described in section 2.2.4. Using this method, a highly enriched parietal cell fraction was obtained containing, on average, $1.8 \pm 0.3 \times 10^7$ cells (5) of which $92.8 \pm 1.6\%$ (5) were parietal, and $90.4 \pm 2.0\%$ (5) excluded trypan blue.

Using an elutriator rotor to produce an enriched parietal cell fraction from a dog gastric mucosal preparation, Soll (1978a) recovered between 10% and 25% of the parietal cells loaded, which is similar to the recovery of parietal cells in this study. Soll (1978a) ultimately obtained a preparation containing 85% parietal cells.

Schepp et al., (1983a), utilized isopycnic centrifugation in Percoll to obtain an enriched parietal cell fraction from the rat gastric mucosa. Using this technique a yield of 5×10^6 cells/stomach was achieved, consisting of between 65% and 85% parietal cells and of these 95% were

viable. Using a discontinuous Percoll gradient Ecknauer et al., (1981) recovered between 30% and 50% of the parietal cells present in a partially purified preparation of rat gastric mucosal cells, with an average parietal cell content of 70%. Up to 90% of the parietal cells present in the enriched fraction excluded trypan blue.

Theoretically it is possible that a cell-type of a similar density to the parietal cell may co-purify with the parietal cell when using isopycnic centrifugation techniques e.g. histamine containing cells of the canine fundic mucosa (Soll, 1981a). However, co-purification is prevented by a further velocity separation step (Ecknauer et al., 1981), which separates the larger parietal cells from other cell-types. This technique was employed in this study in the preparation of a highly enriched parietal cell preparation. This further separation step also serves to wash any Percoll from the cells.

Although Percoll is not cytotoxic (Pertoft et al., 1977), it is possible that parietal cell enrichment by centrifugation at 2,000g (2.2.3) may have caused some membrane damage, since the average viability of the cells present in the enriched fraction ($92.6 \pm 1.2\%$), as assessed by trypan blue exclusion, was significantly less than that of cells present in the original gastric mucosal cell preparation (unpaired t-test, $P < 0.001$). Despite this reduced viability the value obtained in this study is comparable with that of other workers using similar enrichment techniques (Schepp et al., 1983a, b). Further work on the mechanism of gastric acid secretion using these enrichment procedures should produce valid results which may be compared with those of other workers in this field.

2.3.2 Cell responsiveness and aminopyrine accumulation.

The suitability of isolated cells as a tool for the study of acid secretion can be assessed by their responsiveness to secretagogues. Parietal cells isolated (2.2.2) and enriched (2.2.3) from the rat gastric mucosa respond to secretagogues by the accumulation of [^{14}C] aminopyrine.

Assuming a pH of 1.2 for the intracellular spaces where aminopyrine accumulates (Sack and Spenny, 1982), a theoretical maximum aminopyrine accumulation ratio of 6300 may be attained. This theoretical aminopyrine accumulation ratio is never achieved, and possible explanations may be suggested:

1. Assay conditions are sub-optimal. Optimal assay conditions have been described by Sack and Spenny (1982), who demonstrated that the effective dispersion of the cell/gland preparation, and the geometry of the incubation vessel were of primary importance.

2. Diffusion of acid out of the secretory canaliculi down its concentration gradient, may also prevent achievement of the theoretical maximum aminopyrine accumulation ratio.

3. The isolation procedure may cause damage to the secretory mechanism, such that the theoretical maximum aminopyrine accumulation ratio is not attained.

4. The theoretical maximum aminopyrine accumulation ratio assumes that aminopyrine is accumulated by all the cell-types present in the preparation. This is not true since aminopyrine is only accumulated by parietal cells, which on average constitute only approximately 27% of the total cell volume in an unenriched preparation. In addition, aminopyrine is only accumulated within the secretory canaliculi and the tubulovesicles of the acid-secreting parietal cell, which constitute only approximately 50% of the parietal cell volume (Zalewsky and Moody, 1977). Using these volume corrections the theoretical maximum aminopyrine accumulation becomes 850. The maximum aminopyrine accumulation ratio obtained in this study with rat isolated parietal cells was 198. No correction was made for the intracellular volume not associated with acid secretion, since this made a constant and small contribution to the aminopyrine accumulation ratio for each treatment.

The aminopyrine accumulation test is a simple and sensitive index of acid secretion which has been used by many workers prior to this study. Table 2.2 compares aminopyrine accumulation by rat isolated parietal cells (2.2.2) with other preparations.

Table 2.2 Aminopyrine accumulation by several in vitro systems in response to stimulation with histamine

Author	Preparation	Secretagogue	Aminopyrine accumulation ratio		
			Basal	Stimulated	Enhancement
Present study	Rat isolated parietal cells	0.5mM-histamine + 0.1mM-IBMX	2.2 ± 0.4(7)	59.8 ± 17.5(7)	27.7
Schepp et al., (1983a)	Rat isolated parietal cells	1mM-histamine + 0.1mM-IBMX	1.35	6.53	4.8
Soll et al., (1980a)	Canine isolated parietal cells	10µM-histamine 10µM-IBMX	1.8 ± 0.3	44.7 ± 6.1	24.43
Chew, (1983) †	Rabbit isolated parietal cells	100µM -histamine	52	320	6.2
Dial et al., (1981)	Rat isolated parietal cells	0.5mM-histamine + 0.1mM-IBMX	3.2	12.6	3.9
Batzri and Dyer, (1981)	Guinea pig parietal cells	10µM-histamine + 100µM-Ro 20-1724	0.6	2.5	4.2
Berglindh (1985) †	Rabbit isolated parietal cells	10µM-histamine	75-169	380-1733	5-10

† Since a high basal tone was observed in both rabbit preparations this phenomenon appears to be species specific.

CHAPTER 3.

SUBSTRATE-DEPENDENCY OF AMINOPYRINE ACCUMULATION BY RAT PARIETAL CELLS.

3.1 INTRODUCTION.

3.1.1 In vitro preparations used to study substrate utilization by parietal cells.

In order to secrete sufficient quantities of H^+ against a million-fold concentration gradient, the parietal cell utilizes vast amounts of energy in the form of ATP (Hersey and Miller, 1981). The production of this energy from substrate metabolism is tightly linked to the acid-secretory process, since in mammalian preparations e.g. rabbit gastric glands (Hersey, 1981) and piglet gastric mucosa, (Forte et al., 1980), acid secretion is dependent on exogenous substrate, and acid production is inhibited by agents which inhibit oxidative metabolism such as amytal (Sachs et al., 1967).

The interaction between substrate metabolism and gastric acid secretion has been investigated using amphibian and mammalian stomach preparations in vitro. The use of such in vitro preparations enables metabolic studies to be carried out without interference from changes in blood flow which could alter substrate availability.

3.1.1.1 Amphibian stomach preparations.

Using two distinct in vitro frog gastric mucosal preparations, Alonso et al., (1967) demonstrated that this tissue preferred ketone bodies and fatty acids rather than glycolytic intermediates or glucose as substrates for acid secretion. Alonso et al., (1967) observed a significant

increase in oxygen consumption by a preparation of frog gastric mucosal cells stripped from the muscularis layer ($p < 0.01$), and a significant increase in acid secretion from a gastric mucosal preparation mounted as a diaphragm ($p < 0.01$), in the presence of these preferred substrates. Subsequent work (Hersey, 1977) using $^{14}CO_2$ production by a frog gastric mucosal preparation *in vitro* as an index of acid secretion, demonstrated that this tissue utilized 1mM-butyrate in preference to the same concentration of glucose or pyruvate. The β-oxidation of fatty acids is now known to play a crucial role in the production of energy for acid secretion in the frog gastric mucosa (Harris *et al.*, 1975). Chacin *et al.*, (1980) demonstrated that the toad gastric mucosa utilized octanoate (1mM) in preference to glucose (1mM) and pyruvate (1mM) when stimulated to secrete acid with histamine or theophylline. In the presence of 4-pentenoate (20mM), an inhibitor of β-oxidation, this acid-secretory response was considerably reduced, indicating that β-oxidation may be intimately connected with the mechanism of acid secretion in amphibians. In general, the amphibian systems used to study the role of substrate metabolism in gastric acid secretion, exhibit a preferential utilization of fatty acids rather than carbohydrates. However, the presence of endogenous substrates, which can support considerable rates of acid secretion despite the lack of added substrates, makes valid conclusions difficult using amphibian systems. This problem can be overcome by prolonged pre-incubation to deplete the endogenous supplies of substrate (Kasbekar, 1976). Using an endogenous substrate-depleted frog gastric mucosal preparation, Kasbekar (1976) confirmed the metabolic preference of this tissue for fatty acids rather than carbohydrates.

3.1.1.2 Mammalian stomach preparations.

Extrapolation of the results obtained using amphibian preparations to man is not possible since the feeding habits of amphibians and mammals are quite distinct, and the metabolic pathways present in each case may also vary. In fact, amphibians in general may often be kept for long periods without feeding and may therefore have become metabolically adapted to the efficient utilization of fats rather than carbohydrates. In addition, unlike mammalian stomach preparations, amphibian preparations can maintain a secretory response even in the absence of exogenous secretagogues. In the mammalian stomach, chief cells secrete pepsinogen and the parietal cells secrete acid, whereas in frogs there is a single oxyntic cell secreting both pepsinogen and acid. For these reasons, most of the recent work has been performed using *in vitro* mammalian preparations, although somewhat conflicting results have been obtained.

Hersey (1981), using substrate-depleted rabbit gastric glands found that short chain fatty acids were more effective than carbohydrates in restoring the responsiveness of this preparation to histamine, as assessed by changes in oxygen consumption. Although supranormal substrate concentrations were used in this study (10mM), the maximal effect was demonstrated at concentrations below 3mM. Subsequent work (Hersey *et al.*, 1982) has indicated that the primary role of glucose in rabbit gastric glands is to provide pyruvate for oxidation via the citric acid cycle. The increase in citric acid cycle activity, observed with the onset of acid secretion *in vivo* by measurements on parietal cell-enriched dog gastric biopsies (Sarau *et al.*, 1977), is consistent with the hypothesis proposed by Hersey *et al.*, (1982). In

addition, the increased activity of glycolysis also observed by Sarau et al., (1977) which may generate pyruvate equivalents for subsequent oxidation, is also in accord with the hypothesis proposed by Hersey et al., (1982). Glucose, L-glutamine and L-isoleucine metabolism in guinea-pig gastric glands were increased under acid-secreting conditions (Saladino et al., 1982), indicating that carbohydrates and amino acids may serve as important sources of metabolic energy for the acid-secretory response in this tissue, although the role of fatty acids was not investigated. Carbohydrates rather than fatty acids also seem to be the preferred exogenous substrate for the piglet gastric mucosa (Forte et al., 1980), although this metabolic preference may change with maturation (Wittels and Bressler, 1965).

Very few studies have been carried out to investigate the interaction between potential metabolic substrates [Forte et al., (1980) and Hersey, (1981)]. Such work could yield valuable information regarding the normal physiological situation, since in vivo the parietal cell must be exposed to a range of substrates. However, to date, these studies have involved only a limited number of substrate combinations, and often the gastric preparations have been exposed to these substrates at non-physiological concentrations. In particular, no experiments had been performed previously in which the fatty acids tested were of physiological chain length and bound to albumin as is the situation in the plasma.

The range of substrates to be tested in this study was derived from the results of other workers in vitro (see above) and the results of Anderson and Hanson (1983 a,b) in vivo, which suggested a possible role for glucose, D-3-hydroxybutyrate, L-glutamine, and branched-chain amino acids in the provision of energy for acid secretion. Even though the butyrate concentration of arterial blood is

negligible, this fatty acid was tested at a concentration of 0.25mM to enable comparison with other workers who have shown that butyrate can support acid secretion *in vitro* (Hersey *et al.*, 1982).

Throughout this section the aminopyrine accumulation within the acidic spaces of the parietal cell was used as an indirect index of secretory activity. The current work is the first to utilize parietal cells isolated from an adult omnivorous mammal, and to investigate the substrate-dependency of acid secretion using physiological concentrations. The aim of this work was to attempt to resolve the contradictions encountered in previous reports, and to answer the following questions:

(1) What are the substrates that the rat parietal cells can utilize, when present at physiological concentrations, as sources of energy for acid secretion?

(2) Do changes in substrate concentration affect their ability to support acid secretion?

(3) Does the presence of combinations of substrates at physiological concentrations result in a higher level of acid secretion than that obtained with single substrates?

In addition, an investigation of the distribution of glutaminase (EC 3.5.1.2) activity between parietal cells and other cells in the gastric mucosa was undertaken, since contrary to expectations induced by results *in vivo* (Anderson and Hanson, 1983a,b) L-glutamine did not appear to support acid secretion.

3.2 METHODOLOGY.

3.2.1 Incubation of cell suspensions.

A parietal cell suspension (approximately 7×10^6 cells/ml) was prepared (section 2.2.2) in Krebs-Ringer bicarbonate medium containing dialysed fatty acid-free bovine serum albumin (3g/100ml) (appendix A2.1). Aliquots (1.2ml-1.5ml) of this suspension were added to siliconized incubation vials (appendix A3) containing histamine (0.5mM), carbachol (0.1mM), IBMX (0.1mM), [^{14}C] aminopyrine (0.9μM; 0.1μCi/ml), [^{3}H] polyethyleneglycol (0.4μCi/ml), and where appropriate, the required concentration of substrate dissolved in a small volume of saline (NaCl, 9g/l). Each substrate (usually 30μl) was added to the incubation vials from a freshly-prepared concentrated stock solution. Oleate, when required, was first complexed with bovine serum albumin (appendix A5.1) before addition to the incubation vials, but was omitted from the experiments with higher substrate concentrations due to the increased viscosity of the stock solution (0.2M) and the consequent difficulty in forming a complex with albumin. A sodium acetoacetate stock solution was prepared (appendix A5.2) and assayed for purity (appendix A5.3) before use. The stock solution was stored at -20^0C between experiments. Each vial was gassed with 95%O_2 : 5%CO_2, capped and incubated at 37^0C with continual shaking (120 cycles/min) for 30 minutes, and the aminopyrine accumulation measured as described by Berglindh et al., (1976) (2.2.5).

3.2.2 Preparation of a gastric mucosal homogenate.

The stomach was removed from an anaesthetized rat (2.2.1), and the glandular region carefully dissected from the non-glandular region. The glandular region was then cut longitudinally to give a flat sheet, which was shaken vigourously in saline (9g/l NaCl) at 4^0C. The stomach sheet was placed on a glass plate over ice, blotted with filter paper and the oxyntic mucosa was scraped off using a scalpel blade. The oxyntic mucosa was carefully weighed and resuspended in a volume of 10 times its weight (g) of homogenization buffer (3.2.3) at 4^0C. The mucosal preparation was then homogenized at near maximal speed using an ultraturrax homogenizer (Janke and Kunkel) and assayed for glutaminase (3.2.4).

3.2.3 Preparation of parietal cell homogenates.

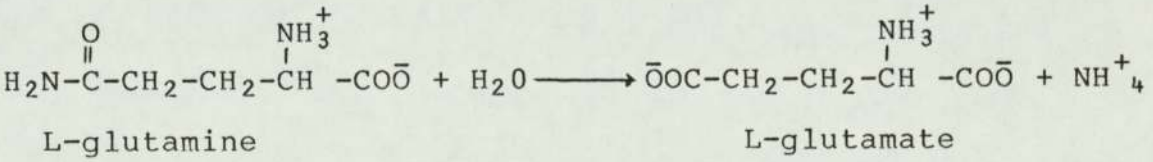
Both parietal cell-enriched and depleted fractions (2.2.3) were centrifuged at 10,000g for 30s (Beckman Microfuge). The parietal cell enriched fraction (B) was sonicated at 4^0C (MSE Soniprep, 4W, 6 x 5s) in a known volume of homogenization buffer [Tris (hydroxy-methyl) methylamine (Tris-HCl) (25mM), Sucrose (250mM), and Dithiothreitol (2mM) pH 7.6], and the parietal cell-depleted fraction (A) was treated similarly to produce an homogenate containing 100mg wet weight tissue/ml.

3.2.4 Assay of glutaminase.

A sample of homogenate (50µl) was added to 0.8ml of assay buffer [Tris-HCl (62.5mM), KH_2PO_4 (187.5mM) pH 8.1] and 0.2ml of glutamine (150mM), and was incubated for 30 minutes at 30^0C. After incubation, the reaction was stopped by the addition of 0.15ml of ice-cold perchloric acid (42% w/v). Standard tubes containing 50µl of glutamate (3mM), in lieu of glutamine, were added to the assay system, to which no sample was added, together with control tubes in which 50µl of cell fraction A was added to the assay system after the stage at which perchloric acid was added.

Control, standard and sample tubes were centrifuged (MSE. Chilspin) at 1600g for 5 min at 15^0C. A 1.0ml aliquot was taken from each tube and then added to 10µl of universal indicator. This mixture was continually stirred while 20µl aliquots of KOH (4M) were added to it until the colour indicated a pH between 5 and 8, and the volume of KOH required was noted. When the crystals of potassium perchlorate had settled, 0.8ml was added to a cuvette for the glutamate assay (3.2.5). All procedures after the addition of perchloric acid were carried out at 4^0C. The reaction catalysed by glutaminase is shown in reaction 3.1.

Reaction 3.1

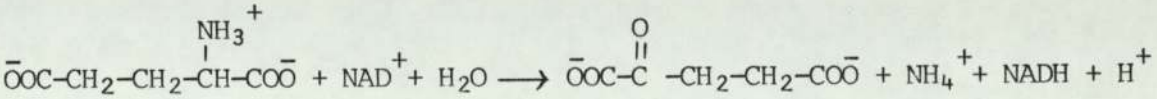


L-glutamine L-glutamate

3.2.5 Assay of glutamate.

Glutamate was assayed using the method of Bernt and Bergmeyer (1974) incorporating the reaction catalysed by glutamate dehydrogenase (EC1.4.1.3), reaction 3.2.

Reaction 3.2.



L-glutamate 2-oxoglutarate

A sample for assay (0.8ml) was added to a cuvette containing 1.0ml of hydrazine buffer [hydrazine hydrate (714mM), glycine (903mM) pH 9.0], 0.1ml of [NAD (18.9mM), ADP (7.2mM)], and 0.2ml of distilled water. The absorbance of each cuvette was measured at 340nm (Pye Unicam SP30 spectrophotometer), and 10µl of glutamate dehydrogenase (specific activity 1200U/ml) was added. The absorbance was noted every 15 minutes until no further changes occurred, or until the blank and sample cuvettes changed at the same rate. The glutamate concentration in the sample was calculated from the corrected change in absorbance and the extinction coefficient of NADH at 340nm (equation 3.1). Correction for the glutamate produced in the absence of homogenate was made on each occasion. The internal glutamate standard was used to check the functioning of the glutamate assay.

Equation 3.1:

$$\text{Glutamate Concentration } (\mu\text{moles/ml}) = \frac{\Delta A}{6.3} \times \frac{1.2}{0.05} \times \frac{2.11}{0.8} \times \left[\frac{1 + 0.01 + \text{Vol.KOH(ml)}}{1}\right]$$

3.2.6 Validation and optimization of the glutaminase assay.

In order to achieve maximum sensitivity of the glutaminase assay used routinely in this study (3.2.4), the effect of sonication, duration of incubation, and volume of homogenate added to the assay medium on subsequent glutamate formation were first investigated using a gastric mucosal homogenate. Sonication was necessary to disrupt cellular membranes and expose glutaminase to the assay buffer system, however, increasing the sonication power was shown to attenuate enzyme activity (fig 3.1). Therefore, in this study a level of sonication just sufficient to break cellular membranes was used (6 x 5s at 4 watts). This was determined by light microscopic examination following sonication. The levels of glutamate produced during the glutaminase assay were shown to be directly related to the duration of incubation (fig 3.2), and to the volume of homogenate added to the assay system (fig 3.3). For the purpose of this study a 50µl sample of homogenate was added to the assay system and incubation was for 30 minutes. Some workers (Watford et al., 1984; and McGivan et al., 1980) incorporate glutamine into the assay buffer, but degradation of glutamine to glutamate during storage led to high blanks, and this procedure was therefore not adopted. As an alternative, the assay system was prepared in two parts, [Tris-HCl, KH_2PO_4 pH 8.1, and glutamine (150mM)] which were added to the assay system separately.

Figure 3.1

The effect of the power of sonication on the glutaminase activity of a gastric mucosal homogenate (section 3.2.2). At each intensity the sample was exposed to sonication for six periods of five seconds (single experiment).

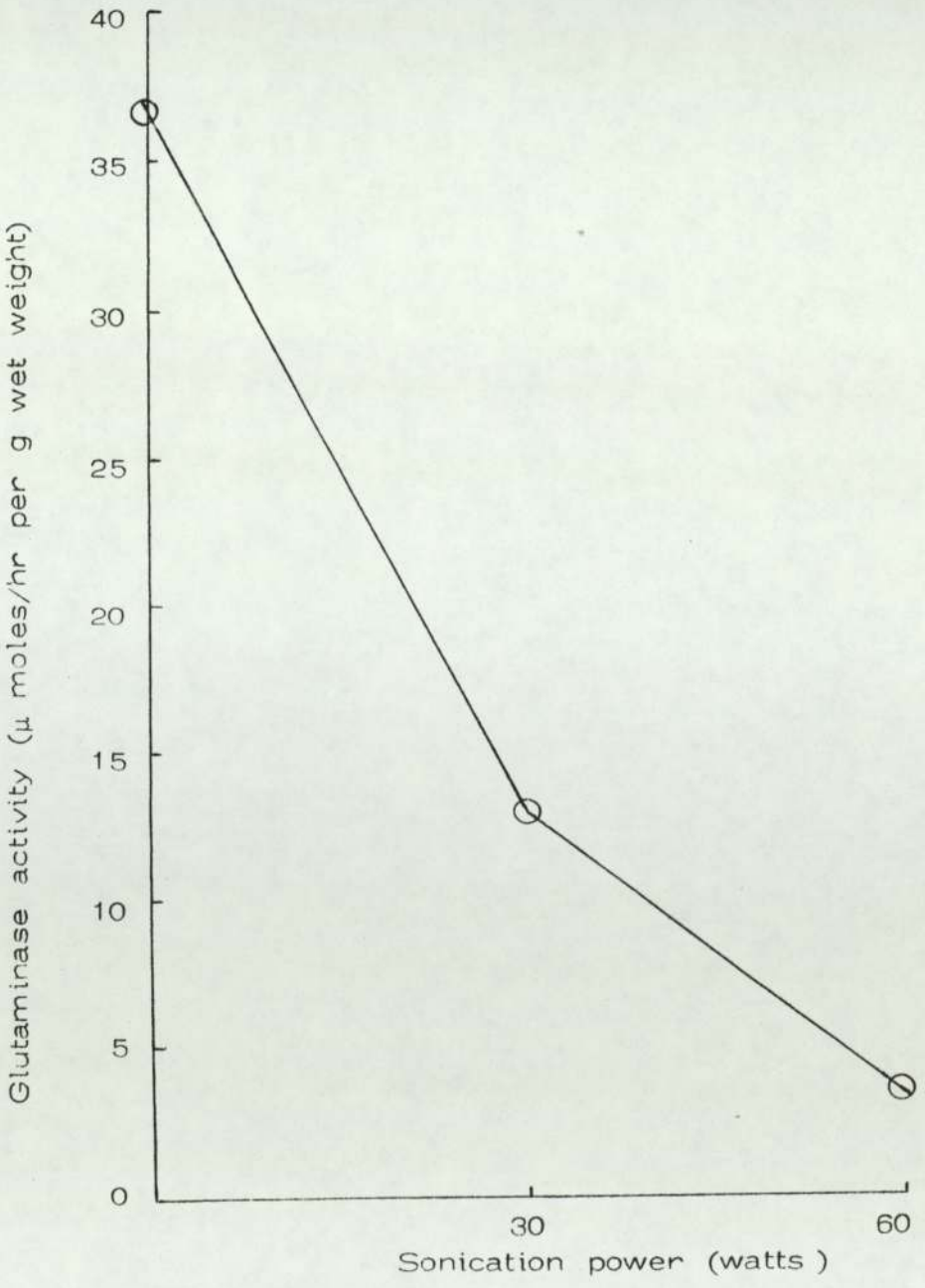


Figure 3.2

The effect of the duration of incubation on the amount of glutaminase activity in a gastric mucosal homogenate (single experiment). The equation of the straight line (not given) was determined by computer programme (linear regression analysis).

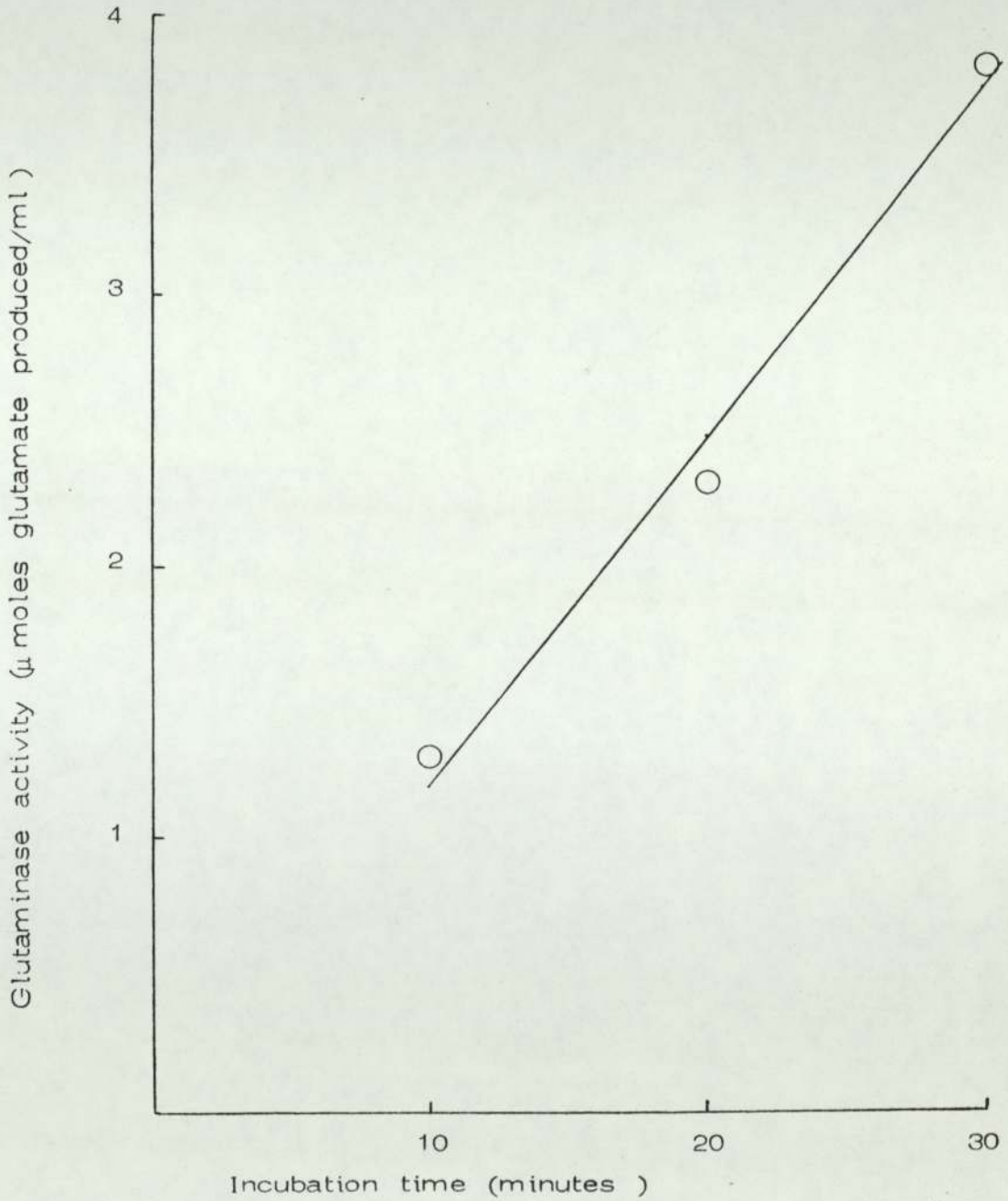
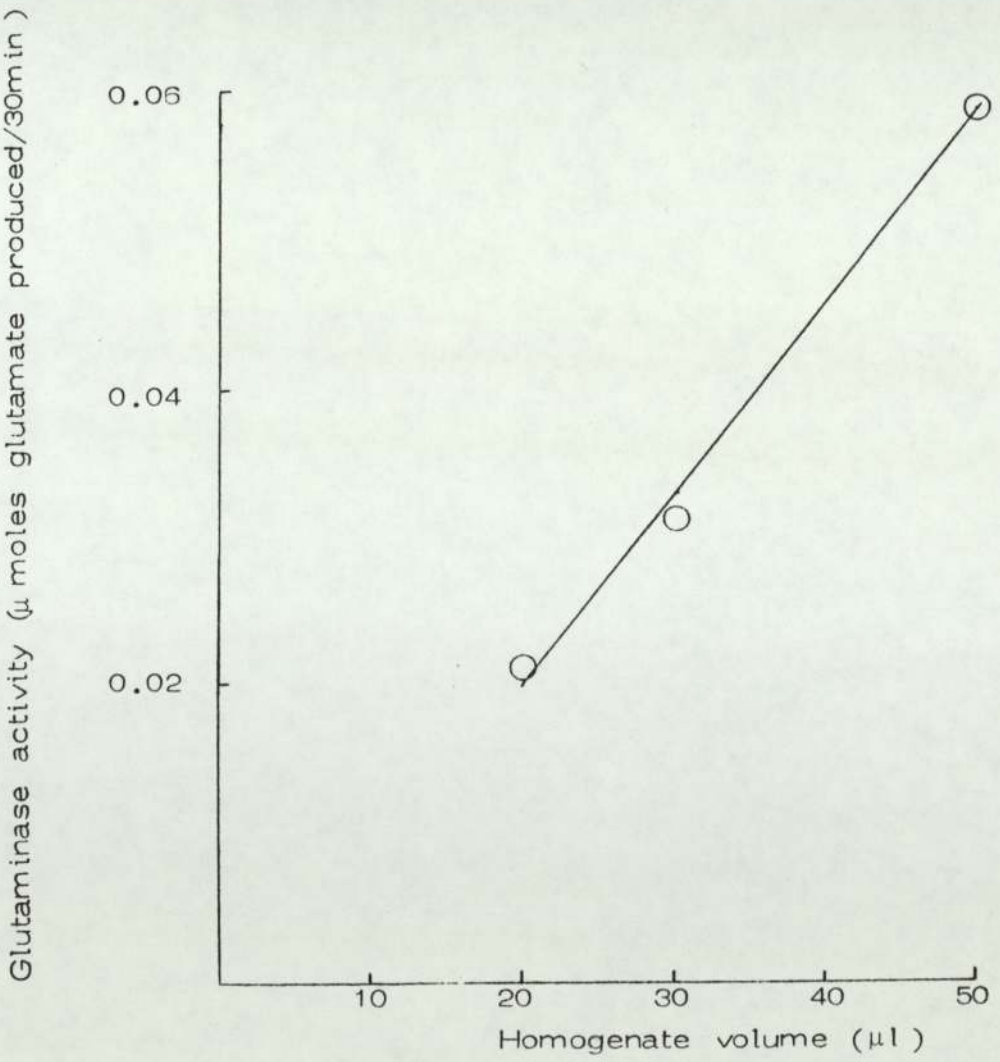


Figure 3.3

The effect of the volume of homogenate added to the assay medium on glutaminase activity (single experiment). The equation of the straight line (not given) was determined by linear regression.



3.2.7 Statistical treatment and presentation of data.

Statistical analysis of the aminopyrine accumulation data was performed by two-way analysis of variance followed by a multiple comparison test [Student Newman-Keuls multiple comparison test or Dunnett's test, Zar (1974)], thereby separating the effect of variable responsiveness between experiments, which can be considerable (table 3.1 and Soll, 1980a), from that due to substrates. Tukey's test of non-additivity (Snedecor and Cochran, 1976) was used to determine if a logarithmic transformation of the data was required before the analysis of variance was performed. Although the aminopyrine accumulation ratios are given for each treatment (e.g. table 3.1), presentation of the data was improved and comparison between different sets of experiments facilitated by normalization of the data (e.g. table 3.2). Such normalization procedures are commonly employed by others (see Soll, 1980a). Statistical tests were performed on the untransformed data.

3.3 RESULTS.

3.3.1 The effect of some potential substrates at physiological concentrations on aminopyrine accumulation by rat parietal cells.

Parietal cells incubated in the presence of glucose (5mM), oleate (0.6mM), butyrate (0.25mM), lactate (0.6mM), D-3-hydroxybutyrate (0.55mM), L-isoleucine (0.068mM), acetoacetate (0.35mM), and L-valine (0.117mM), and stimulated by secretagogues (table 3.1), accumulated aminopyrine to a significantly greater extent than cells incubated without exogenous substrate (Newman Keul's test; $P < 0.05$, table 3.2). However, parietal cells incubated in the presence of L-leucine (0.095mM), L-glutamine (0.5mM) and acetate (0.25mM) failed to accumulate aminopyrine significantly above the levels found in the absence of exogenous substrate. The aminopyrine accumulation ratio achieved in the presence of glucose (5mM) was shown to be consistently higher than that achieved in the presence of any other substrate or with no substrate (table 3.1).

3.3.2 The effect of changes in L-isoleucine and D-3-hydroxybutyrate concentration on aminopyrine accumulation by rat parietal cells.

The relationship between the concentration of L-isoleucine and aminopyrine accumulation by parietal cells (fig 3.4) fitted an equation of a form similar to the Michaelis-Menten equation, and $K_{0.5}$ was estimated (Wilkinson, 1961) to be 0.15 ± 0.002mM (n=4). There was an approximate linear relationship between D-3-hydroxybutyrate concentration and aminopyrine accumulation by parietal cells (fig 3.5) below 2mM. Further increases in D-3-

Table 3.1

The effect of some potential substrates at physiological concentrations on aminopyrine accumulation by rat parietal cells stimulated with histamine (0.5mM), carbachol (0.1mM) and IBMX (0.1mM).

Non-transformed data are given as aminopyrine accumulation ratios from 5 experiments with the transformed data given in table 3.2.

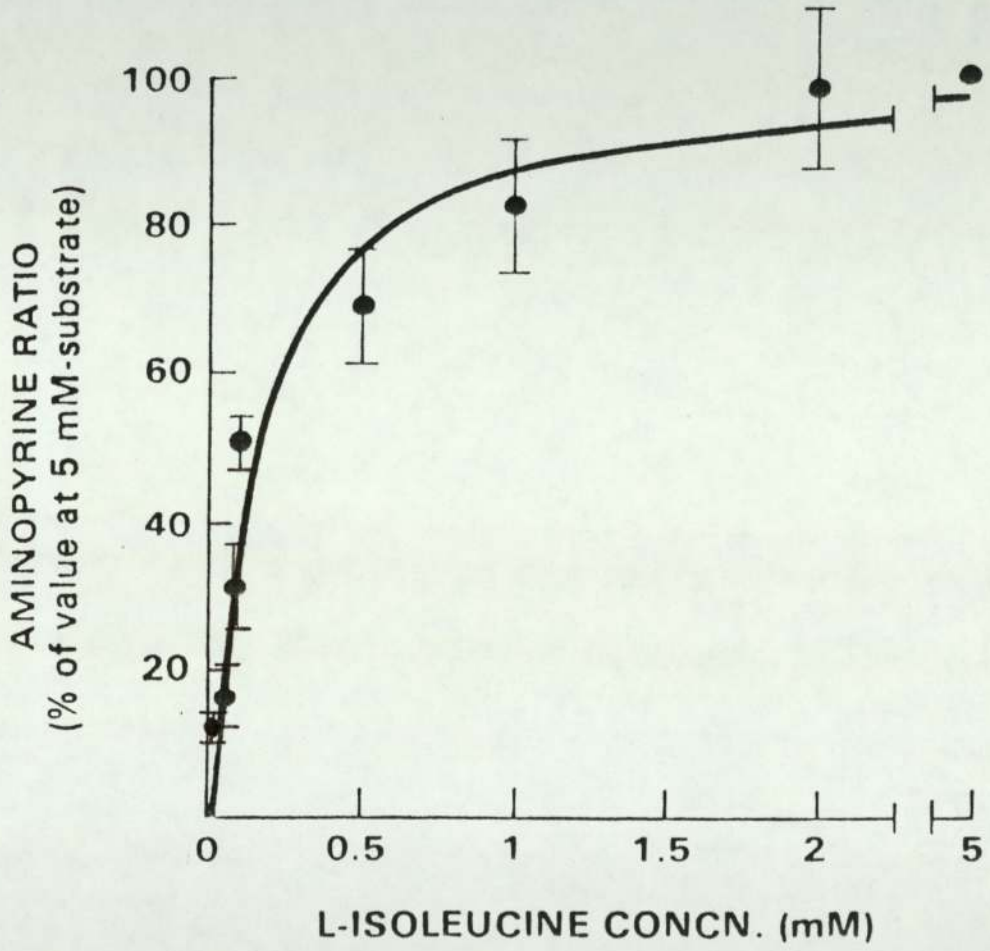
Substrate (mM)	Aminopyrine Accumulation Ratio					mean
	Experiment number					
	1	2	3	4	5	
Glucose (5)	31.1	19.5	18.6	18.8	27.3	23.0
Oleate (0.6)	29.1	15.3	16.8	12.6	17.0	18.1
Butyrate (0.25)	25.6	17.4	13.4	13.4	12.7	16.5
Lactate (0.6)	28.2	12.4	18.7	10.9	11.3	16.3
3-D-hydroxybutyrate (0.55)	29.6	13.2	15.9	9.1	10.6	15.6
L-isoleucine (0.068)	26.7	12.3	16.6	7.0	9.7	14.5
Acetoacetate (0.35)	26.8	9.1	11.1	9.3	8.8	13.0
L-Valine (0.117)	18.1	8.4	12.7	7.0	10.8	11.4
Acetate (0.25)	15.2	8.3	11.1	7.2	9.0	10.1
L-Leucine (0.095)	11.9	5.6	10.2	5.6	9.5	8.6
L-glutamine (0.5)	15.0	5.6	7.3	4.7	6.0	7.7
None	17.8	6.0	9.5	5.5	6.8	9.1

Table 3.2 Effect of physiological concentrations of potential substrates on accumulation of aminopyrine by rat parietal cells.

Exogenous substrate (mM)	Aminopyrine accumulation ratio (normalized)
Glucose(5)	100 **
Oleate(0.6)	78 ± 6** †
Butyrate(0.25)	72 ± 7** †
Lactate(0.6)	71 ± 11** ††
D-3-hydroxybutyrate(0.55)	67 ± 10** ††
L-isoleucine(0.068)	62 ± 11** ††
Acetoacetate(0.35)	55 ± 9* ††
L-valine(0.117)	49 ± 6* ††
Acetate(0.25)	44 ± 5 ††
L-leucine(0.095)	37 ± 5 ††
L-glutamine(0.5)	33 ± 5 ††
None	39 ± 6 ††

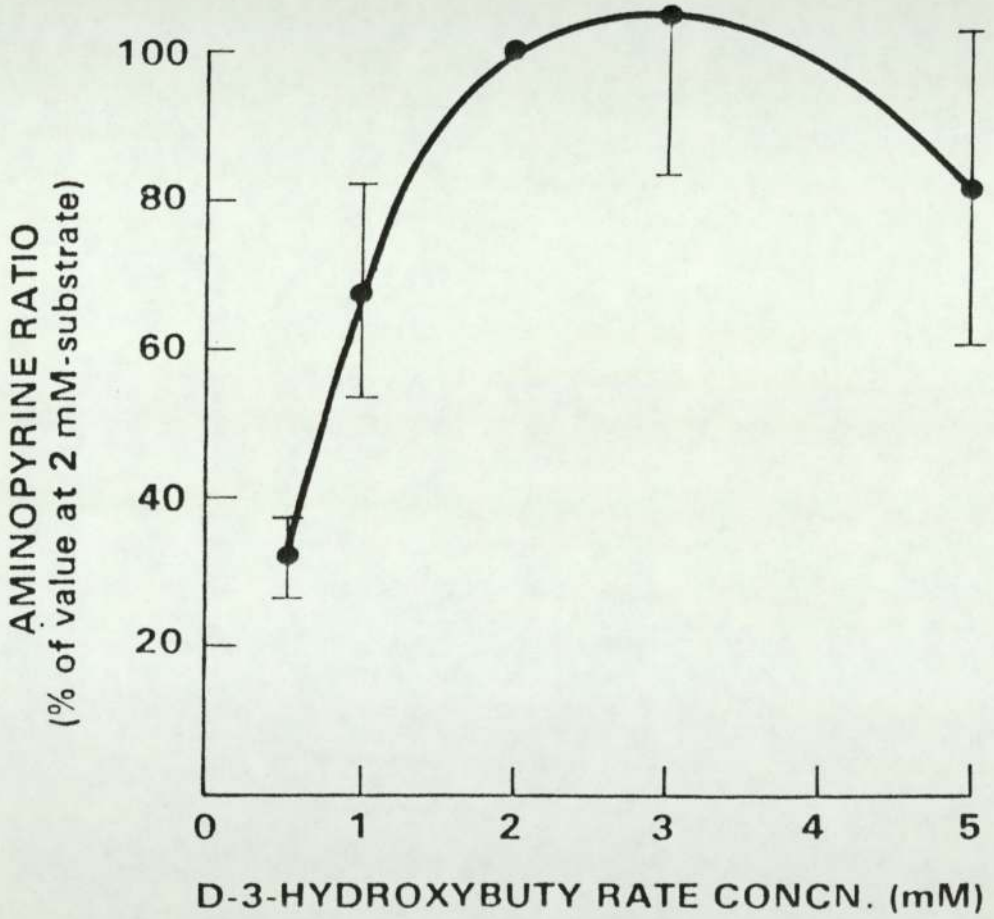
Results from 5 experiments were presented as mean ± S.E.M. and have been normalized by expressing the data from each experiment relative to the aminopyrine accumulation ratio obtained with glucose, which was made 100. The actual aminopyrine accumulation ratio obtained with 5mM-glucose was 23 ± 3. The concentrations of secretagogues are given in table 3.1 Significant differences from the result with no exogenous substrate are denoted: *$P<0.05$, **$P<0.01$; significant differences from the result with glucose are denoted: † $P<0.05$, †† $P<0.01$. Statistical analyses were performed by analysis of variance and follow-up procedures (Zar, 1974).

Fig 3.4 Effect of concentration of L-isoleucine on the accumulation of aminopyrine by rat parietal cells.



Data from 4 experiments have been expressed as a percentage of the aminopyrine accumulation ratio with L-isoleucine(5mM) which was 38 ± 14. Results are expressed as means ± S.E.M.
The concentration of secretagogues are given in table 3.1.
The graph was plotted using the method of Wilkinson (1961).

Fig 3.5 Effect of concentration of D-3-hydroxybutyrate on the accumulation of aminopyrine by rat parietal cells.



Data from 4 experiments have been expressed as a percentage of the aminopyrine accumulation ratio obtained with D-3-hydroxybutyrate (2mM) which was 22±9. Results are expressed as means ± S.E.M. The concentrations of secretagogues are given in table 3.1.

hydroxybutyrate concentration produced no further increase in aminopyrine accumulation.

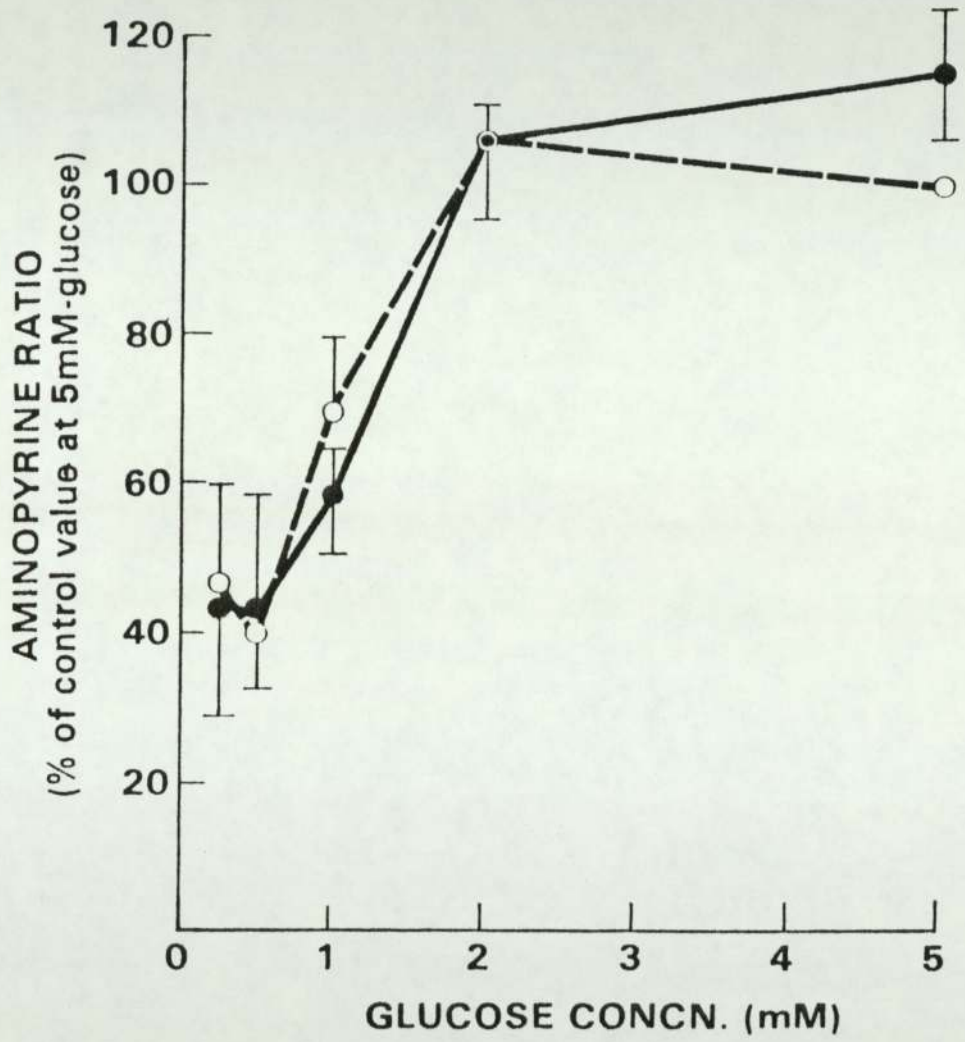
3.3.3 The effect of glucose concentration on aminopyrine accumulation by rat parietal cells in the presence and absence of insulin (100nM).

Increasing the concentration of glucose in the incubation medium served to enhance aminopyrine accumulation by parietal cells stimulated with histamine (0.5mM), IBMX (0.1mM) and carbachol (0.1mM). This effect of glucose concentration on aminopyrine accumulation was unaltered by the presence of 100nM-insulin (fig 3.6).

3.3.4 The effect of some potential substrates at supranormal concentrations on aminopyrine accumulation by isolated parietal cells.

The results from 5 experiments are given in table 3.3. This data was normalized as described in table 3.4 to facilitate comparison with other results. In the presence of L-isoleucine (5mM) and lactate (5mM) aminopyrine accumulation by parietal cells was significantly greater (Newman Keuls test, $P < 0.01$) than all other substrates tested at supraphysiological concentrations and no substrate (table 3.4). Glucose (5mM) was shown to be as effective as D-3-hydroxybutyrate (2mM), butyrate (5mM), acetate (5mM), L-valine (5mM), and acetoacetate (2mM), but more effective than L-leucine (5mM), L-glutamine (5mM), and no substrate at supporting aminopyrine accumulation. L-glutamine (5mM) did not stimulate aminopyrine accumulation above the level achieved in the absence of exogenous substrate.

Fig 3.6 Effect of glucose concentration in the presence and absence of insulin (100nM) on the accumulation of aminopyrine by rat parietal cells.



Data from 4 experiments have been normalized as a percentage of the aminopyrine accumulation ratio obtained with 5mM-glucose and no insulin, which was 46 ± 18. Results are presented as means ± S.E.M. O—O, no insulin added; ●—●, 100nM-insulin added. The concentration of secretagogues are given in section 3.3.3.

Table 3.3

The effect of some potential substrates at supranormal concentrations on aminopyrine accumulation by rat parietal cells stimulated with histamine (0.5mM), carbachol (0.1mM) and IBMX (0.1mM).

Non-transformed data are given as aminopyrine accumulation ratios from 5 experiments, with the transformed data given in table 3.4.

Substrate (mM)	Aminopyrine Accumulation Ratio					mean
	Experiment number					
	1	2	3	4	5	
Glucose (5)	8.4	16.6	12.3	25.0	65.0	25.5
Lactate (5)	39.5	49.1	16.2	71.0	98.1	54.8
L-isoleucine (5)	35.5	57.0	20.7	52.3	99.8	53.1
D-3-Hydroxybutyrate (2)	26.3	19.9	14.4	35.9	26.7	24.7
Butyrate (5)	18.9	15.5	11.3	15.7	16.3	15.5
Acetate (5)	20.4	14.3	12.7	8.1	23.0	15.7
Acetoacetate (2)	18.2	6.6	10.7	12.1	12.0	11.8
L-Valine (5)	9.5	16.3	9.0	19.7	21.1	15.1
L-leucine (5)	7.3	11.9	5.0	10.2	11.9	9.3
L-glutamine (5)	4.7	5.6	4.3	10.2	7.4	6.4
None	2.9	5.0	6.5	8.9	2.4	5.1

Table 3.4 The effect of substrates at supranormal concentrations on the accumulation of aminopyrine by rat parietal cells.

Substrate (mM)	Normalized aminopyrine accumulation ratio
Glucose(5)	100 **
Lactate(5)	266 ± 61** ††
L-isoleucine(5)	260 ± 53** ††
D-3-hydroxybutyrate(2)	147 ± 45**
Butyrate(5)	100 ± 34**
Acetate(5)	100 ± 38**
Acetoacetate(2)	82 ± 35**
L-valine(5)	79 ± 14**
L-leucine(5)	52 ± 12* †
L-glutamine(5)	35 ± 7 ††
None	31 ± 8 ††

Results from 5 experiments were presented as means ± S.E.M. The data was normalized as a percentage of the aminopyrine accumulation ratio obtained in the presence of 5mM-glucose alone. Significant differences from this value are denoted: † $P<0.05$, †† $P<0.01$, and significant differences from the result with no exogenous substrate are denoted: *$P<0.05$, **$P<0.01$, as determined by analysis of variance and follow-up procedures (Zar,1974). The concentration of secretagogues are given in table 3.3.

3.3.5 The effect of combinations of some potential substrates at physiological concentrations on aminopyrine accumulation by rat parietal cells.

Parietal cells incubated in the presence of combinations of potential substrates and stimulated with secretagogues (table 3.5) accumulated aminopyrine. Using this data, normalized as indicated in table 3.6, the following combinations of substrates were shown to give significantly higher aminopyrine accumulation ratios than that obtained with glucose (5mM) alone (Dunnetts test $P < 0.05$).

(I) Glucose(5mM) + L-isoleucine(0.068mM) + D-3-hydroxybutyrate(0.55mM)

(II) Glucose(5mM) + Lactate(0.6mM) + L-isoleucine (0.068mM)

(III) Glucose(5mM) + Lactate(0.6mM) + L-isoleucine (0.068mM) + D-3-hydroxybutyrate(0.55mM)

None of the combinations of substrates tested at physiological concentrations could consistently improve on the aminopyrine accumulation achieved in the presence of L-isoleucine (5mM).

Table 3.5

The effect of combinations of some potential substrates at physiological concentrations on aminopyrine accumulation by rat parietal cells stimulated with histamine(0.5mM), carbachol (0.1mM) and IBMX (0.1mM).

Non-transformed data are given as aminopyrine accumulation ratios from 8 experiments with the transformed data given in table 3.6.

Key: G = Glucose(5mM),
D-3-h = D-3-hydroxybutyrate(0.55mM),
Lac = Lactate(0.6mM),
L-ileu = L-isoleucine(0.068mM).

Substrate Combinations (mM)	Aminopyrine Accumulation Ratio Number of Experiments								mean
	1	2	3	4	5	6	7	8	
G	25.7	24.6	45.4	58.6	17.2	11.3	18.0	33.0	29.2
G + L-ileu	28.4	43.0	45.4	57.9	22.2	13.5	26.0	24.4	32.6
G + Lac	20.5	55.9	77.7	43.1	16.9	13.2	18.6	48.0	36.8
G + D-3-h	31.0	41.1	47.0	43.6	14.9	9.5	17.5	41.0	30.7
G + D-3-h + Lac	26.0	38.7	28.8	73.3	17.8	7.8	19.2	38.0	31.2
G + D-3-h+L-ileu	32.0	72.4	108.9	57.5	26.0	17.0	28.1	35.5	47.2
G + L-ileu + Lac	28.0	72.7	86.0	69.0	21.5	12.3	20.7	50.6	45.1
G + L-ileu + D-3-h + Lac	31.0	64.5	72.6	75.3	21.5	16.2	22.0	45.3	43.0
L-ileu (5)	33.2	101.0	181.8	97.4	30.8	12.8	23.5	46.8	65.9

Table 3.6 The effect of combinations of substrates at physiological concentrations on the accumulation of aminopyrine by isolated rat parietal cells

Substrate Concentrations(mM)	Aminopyrine Ratio as % Glucose alone
Glucose(5)	100
Glucose + L-isoleucine(0.068)	119 ± 11
Glucose + Lactate(0.6)	127 ± 18
Glucose + D-3-hydroxybutyrate(0.55)	107 ± 10
Glucose + D-3-hydroxybutyrate(0.55) + Lactate(0.6)	105 ± 11
Glucose + D-3-hydroxybutryate(0.55) + L-isoleucine(0.068)	165 ± 24**
Glucose + L-isoleucine(0.068) + Lactate(0.6)	152 ± 23*
Glucose + L-isoleucine(0.068) + Lactate(0.6) + D-3-hydroxybutyrate(0.55)	147 ± 15*
L-isoleucine(5)	209 ± 44**

The results were presented as means ± S.E.M. from 8 experiments.
Significant differences from the result with 5mM-glucose alone, determined by Dunnett's test (Zar, 1974), are denoted *, $P<0.05$, **, $P<0.01$.
The data were normalized by expression as a percentage of 5mM-glucose alone.
The concentrations of secretagogues are given in table 3.5.

3.3.6 The effect of L-glutamine (0.5mM) alone, and in combination with lactate (0.6mM) on the accumulation of aminopyrine by rat parietal cells exposed to glucose (5mM).

The addition of lactate (0.6mM) and L-glutamine (0.5mM), or L-glutamine (0.5mM) alone to parietal cells exposed to glucose (5mM) had no significant effect on aminopyrine accumulation. The data from 3 experiments are shown in table 3.7. This data was expressed as a percentage of the aminopyrine accumulation ratio obtained with glucose (5mM) alone (table 3.8) to facilitate comparison with previous results.

3.3.7 The effect of phenylpyruvate (2mM) and L-glutamine (5mM) on the accumulation of aminopyrine by rat parietal cells.

The data expressed as aminopyrine accumulation ratios from 4 experiments (table 3.9) was normalized as shown in table 3.10 to facilitate comparison with data obtained previously. Parietal cells incubated in the presence of phenylpyruvate (2mM) produced aminopyrine accumulation ratios which were significantly less than those cells incubated in the presence of L-glutamine (5mM) (Newman Keul's test, $P < 0.05$ and table 3.10). However, with the addition of both phenylpyruvate and L-glutamine in combination to the incubation medium, aminopyrine accumulation increased to a value significantly greater (Newman Keul's test, $P < 0.05$) than with phenylpyruvate alone, but not significantly different from that found with L-glutamine alone (table 3.10). Since the aminopyrine accumulation ratio in the presence of L-glutamine (5mM) was equivalent to that in the absence of exogenous substrate (tables 3.3 and 3.4), phenylpyruvate (2mM) can be said to

Table 3.7

The effect of glutamine (0.5mM) alone, and in combination with lactate (0.6mM) on aminopyrine accumulation by rat parietal cells exposed to glucose (5mM) and stimulated with histamine (0.5mM), carbachol (0.1mM) and IBMX (0.1mM).

Non-transformed data are given as aminopyrine accumulation ratios from 3 experiments with the transformed data given in table 3.8.

Key: G = Glucose(5mM), L-gln = L-glutamine(0.5mM), Lac = Lactate(0.6mM)

Substrate Combination	Aminopyrine Accumulation Ratio Experiment number			mean
	1	2	3	
G	58.6	17.2	11.2	29.0
G + L-gln	61.7	11.2	11.0	28.0
G + L-gln + Lac	55.7	12.7	12.7	27.0

Table 3.8 The effect of glutamine (0.5mM) alone, and in combination with lactate(0.6mM), on accumulation of aminopyrine by rat parietal cells exposed to glucose(5.0mM).

Exogenous Substrate(mM)	Normalized Aminopyrine Accumulation Ratio
Glucose(5)	100
Glucose(5) + L-glutamine(0.5)	89 ± 12
Glucose(5) + L-glutamine(0.5) + Lactate(0.6)	94 ± 11

The results were presented as mean ± S.E.M. from 3 experiments, and have been normalized so that the aminopyrine ratio obtained with glucose alone, 29 ± 15 = 100.

The concentrations of secretagogues are given in table 3.7

Table 3.9

The effect of phenylpyruvate (2mM) and L-glutamine (5mM) on aminopyrine accumulation by rat parietal cells stimulated with histamine (0.5mM), carbachol (0.1mM) and IBMX (0.1mM).

Non-transformed data are given as aminopyrine accumulation ratios for 4 experiments, with the transformed data given in table 3.10.

Key: L-gln = L-glutamine (5mM),
PP = Phenylpyruvate (2mM)

Substrate Combination	Aminopyrine Accumulation Ratio				Mean
	Experiment number				
	1	2	3	4	
L-gln	3.7	5.9	23.3	6.0	9.7
PP	0.9	2.8	11.3	4.5	4.9
L-gln + PP	1.6	4.9	21.4	5.8	8.4

Table 3.10 The effect of phenylpyruvate(2.0mM) and glutamine(5.0mM) on the accumulation of aminopyrine by rat parietal cells.

Exogenous Substrate(mM)	Normalized Aminopyrine Ratio
1. L-glutamine(5)	100
2. Phenylpyruvate(2)	48 ± 10*
3. L-glutamine(5) + Phenylpyruvate(2)	79 ± 12 †

Results were presented as mean ± S.E.M. from 4 experiments, and have been normalized so that the aminopyrine ratio obtained with glutamine alone, 9.7 ± 4.5 = 100.

For comparison of line 1 with line 2 *, indicates $P<0.05$.

For comparison of line 2 with line 3 †, indicates $P<0.05$, by analysis of variance and follow-up procedures (Zar, 1974).

have an inhibitory effect on aminopyrine accumulation. This effect of phenylpyruvate can be ameliorated by the addition of L-glutamine to the incubation medium.

3.3.8 The distribution of glutaminase activity between cell fractions isolated from rat fundic mucosa.

Glutaminase activity cannot be said to be concentrated within the parietal cell-enriched fraction of mucosal cells, since the production of an enriched parietal cell fraction (B) failed to generate glutaminase activity greater than that in a fraction depleted of parietal cells (A) (table 3.11). However, parietal cells do possess some glutaminase activity and this was calculated (3.3.9) using two equations derived from table 3.11 and assuming that all non-parietal cells have similar glutaminase activity, to be 58.8µmoles/ hr/g wet weight. This value is approximately 10% of the glutaminase activity in intestinal epithelial cells (Pinkus and Windmueller, 1977).

Table 3.11 Distribution of glutaminase activity between cell fractions isolated from the rat fundic mucosa.

Cell Fraction	Number of cell batches	Proportion of Parietal cells (%)	Glutaminase Activity (μmol/hr per 10^8 cells	Proportion of aggregated cell volume due to parietal cells	Glutaminase Activity (μmol/hr/g wet wt)
A	6	23.8 ± 2.8	8.3 ± 1.3	38.6 ± 3.7	90.4 ± 14.6
B	4	69.5 ± 1.2	9.2 ± 1.8	82.2 ± 0.7	68.2 ± 15.9

Results are presented as means ± S.E.M.

The preparation of the cell fractions and assay of glutaminase activity are described in sections 2.2.3 and 3.2.4.

Values of 14 and 11μm, for the average diameter of parietal and 'non-parietal' cells respectively, have been used to calculate cell volumes and enzyme activity expressed per g wet wt.

3.3.9 Determination of parietal cell glutaminase activity.

Total glutaminase activity =
(Proportion of cell volume due to parietal cells x Parietal cell activity) + (Proportion of cell volume due to non-parietal cells x non-parietal cell activity).

For fraction A.

$$90 = (0.39 \times \text{parietal cell activity}) + (0.61 \times \text{non-parietal cell activity}). \quad - (1)$$

For fraction B.

$$68 = (0.82 \times \text{parietal cell activity}) + (0.18 \times \text{non-parietal cell activity}). \quad - (2)$$

From (1) Non-parietal cell activity =

$$\frac{90 - (0.39 \times \text{parietal cell activity})}{0.61}$$

Substituting in (2)

$$68 = (0.82 \times \text{parietal cell activity}) + 0.18 \left[\frac{90 - (0.39 \times \text{parietal cell activity})}{0.61}\right]$$

$$68 = (0.82 \times \text{parietal cell activity}) + 26.56 - (0.115 \times \text{parietal cell activity})$$

$$41.44 = 0.705 \times \text{parietal cell activity}.$$

Parietal cell activity = 58.78µmole/hr/g. wet weight.

Non-parietal cell activity = 109.96µmole/hr/g. wet weight.

3.4 DISCUSSION.

3.4.1 Metabolism of potential metabolic substrates by rat parietal cells.

Throughout this study aminopyrine accumulation has been used as a reliable, yet indirect index of acid secretion (Soll, 1980a). It is assumed in this chapter that any effect of the substrates tested on aminopyrine accumulation reflects their ability to provide energy, by their metabolism, for the acid-secretory process. Non-substrate-depleted cells were used in this study, since substrate depletion was shown to have no effect on the relative importance of subsequently added substrates (Shaw *et al.*, 1985).

3.4.1.1 Glucose metabolism.

When present at physiological concentrations glucose alone was able to support aminopyrine accumulation to a greater extent than any fatty acid, ketone body or branched-chain amino acid tested (table 3.2). This observation is consistent with recent work by Anderson and Hanson (1983a) who demonstrated a significant uptake of glucose at physiological concentrations by the acid-secreting rat stomach *in vivo*, and Saladino *et al.*, (1982) who demonstrated significant amounts of CO_2 production by guinea pig gastric glands in the presence of 5mM-glucose. Any variation in the concentration of glucose within the physiological range (approx. 2-6mM) did not affect the aminopyrine accumulation (fig 3.6).

The parietal cell has the capacity to generate large amounts of energy, in the form of ATP, from the metabolism of glucose via glycolysis and the citric acid cycle. Two molecules of ATP are generated from the conversion of glucose to pyruvate in glycolysis, and thirty six molecules of ATP are generated as a result of citric acid cycle activity. Although the parietal cell is theoretically capable of generating large amounts of energy from the metabolism of exogenous glucose, this substrate alone has been shown to be insufficient to support maximal rates of acid secretion (table 3.4). Glucose may provide insufficient energy from its metabolism to achieve maximal rates of acid secretion due to its inability to traverse the parietal cell membrane. Alternatively, certain glycolytic or citric acid cycle enzymes may be of inherently low activity or may be inhibited in some way.

Since insulin (7nM) stimulates glucose transport into rat adipose cells by increasing the number of glucose carrier systems (Wardzala et al., 1978), 100nM-insulin was incorporated into the incubation medium in an attempt to enhance the aminopyrine accumulated by parietal cells in the presence of glucose. Insulin (100nM) was unable to improve aminopyrine accumulation by parietal cells in the presence of glucose (fig 3.6). This may be explained by; (i) the absence of insulin receptors on the parietal cell surface or the occupancy of such receptors by insulin having no effect on glucose transport, (ii) the removal/disruption of the insulin receptors during cell isolation, or (iii) the fact that the number of glucose transporting systems on the parietal cell surface may not limit glucose metabolism by the parietal cell.

Several key glycolytic enzymes have been demonstrated in various mucosal preparations of the gastrointestinal tract. Hanson and Carrington (1981) established hexokinase (EC 2.7.1.1) activity in the rat gastric mucosa, and Orwell et al., (1975) have shown pyruvate kinase (EC.2.7.1.40) activity in the fundic and antral gastric mucosa of gastric and duodenal ulcer patients. However, since acid secretion is highly sensitive to inhibition by anoxia (Fromm et al., 1975) it is probable that these glycolytic enzymes are of low maximal activity, thereby effectively limiting glucose metabolism.

Alternatively, glucose metabolism may be limited by inhibition of these glycolytic or citric acid cycle enzymes due to the metabolism of endogenous substrates, e.g. rabbit gastric glands can metabolize non-esterified fatty acids (Hersey, 1981), and the elevated citrate concentration resulting from this β-oxidation may limit glucose metabolism by allosteric inhibition of phosphofructokinase (EC 2.7. 1.11). Alternatively, the inhibition of pyruvate dehydrogenase (EC 1.2.4.1, EC 2.3.1.12, EC 1.6.4.3) by acetyl-CoA and NADH, which are also products of fatty acid metabolism, may also limit glucose metabolism. However, since in the absence of exogenous substrates aminopyrine accumulation by rat parietal cells is substantially reduced (table 3.2), the metabolism of endogenous substrates is probably minimal.

3.4.1.2 Metabolism of Lactate.

Although at a physiological concentration, lactate metabolism was able to support acid secretion in vitro (table 3.2), the acid-secretory rates obtained in the presence of glucose (5mM) were not achieved. By increasing the concentration to supraphysiological levels (5mM),

lactate metabolism was able to generate sufficient energy to support significantly higher (Newman Keuls test, $P < 0.01$) rates of acid secretion than glucose (table 3.4).

The ability of such supraphysiological levels of lactate to support acid secretion more effectively than glucose *in vitro*, is consistent with data obtained by Anderson and Hanson (1983a) who demonstrated a net uptake of lactate by the rat stomach *in vivo* only when the arterial lactate concentration was above 1.3mM [a concentration only normally achieved during exercise (Newsholme and Leech, 1983)]. However, since Anderson and Hanson (1983a) were unable to discriminate between lactate utilization by the individual cell types of the fundic mucosa it is possible that at physiological concentrations lactate uptake by parietal cells is masked by a greater lactate output by another cell type.

Lactate must be oxidized to pyruvate by lactate dehydrogenase (EC 1.1.1.27) before it can be metabolized, and the pyruvate produced is then converted to acetyl-CoA by the pyruvate dehydrogenase complex. There are two possible explanations why a supraphysiological concentration of lactate, metabolized in this way, could support acid secretion more effectively than a physiological concentration of glucose. 1) Glucose metabolism supplies insufficient pyruvate, probably due to a low glycolytic activity, to support maximal rates of acid secretion. The metabolism of 5mM-lactate serves to increase the supply of pyruvate for subsequent oxidation and therefore a greater rate of acid secretion could be achieved. 2) The elevated level of pyruvate resulting from the metabolism of 5mM-lactate may activate a pyruvate dehydrogenase phosphatase which converts pyruvate dehydrogenase to the active dephosphorylated form. In this way, pyruvate may be more efficiently oxidized and a greater level of acid secretion achieved.

3.4.1.3 Metabolism of fatty acids.

At the concentrations tested, the fatty acids oleate and butyrate are able to support levels of acid secretion significantly greater than those achieved in the absence of exogenous substrate (table 3.2). Oleate could support acid secretion *in vitro* at a concentration found normally in the blood. Butyrate was also able to support acid secretion, but only at a concentration which was considerably higher than that found physiologically (Remesey and Demigne, 1974). At a low concentration (0.25mM) the short-chain fatty acid, acetate, was unable to support acid-secretory rates significantly above basal levels, possibly due to the absence of a short-chain fatty acyl-CoA synthetase (EC 2.6.1.1) in parietal cells.

Increasing the concentration of acetate and butyrate to supraphysiological levels improved the ability of both fatty acids to support acid secretion to the level found with 5mM-glucose (table 3.4), thereby indicating that substrate availability was limiting metabolism. At supra-physiological levels acetate may have been activated by the medium chain fatty acyl-CoA synthetase, thereby facilitating metabolism at higher concentrations. However, butyrate (5mM) and acetate (5mM) were less effective than lactate (5mM) in supporting acid secretion, indicating that either β- oxidation was limited in some way or that when incubated in the presence of fatty acids alone, acetyl-CoA metabolism in parietal cells is limited by the availability of oxaloacetate.

3.4.1.4 Metabolism of ketone bodies.

At physiological concentrations D-3-hydroxybutyrate and acetoacetate were able to support rates of acid secretion significantly above basal levels (table 3.2). They may also function as respiratory fuels in the post-absorptive rat small intestine (Windmueller and Spaeth, 1978; Hanson and Parsons, 1978). However, neither of these ketone bodies at physiological concentrations was able to support acid secretion to the level achieved in the presence of 5mM-glucose (table 3.2).

The approximately linear relationship between the concentration of D-3-hydroxybutyrate and aminopyrine accumulation, up to 2mM, (fig 3.5) is consistent with passive diffusion limiting entry into the parietal cell. Similarly a linear relationship between D-3-hydroxybutyrate concentration (up to 1mM) and uptake by the pentagastrin-stimulated rat stomach *in vivo* has been demonstrated (Anderson and Hanson, 1983a). Increasing the concentration of both ketone bodies up to 2mM (table 3.4) improved their ability to support acid secretion to the level of glucose (5mM), although the level of acid secretion obtained in the presence of lactate (5mM) was not achieved. Any further increase in the concentration of D-3-hydroxybutyrate above 2-3mM reduced aminopyrine accumulation (fig 3.5), possibly due to an adverse affect on cellular metabolism derived from perturbation of the mitochondrial $\frac{NADH}{NAD}$ ratio.

Both *in vivo* and *in vitro* data indicate that the ability of ketone bodies to support acid secretion is concentration-dependent. The concentration of ketone bodies in the blood is elevated after an overnight fast [acetoacetate (0.35mM), D-3-hydroxybutyrate (0.55mM)] and during starvation. Therefore, the ability of ketone bodies

to provide energy for acid secretion may also be influenced by the nutritional status.

The degradation of ketone bodies to provide energy for acid secretion occurs in the mitochondrial matrix. D-3-hydroxybutyrate is oxidized to acetoacetate by D-3-hydroxybutyrate dehydrogenase (EC 1.1.1.30), and the acetoacetate produced is activated in a reaction catalysed by 3-oxo acid CoA-transferase (EC 2.8.3.5). The acetoacetyl-CoA generated is cleaved by acetoacetyl-CoA thiolase (EC 2.3.1.9) to produce two molecules of acetyl-CoA which enter the citric acid cycle. D-3-hydroxybutyrate dehydrogenase, and 3-oxo acid CoA-transferase activity have been demonstrated in the rat gastric mucosa (Hanson and Carrington, 1981). In fact, the activity of 3-oxo acid CoA-transferase in the glandular mucosa of the rat stomach was 2-4 times greater than in other regions of the gastrointestinal tract (Hanson and Carrington, 1981). Since supraphysiological concentrations of ketone bodies were unable to support acid secretion as effectively as 5mM-lactate (table 3.4) it is possible that the entry of acetyl-CoA (produced from ketone body metabolism) into the citric acid cycle is limited by the availability of oxaloacetate.

3.4.1.5 Metabolism of branched-chain amino acids.

At physiological concentrations L-isoleucine and L-valine were able to support acid secretion *in vitro*, while L-leucine was unable to significantly increase acid secretion above the level achieved without exogenous substrate (table 3.2). Although all the branched-chain amino acids are transported into the cell via the Na^+-independent system L (Guidotti *et al.*, 1978), a lower affinity of system L for L-isoleucine than for L-leucine

could account for its improved ability to support acid seretion. Thus a high Km for L-leucine transport such as the value of 3.2mM for transport into intestinal epithelial cells (Reiser and Christiansen, 1971) could account for the inability of this amino acid to support acid secretion *in vitro* at low concentrations. Increasing the concentration of L-isoleucine (fig 3.4) indicated that changes in plasma concentration would probably influence the ability of this branched-chain amino acid to support acid secretion *in vivo*. This is substantiated by the observation that branched-chain amino acids are removed from the circulation by the rat stomach *in vivo* at a rate dependent on the plasma concentration (Anderson and Hanson, 1983b). The high activity of branched-chain amino acid aminotransferase (EC 2.6.1.42) in the rat gastric glandular mucosa (Ichihara *et al.*, 1975) also indicates that this group of amino acids may be important in the provision of energy for acid secretion.

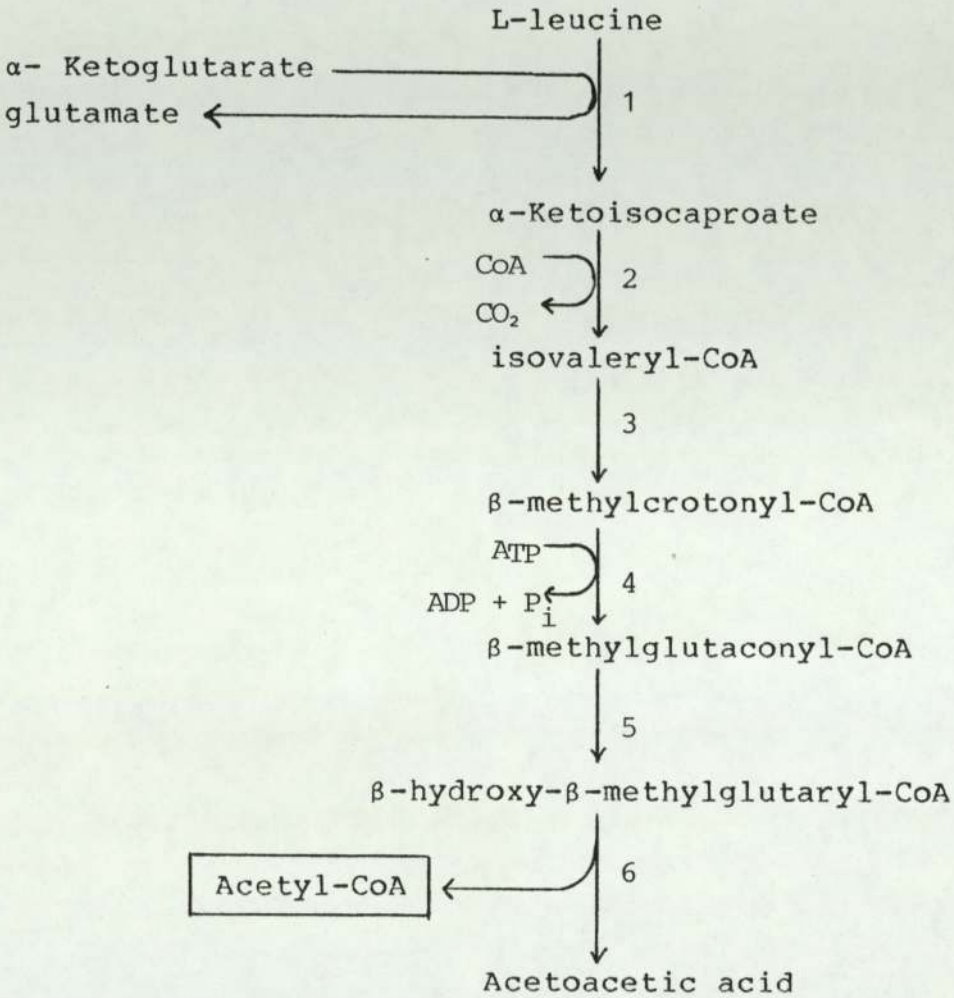
At supranormal concentrations the ability of branched-chain amino acids to support acid secretion was considerably enhanced, indicating that amino acid availability may have been limiting metabolism. In fact, 5mM-L-isoleucine was significantly more effective than glucose (5mM) at supporting acid secretion (table 3.4).

In addition to substrate availability limiting amino acid metabolism it is also possible that the variation in the ability of branched-chain amino acids to support acid secretion was due to differences in their degradative pathways. Thus L-leucine, a purely Ketogenic amino acid, is ultimately degraded to acetyl-CoA (fig 3.7) which can enter the citric acid cycle (fig 3.8). As low concentrations of L-leucine are unable to support acid secretion significantly above the level achieved in the absence of added substrates, the activity of at least one enzyme involved in its metabolism up to acetoacetate may

either be inhibited or of inherently low activity. L-valine is purely glucogenic and generates succinyl-CoA when metabolized (fig 3.9) which can also enter the citric acid cycle (fig 3.8). L-isoleucine is both ketogenic and glucogenic and produces succinyl-CoA and acetyl-CoA when metabolized (fig 3.10). The production of these two metabolites which can potentially generate large amounts of energy, may provide one explanation for the ability of 5mM-L-isoleucine to support a level of acid secretion higher than all the other substrates tested (table 3.4). In addition, several of the enzymes involved in L-isoleucine metabolism are also responsible for the β-oxidation of fatty acids and are therefore likely to be highly active. The observation that a supraphysiological concentration of L-isoleucine can support maximal rates of acid secretion has not been demonstrated previously in any stomach preparation. It appears that L-isoleucine and L-valine may contribute some energy from their metabolism to the acid-secreting parietal cell <u>in vivo</u> or else potentiate the utilization of endogenous fuels.

Fig 3.7

A possible pathway for the metabolism of L-leucine in the rat parietal cell.



Enzyme Key

1. L-leucine transaminase (EC 2.6.1.6)
2. α-Ketoisovaleric acid dehydrogenase (EC 1.2.1.25)
3. Acyl-CoA dehydrogenase (EC 1.3.99.3)*
4. Methylcrotonyl-CoA carboxylase (EC 6.4.1.4)
5. Methylglutaconyl-CoA hydratase (EC 4.2.1.18)
6. Hydroxymethylglutaryl-CoA lyase (EC 4.1.3.4)

* Enzymes involved in the β-oxidation of fatty acids.

Fig 3.8 Metabolism of branched-chain amino acids via the citric acid cycle.

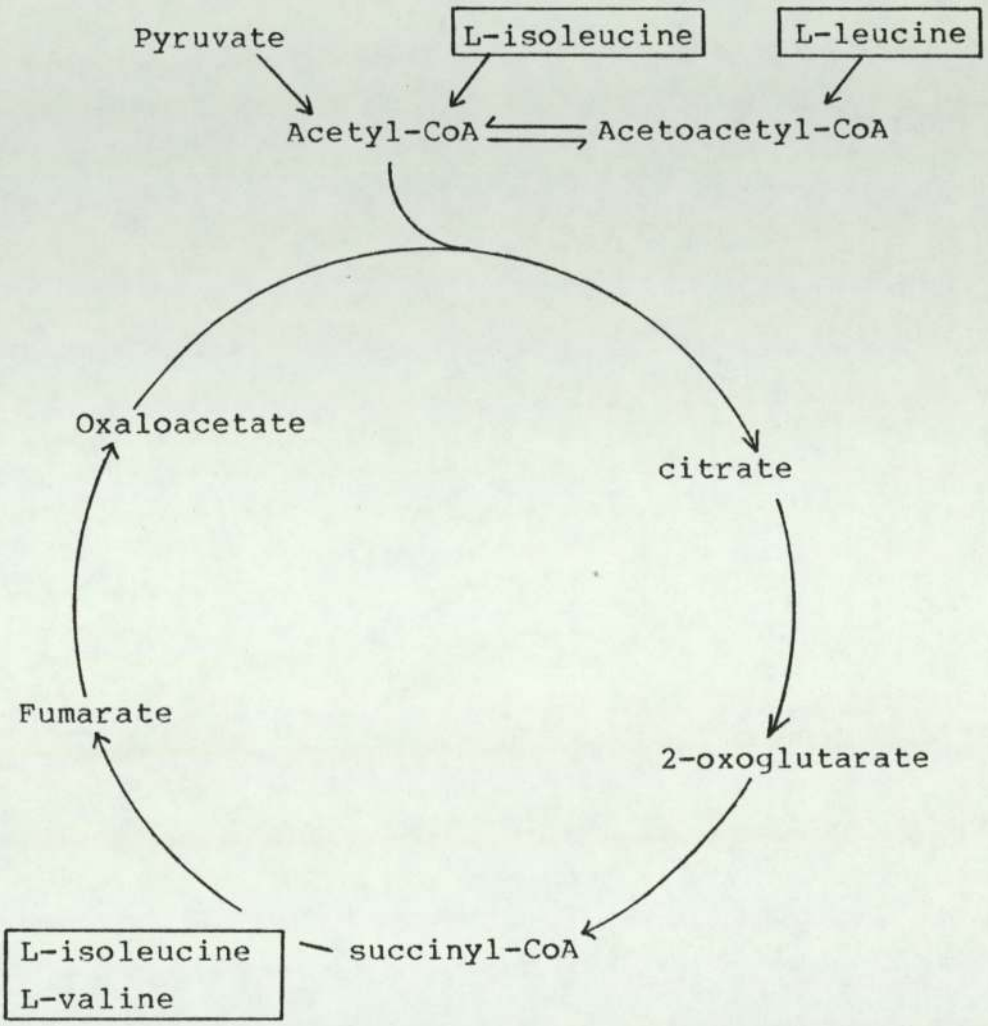
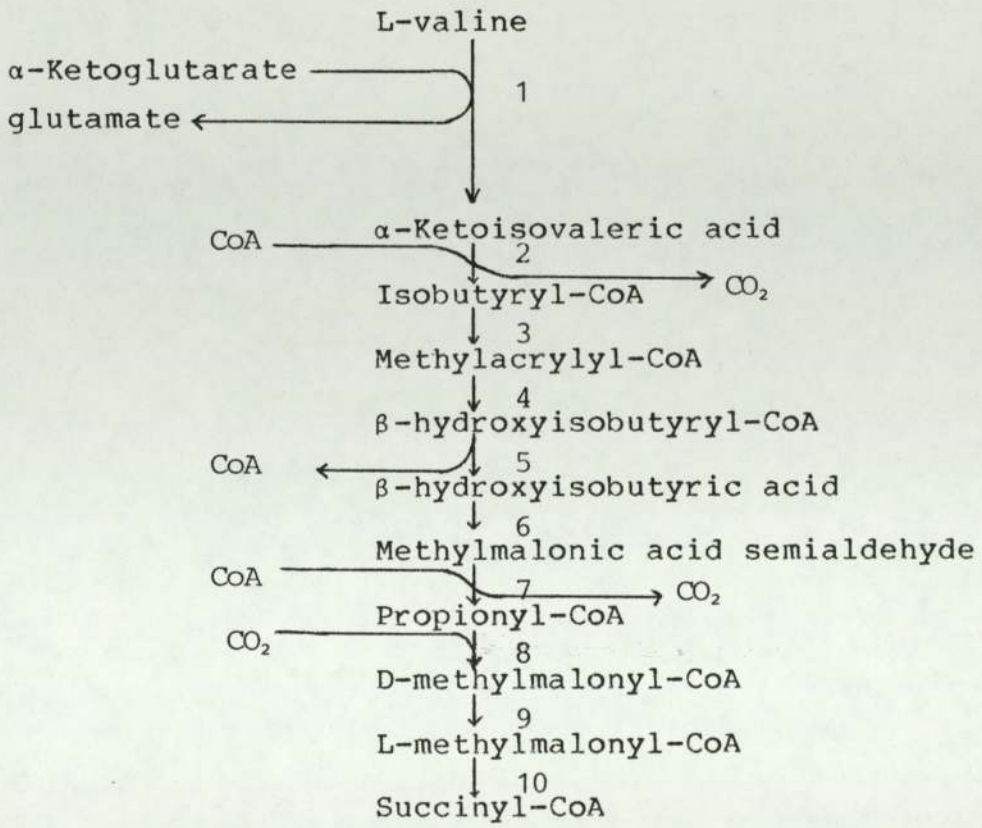


Fig3.9 A possible pathway for the metabolism of L-valine in the rat parietal cell.

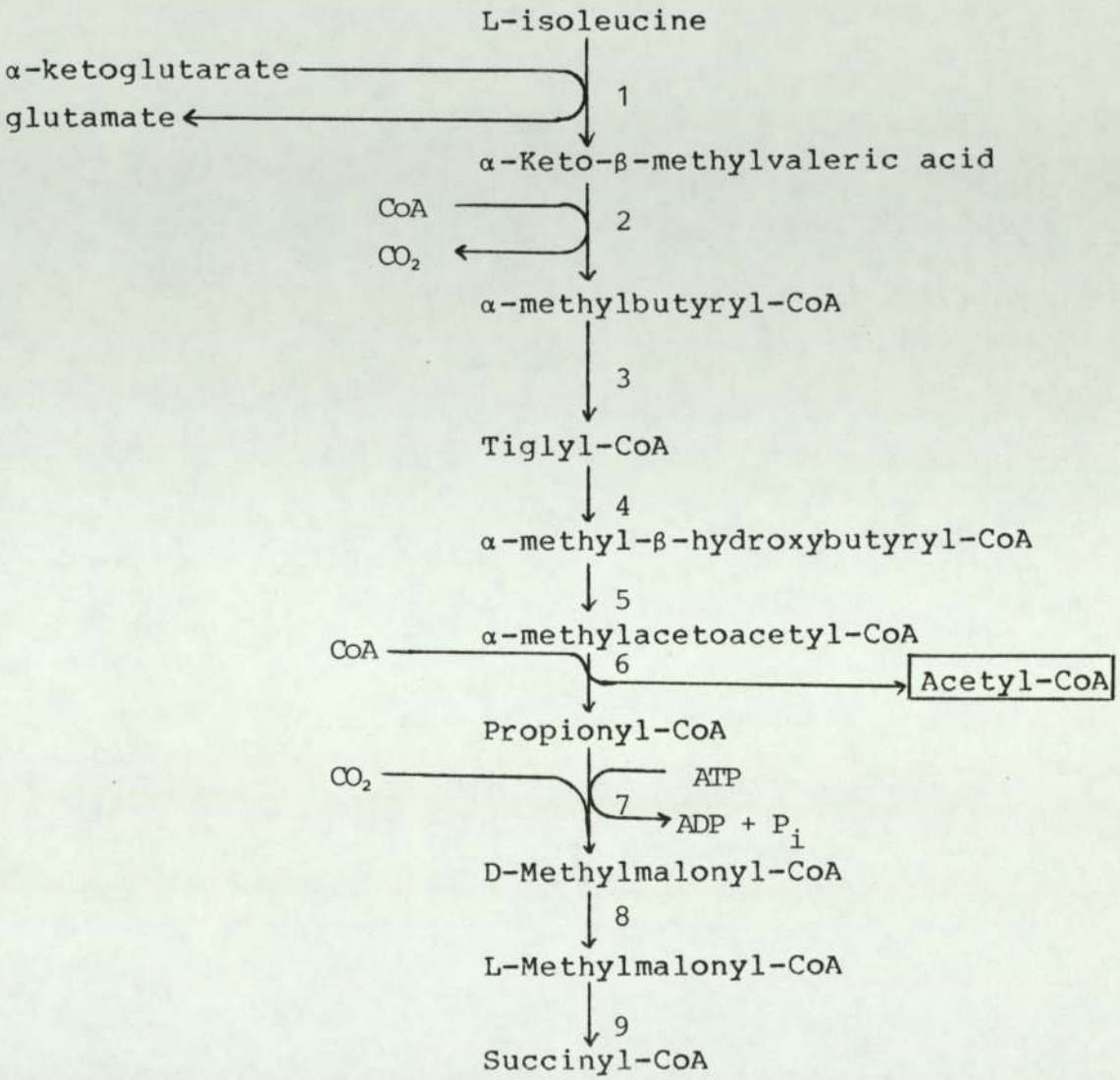


Enzyme Key

1. Branched-chain amino acid transaminase (EC 2.6.1.32)
2. α-Ketoisovaleric acid dehydrogenase (EC 1.2.1.25)
3. Acyl-CoA dehydrogenase (EC 1.3.99.3)*
4. Enoyl-CoA hydratase (EC 4.2.1.17)*
5. β-hydroxyisobutyryl-CoA hydrolase (EC 3.2.1.4)
6. β-hydroxyisobutyric acid dehydrogenase (EC 1.1.1.31)
7. Methylmalonic acid semialdehyde dehydrogenase (EC 1.2.1.27)
8. Propionyl-CoA carboxylase (EC 6.4.1.3)
9. Methylmalonyl-CoA racemase (EC 5.1.99.1)
10. Methylmalonyl-CoA mutase (EC 5.4.99.2)

* Enzymes also involved in the β-oxidation of fatty acids.

Fig 3.10 A possible pathway for the metabolism of L-isoleucine in the rat parietal cell.



Enzyme Key

1. Branched-chain amino acid transaminase (EC 2.6.1.42)
2. α-Ketoisovaleric acid dehydrogenase (EC 1.2.1.25)
3. Acyl-CoA dehydrogenase (EC 1.3.99.3)*
4. Enoyl-CoA hydratase (EC 4.2.1.17)*
5. β-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35)*
6. Acetyl-CoA acyltransferase (EC 2.3.1.16)*
7. Propionyl-CoA carboxylase (EC 6.4.1.3)
8. Methylmalonyl-CoA racemase (EC 5.1.99.1)
9. Methylmalonyl-CoA mutase (EC 5.4.99.2)

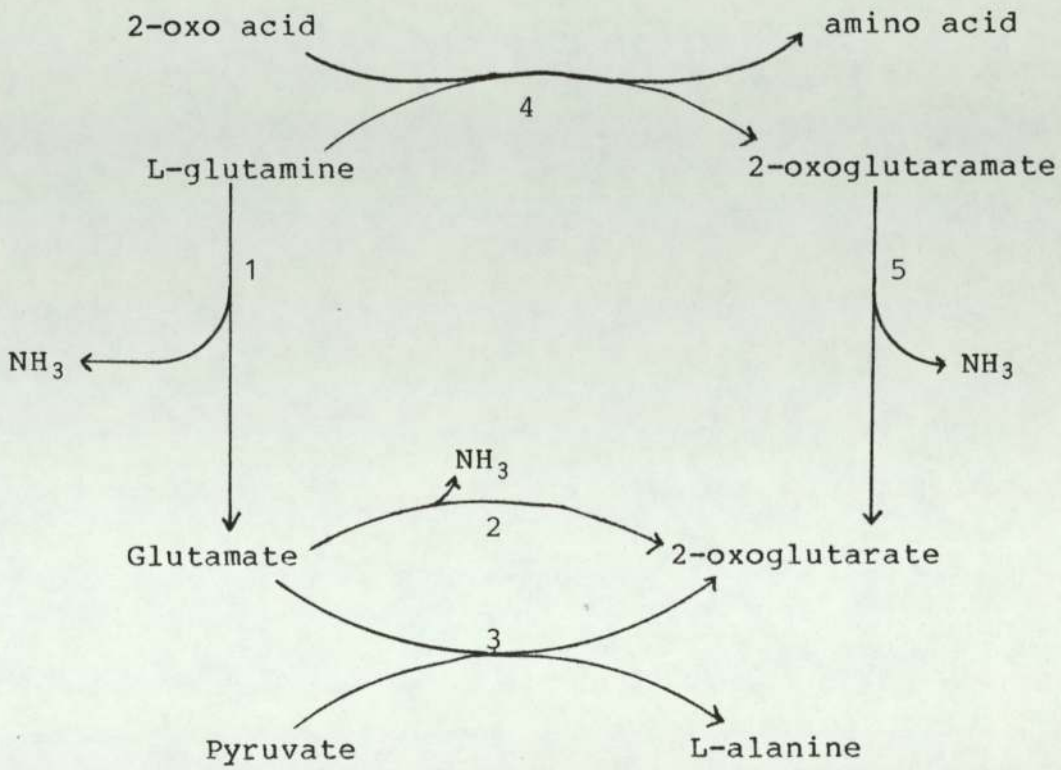
* Enzymes also involved in the β-oxidation of fatty acids.

3.4.1.6 Metabolism of L-glutamine.

L-glutamine was unable to support acid secretion at physiological or supraphysiological concentrations *in vitro* (tables 3.2 and 3.4), even though small amounts of L-glutamine were taken up by the pentagastrin-stimulated rat stomach *in vivo* (Anderson and Hanson, 1983b). This data indicates the problems of using *in vivo* preparations, since although L-glutamine was taken up by the acid-secreting rat stomach it was not possible to determine the specific cell type responsible.

The inability of L-glutamine to support acid secretion *in vitro* may be explained by the absence of an efficient transport mechanism or alternatively the enzymes involved in L-glutamine metabolism may be absent or of limited activity in the parietal cell (fig 3.11). The entry of L-glutamine into some cells occurs by an active Na^+-dependent transport system-N (Kilberg *et al.*, 1980) which may not be present in the parietal cell. Once inside the parietal cell, energy can theoretically be derived from L-glutamine metabolism upon conversion to 2-oxoglutarate, a citric acid cycle intermediate. L-glutamine is hydrolysed to glutamate in a reaction catalysed by glutaminase (EC 3.5.1.2) and the glutamate produced, converted to 2-oxoglutarate by glutamate dehydrogenase (EC.1.4.1.3). Alternatively, glutamate can be converted to 2-oxoglutarate by transamination with pyruvate (fig 3.11). This inability of L-glutamine to support acid secretion *in vitro* was not due to the absence of glutaminase, since the enzyme was demonstrated in the parietal cell enriched fraction B (table 3.11). However, glutaminase activity in this fraction was only about 10% of the activity of that in intestinal epithelial cells (Pinkus and Windmueller, 1977) which are established as being an important site of glutamine utilization (Windmueller and Spaeth, 1978).

Fig 3.11 Possible pathways by which L-glutamine could be metabolized by rat parietal cells.



Enzyme Key

1. Glutaminase (EC 3.5.1.2)
2. Glutamate dehydrogenase (EC 1.4.1.3)
3. L-alanine aminotransferase (EC 2.6.1.2)
4. L-glutamine-2-oxoacid aminotransferase (EC 2.6.1.15)
5. ω-amidase (EC 3.5.1.3)

Having established that the parietal cell may be capable of converting L-glutamine to glutamate, any limitations on glutamine metabolism must be at a point distal to the reaction catalysed by glutaminase. It is unlikely that such a limitation is due to the availability of pyruvate for alanine aminotransferase (EC 2.6.1.2) since in the presence of glucose (5mM) and lactate (0.6mM), when the pyruvate concentration was probably not limiting, the ability of L-glutamine to support acid secretion was not improved (table 3.8).

The ability of L-glutamine to support acid secretion was not improved in the presence of phenylpyruvate (table 3.10), although phenlypyruvate did improve L-glutamine metabolism in hepatocytes (Lund and Watford, 1976) possibly by serving as a 2-oxo acid acceptor in the reaction catalysed by L-glutamine-2-oxo acid aminotransferase (EC 2.6.1.15). Since the ability of L-glutamine (5mM) to support acid secretion was equal to that of no exogenous substrate (table 3.4), and phenylpyruvate (2mM) reduces aminopyrine accumulation below the level achieved in the presence of L-glutamine (5mM), an inhibitory effect of phenylpyruvate (2mM) alone on acid secretion was indicated (table 3.10). This apparent inhibitory effect could be explained by competition of phenylpyruvate with endogenous pyruvate for transport into the mitochondrion. With the addition of L-glutamine and phenylpyruvate in combination to parietal cells <u>in vitro</u>, aminopyrine accumulation was restored to a value not significantly different from that obtained with L-glutamine alone, indicating that some L-glutamine-2-oxo acid amminotransferase may be present within parietal cells. In general, it appears that L-glutamine metabolism cannot support acid secretion in parietal cells to any significant extent, although since it is taken up by the acid-secreting rat stomach <u>in vivo</u> it is probable that it does serve some physiological function in this organ. L-glutamine is an important source of amide

nitrogen which is used in the synthesis of purine nucleotides by rapidly dividing cells (Krebs, 1980). Since the gastric epithelial mucous cells have a rapid turnover within the stomach, L-glutamine may be used for this purpose in these cells. Alternatively, L-glutamine may be metabolized to produce energy by the epithelial or chief cells of the gastric mucosa.

3.4.1.7 The effect of combinations of potential substrates at physiological concentrations on acid secretion *in vitro*.

Parietal cells exposed to D-3-hydroxybutyrate, lactate and L-isoleucine in addition to glucose (5mM) accumulated significantly more aminopyrine than those cells exposed to 5mM-glucose alone (table 3.6). This indicated that for maximal rates of acid secretion to be achieved, substrates in addition to glucose (5mM) are required. As well as glycolysis limiting glucose utilization by parietal cells, the failure of parietal cells exposed to glucose (5mM) and lactate (0.6mM) to accumulate significantly more aminopyrine than those exposed to 5mM-glucose alone (table 3.6), indicates that oxaloacetate availability may also be limiting. Parietal cells exposed to combinations of three or more substrates including L-isoleucine (0.068mM), accumulate significantly more aminopyrine than parietal cells exposed to the other combinations shown in table 3.6 and 5mM-glucose alone.

In summary then, it appears that in order to derive sufficient energy to support maximal rates of acid secretion, additional energy from the metabolism of other substrates in addition to glucose is required. This is not surprising since under physiological circumstances the parietal cell is exposed to combinations of substrates from

the gastric lumen. The metabolism of other substrates, possibly L-isoleucine, may also promote the breakdown of glucose so that the energy demands of the maximally-secreting parietal cell can be met. In view of the low plasma levels of L-isoleucine in the rat this "catalytic role" is perhaps more likely.

3.5 CONCLUSIONS.

In order to match the questions posed in the introduction, the overall conclusions have been divided into distinct sections.

(1) At normal physiological concentrations the following substrates (in increasing order of effectiveness) could support acid secretion in rat parietal cells: L-valine, acetoacetate, L-isoleucine, D-3-hydroxybutyrate, lactate, butyrate, oleate and glucose. Acetate, L-leucine and L-glutamine were unable to support acid secretion at normal physiological concentrations.

(2) The ability of the following substrates to support acid secretion was improved with an increase in their concentration to supranormal levels:

L-leucine, acetate, L-valine, acetoacetate, L-isoleucine, D-3-hydroxybutyrate, lactate and butyrate. This effect was most notable with L-isoleucine and lactate which could support acid secretion more effectively than glucose (5mM) at supranormal levels.

(3) The following combinations of substrates at physiological concentrations could support acid secretion more effectively than glucose (5mM) alone:

(a) Glucose(5mM) + L-isoleucine(0.068mM) + lactate(0.6mM) + D-3-hydroxybutyrate(0.55mM).

(b) Glucose(5mM) + L-isoleucine(0.068mM) + lactate(0.6mM).

(c) Glucose(5mM) + D-3-hydroxybutyrate(0.55mM) + L-isoleucine(0.068mM).

(4) No single substrate at a physiological concentration could support maximal rates of acid secretion in the rat parietal cell. Combination of the present data with that obtained in vivo after an overnight fast (Anderson and Hanson, 1983a,b) suggests that in addition to glucose, D-3-hydroxybutyrate and L-isoleucine may be of major importance in the metabolic processes which power acid secretion in rats under such circumstances.

CHAPTER 4.

THE EFFECT OF PHORBOL ESTERS ON ACID SECRETION BY RAT PARIETAL CELLS IN VITRO AND THE RAT STOMACH IN VIVO.

4.1 INTRODUCTION.

4.1.1 Protein kinase C Activation.

Phorbol diesters were identified as the active tumour-promoting ingredient of croton oil by Hecker (1971) and subsequently there was found to be a direct correlation between the ability of these phorbol esters to promote tumour production and to stimulate calcium-activated, phospholipid-dependent protein kinase (Protein kinase C, Castagna et al., 1982). Although phorbol esters cause a range of biological effects in vitro (table 4.1 and Fisher et al., 1982) most can be tentatively explained by activation of protein kinase C.

Protein kinase C is composed of a single polypeptide chain of molecular weight 77Kda which can be divided into hydrophobic and hydrophilic domains (Kishimoto et al., 1983). The hydrophobic domain may interact with the plasma membrane, and the hydrophilic domain contains the catalytic active site. Protein kinase C may be permanently activated by limited proteolysis by a calcium-dependent neutral protease. The kinase irreversibly activated in this way is termed protein kinase M (Inoue et al., 1977). Alternatively, protein kinase C can be maximally activated in the presence of diacylglycerol, phospholipid and a low concentration of calcium. Diacylglycerols containing at least one unsaturated fatty acid at position 1 or 2 were found to activate protein kinase C (Nishizuka, 1984). Diacylglycerols containing saturated fatty acids were thought to be less effective and mono - and triacylglycerols ineffective (Mori et al., 1982). However, more recent work (Conn et al., 1985; Schlondorff and Levine, 1985) has suggested that dioctanoylglycerol may also be able to activate protein kinase C.

Table.4.1 Some of the biological effects of 12-0-tetradecanoylphorbol-13-acetate (TPA).

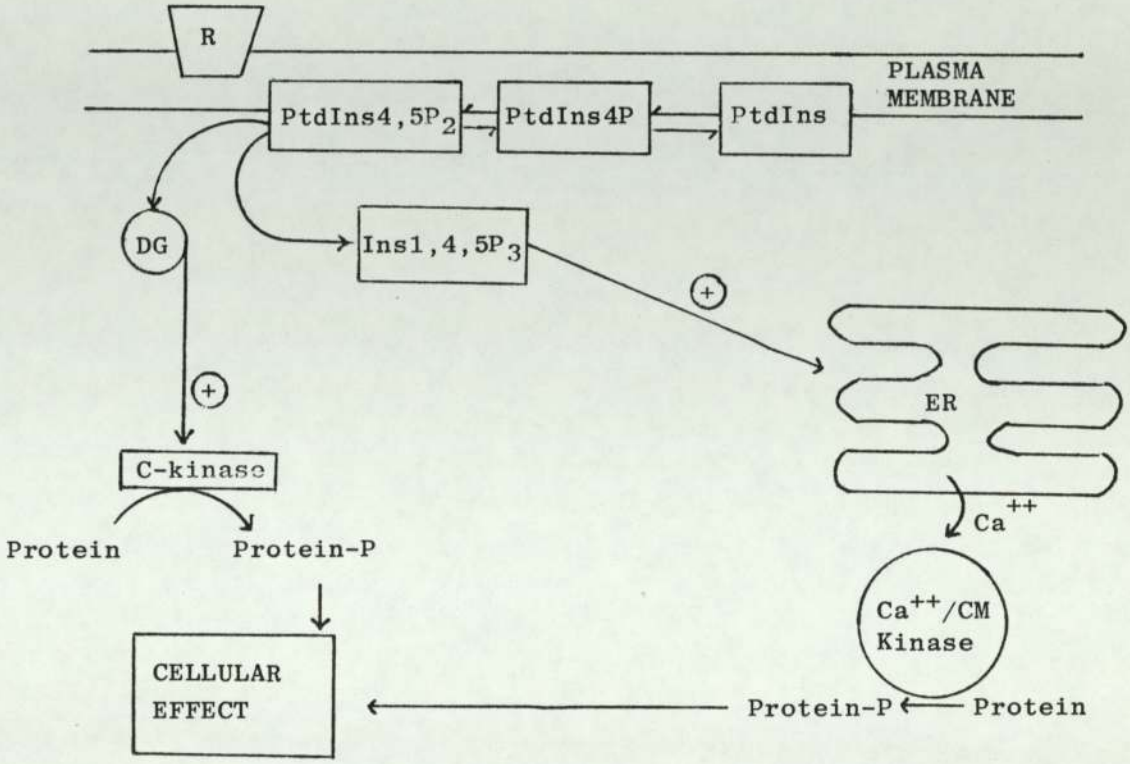
Author.	Observed effect of TPA.
Castagna *et al.*,(1982).	Direct activation of protein kinase C in human platelets *in vitro.*
Ferguson *et al.*,(1982).	Enhancement of lymphocyte proliferation, and inhibition of differentiation.
Ganss *et al.*, (1982).	Stimulation of arachidonic acid release from murine HEL/30 cells *in vitro.*
Grotendorst and Schimmel, (1980).	Increase in cGMP levels and decrease in cAMP levels in cultured myogenic cells.
Gunter, (1981).	Increase in protein discharge from guinea pig pancreatic acini.
O'Brian *et al.*, (1975).	Increased activity of ornithine decarboxylase (EC 4.1.1.50) in mouse epidermis.
Osbourne and Tashyian, (1982).	Reduction in the affinity of EGF, TRH and somatostatin receptors in GH_4C_1 rat pituitary cells.

Under physiological circumstances, the fatty acid at the 2-position is likely to be arachidonic acid, although it may also be oleate or linoleate (Berridge, 1984). Phosphatidylserine can best activate protein kinase C in the presence of unsaturated diacylglycerol and micromolar concentrations of calcium, although other phospholipids can influence enzyme activity (Kaibuchi et al., 1981). Phosphatidylcholine or sphingomyelin can attenuate this effect of phosphatidylserine, and phosphatidylethanolamine can enhance the activation of protein kinase C by phosphatidylserine (Kaibuchi et al., 1981).

Diacyglycerol is transiently produced by the receptor mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate (Rhodes et al., 1983). The hydrolysis of phospatidylinositol 4,5-bisphosphate also yields another second messenger, inositol 1,4,5-trisphosphate which causes the liberation of calcium from the endoplasmic reticulum (Streb et al., 1983; fig 4.1). Agonist-receptor interactions which cause diacylglycerol and inositol 1,4,5-trisphosphate production as part of a bifurcating second messenger pathway, generally also lead to calcium influx across the cell membrane (Berridge, 1981). The elevated levels of calcium and diacylglycerol present within the cell at this point may serve to activate protein kinase C.

Under physiological conditions, protein kinase C is activated by the unsaturated diacylglycerol transiently produced as a result of polyphosphatidylinositol metabolism (Kawahara et al., 1980). The phorbol ester tetradecanoylphorbol-13-acetate (TPA), which has a structural moiety resembling diacylglycerol (fig 4.2), is able to mimic the action of diacylglycerol and can therefore be used as a probe to investigate the involvement of protein kinase C in the regulation of secretion in various cell-types. By mimicking the action of diacylglycerol, TPA can activate

Fig.4.1 The possible role of diacylglycerol and inositol 1,4,5-trisphosphate as second messengers in the rat parietal cell.



Phosphatidylinositol (PtdIns), phosphatidylinositol 4-phosphate (PtdIns 4-P), and phosphatidylinositol 4,5-bisphosphate (PtdIns 4,5-P_2) are kept in dynamic equilibrium by kinases and phosphomonoesterases located on the inner boundary of the plasma membrane. The interaction of an agonist with a receptor (R) on the cell surface stimulates the hydrolysis of PtdIns 4,5-P_2 to yield two second messengers. Inositol 1,4,5-trisphosphate (Ins 1,4, 5,-P_3) serves to liberate calcium from the endoplasmic reticulum (ER), which then may activate calcium, calmodulin-dependent protein kinase (Ca^{++}/CM kinase), while diacylglycerol (DG) is involved in the activation of protein kinase C (C-kinase). The phosphorylation of two distinct groups of proteins by these two kinases may be responsible for the observed cellular effect of the agonist.

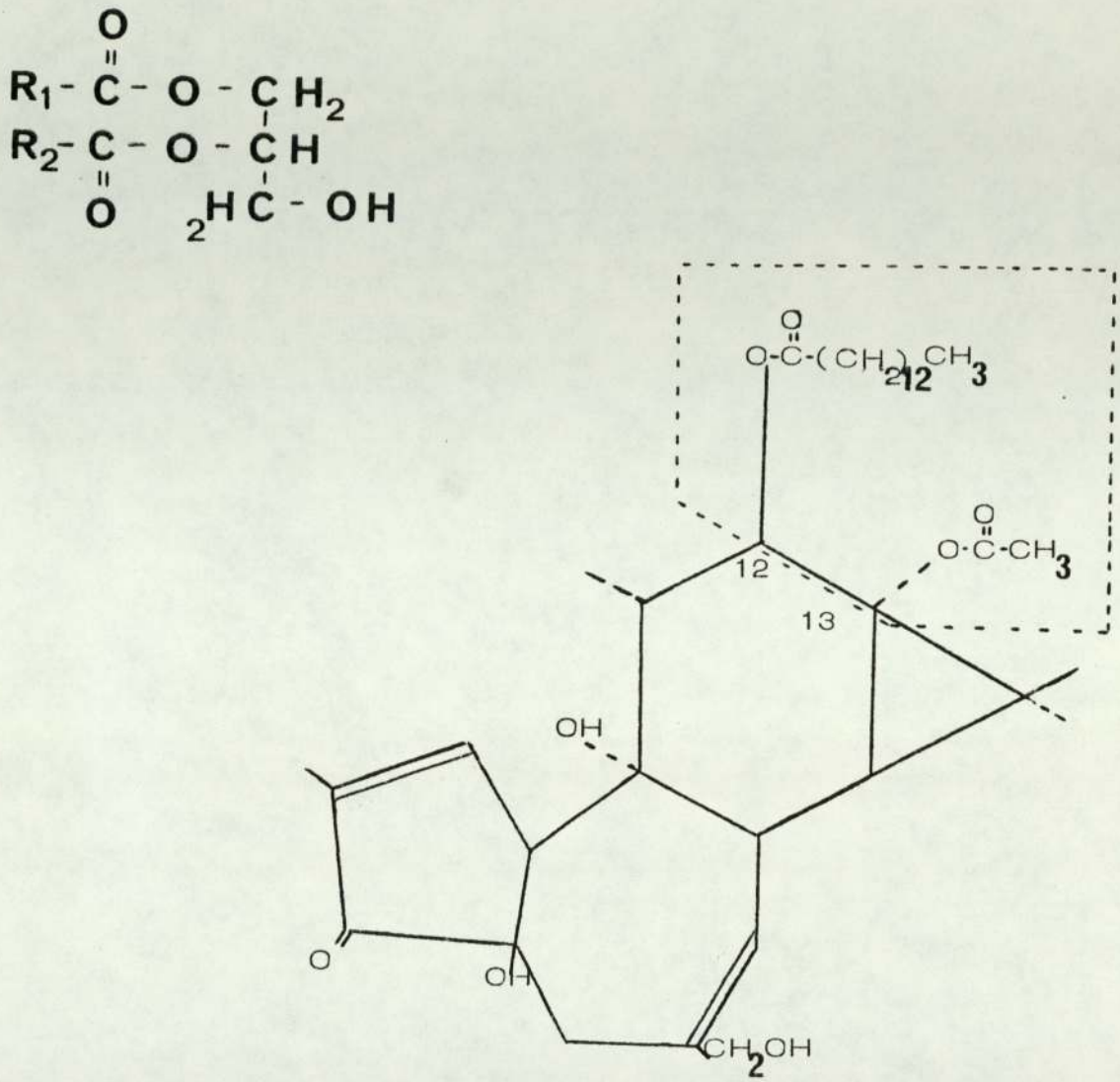


Figure 4.2

Comparison of the structure of the tumour promotor, TPA (bottom) containing a diacylglycerol-like moiety (dotted area), and diacylglycerol itself. R_1 and R_2 represent hydrocarbon chains of fatty acid.

protein kinase C by increasing the affinity of the enzyme for calcium, without causing phosphatidylinositol turnover (Castagna et al., 1982). In fact, the enzyme may be fully activated by TPA without a change in the level of intracellular calcium (Kishimoto et al., 1980; Yamanishi et al., 1983).

A large amount of data exists to support the hypothesis that many of the pleiotrophic actions of phorbol esters are mediated via the activation of protein kinase C. In fact, Niedel et al., (1983) have demonstrated that the phorbol diester receptor and the protein kinase C of rat brain co-purify through several fractionation stages. The apparent dissociation binding constant (Kd) for [^{3}H] phorbol-12,13-dibutyrate has been estimated to be 8nM, which is also the activation constant (Ka) of protein kinase C (Kikkawa et al., 1983), indicating that the phorbol diester binding site and the kinase are possibly the same protein.

Activation of protein kinase C by TPA is associated with a rapid translocation of enzyme activity. Thus thyrotropin-releasing hormone stimulation of GH_3 cells (Drust and Martin, 1985), which transiently increases diacylglycerol levels, induces a rapid translocation of protein kinase C from cytosolic to membrane fractions with concomitant enzyme activation. TPA causes a rapid (within 5 minutes) decrease in cytosolic C-kinase activity accompanied by an increase in enzyme activity in the membrane fraction of parietal yolk sacs (Kraft and Anderson, 1983), an observation consistent with enzyme translocation influencing activation. It is possible that TPA activation of C-kinase may occur because the soluble enzyme is brought into contact with a phospholipid environment (Delclos et al., 1983) (fig 4.3). Therefore it is possible that membrane phospholipid distribution and composition will influence the activity of protein kinase

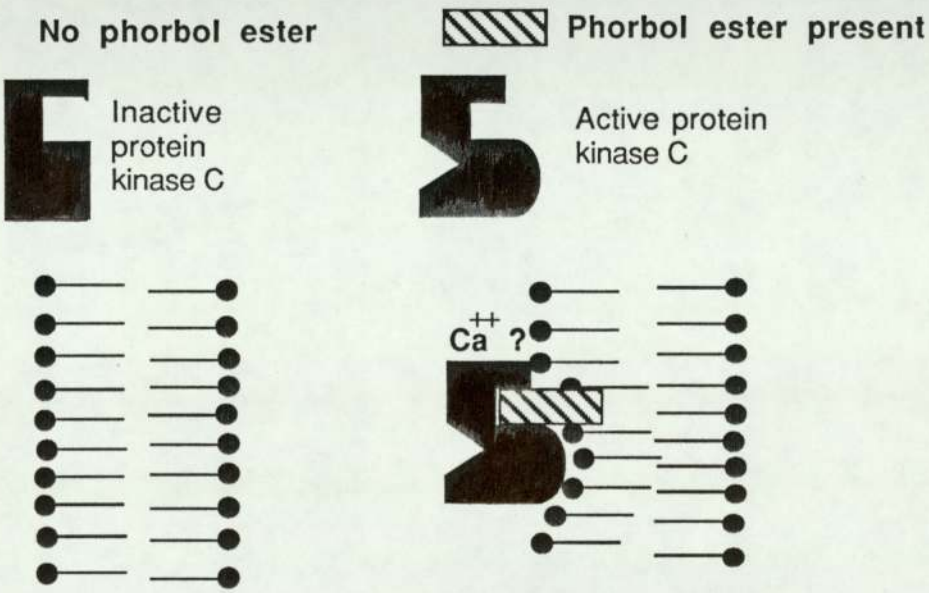


Figure 4.3

Activation of protein kinase C by TPA: The phorbol ester possibly induces protein kinase C activation by causing it to be intercalated into the cell membrane.

C. Protein kinase C translocation to the cell membrane has also been observed in isolated pancreatic acini when treated with TPA (Wooten and Wren, 1984).

4.1.2 The involvement of protein kinase C in cellular secretion.

TPA has been shown to stimulate secretion in a wide variety of cell types. Treatment of isolated pancreatic acini with TPA is associated with an increase in amylase secretion (Wooten and Wren, 1984). Yamamoto et al., (1982) have demonstrated the insulinotrophic effect of the phorbol ester in isolated pancreatic islets, which may be mediated by lipoxygenase products. Katakami et al., (1984) suggested that protein kinase C activation by TPA and calcium mobilization are both involved in the release of histamine from rat peritoneal mast cells. These workers demonstrated that in the presence of TPA and the calcium ionophore A23187, histamine release was markedly increased. Yamanishi et al., (1983) have also demonstrated that at low concentrations both calcium and TPA are required to initiate the physiological release of serotonin from human platelets. Activated protein kinase C may phosphorylate membrane and cytosolic proteins and this phosphorylation has been implicated in the stimulus-secretion coupling mechanism of insulin release in vitro (Hutton et al., 1984). The ability of thrombin to cause the release of serotonin from human platelets in vitro, is also closely associated with the phosphorylation of an endogenous protein by protein kinase C (Castagna et al., 1982).

Although TPA stimulates secretion from a wide range of cell types, (see above) this phorbol ester inhibits aminopyrine accummulation within the tubulovesicles and secretory canaliculi of rat isolated parietal cells stimulated by histamine and dbcAMP (Anderson and Hanson, 1984). TPA could exert this inhibitory action by a direct effect on the parietal cell or alternatively TPA might cause the release of an inhibitor of acid secretion from another cell type. For example TPA (20nM) has been shown to cause the release of somatostatin, a potent inhibitor of acid secretion, from D - cells of the perfused rat stomach (Yamatani et al., 1985). However, it is unlikely that the inhibition of histamine-stimulated aminopyrine accumulation caused by TPA is mediated via somatostatin. Thus, whereas TPA has been shown to substantially inhibit aminopyrine accummulation induced by histamine and IBMX (tables 4.2 and 4.3) and Anderson and Hanson (1984), somatostatin causes only a 15-25% inhibition in an enriched rat parietal cell preparation (Schepp et al., 1983b). Also, TPA causes 58% inhibition of aminopyrine accumulation stimulated by 1mM-dbcAMP (Anderson and Hanson, 1984), but somatostatin does not inhibit dbcAMP-induced acid secretion (Chew, et al., 1983). Although TPA causes the activation of phospholipase A_2 and the liberation of prostaglandins in some cells (Butler-Gralla et al., 1983), it is unlikely that the inhibitory action of TPA on histamine-stimulated aminopyrine accumulation by parietal cells is mediated by prostaglandin production, even though prostaglandins can inhibit acid secretion induced by histamine (Soll, 1980b). Unlike TPA, prostaglandins do not inhibit acid secretion stimulated by dbcAMP (Soll, 1980b) Furthermore, the inhibitory action of TPA was not overcome by indomethacin, a cyclo-oxygenase inhibitor, or nordihydroguaiaretic acid an inhibitor of both lipoxygenase and cyclo-oxygenase pathways (Anderson and Hanson, 1984).

It is more likely that TPA exerts its action by a direct effect on the parietal cell, since results for the effect of TPA concentration on histamine-stimulated aminopyrine accumulation were similar to a classical dose-response curve, and there was no relationship between cell concentration and the action of TPA (Anderson and Hanson, 1985). It has been proposed that TPA activates protein kinase C within the parietal cell thereby inhibiting aminopyrine accumulation (Anderson and Hanson, 1985). This is not unlikely since due to its ubiquitous nature (Kuo et al., 1980), protein kinase C may well be present within the rat parietal cell. More recent work (Chew, 1985) has demonstrated protein kinase C activity in a parietal cell homogenate. Alternatively, TPA may inhibit the adenylate cyclase associated with the histamine receptor on the parietal cell (Coffey and Hadden, 1983), although since TPA also inhibits aminopyrine accumulation stimulated by the stable analogue of cAMP, dibutyryl cAMP (Anderson and Hanson, 1984) a site of action distal to adenylate cyclase is more likely. However, an additional action of TPA on the cyclase cannot be excluded.

The aim of this study was to verify and then to extend the work of Anderson and Hanson (1984). By using a gastric cell preparation containing only 20% parietal cells it is difficult to determine whether TPA exerts its effects directly on the parietal cell or via another cell type (see above). This situation may be clarified by examining the effect of TPA in an enriched parietal cell preparation.

Although the aminopyrine test, as used by Anderson and Hanson (1984), is considered a valid, yet indirect indicator of acid secretion (Berglindh et al., 1976), it does possess several inherent difficulties. Any reduction in aminopyrine accumulation in cells treated with TPA could

be interpreted as an effect of TPA simply on acid retention inside the secretory canaliculi and not on acid secretion across the canalicular membrane, since aminopyrine accumulation is dependent upon the amount of acid sequestered within the secretory canaliculi and is not a direct index of the secretory rate (2.1.4.3). In order to confirm that TPA does in fact inhibit aminopyrine accumulation by reducing acid secretion from the parietal cell and not by stimulating the back diffusion of protons or by enhancing the dissipation of acid, the effect of perfusion of TPA through the rat stomach lumen *in vivo* on acid secretion was studied. This *in vivo* model, which measures acid secretion directly, was described by Ghosh and Schild (1958) and is suitable for the study of agents which cause the inhibition of acid secretion stimulated by carbachol, gastrin, and histamine (Robein *et al.*, 1979).

This *in vivo* preparation was used in preference to an *in vitro* mammalian chambered mucosa for several reasons. With a gastric mucosal preparation *in vitro* (Finke *et al.*, 1985) the time for acid-secretory rates, in response to histamine, to reach maximal levels was in no case less that 2 hours. Carbachol and pentagastrin were either ineffective or had only transitory effects on secretion when used in this preparation. Acetylcholine and pentagastrin also failed to stimulate acid secretion significantly in a piglet gastric mucosal preparation *in vitro* (Forte *et al.*, 1975). Using a rat isolated mucosal preparation, Pearce *et al.*, (1981) were also unable to show a significant stimulation of acid secretion with pentagastrin. In general, it is difficult to make significant conclusions from data obtained using an *in vitro* model of acid secretion which does not respond to physiological stimulants.

The interaction of gastrin, histamine, and acetylcholine with their receptors on the parietal cell has been described previously (1.2.1), and will not be discussed here. However, it is important to note that with this *in vivo* model the site of action of any drugs administered is uncertain, yet when used in conjunction with an isolated cell system it provides useful information which can be related to the physiological situation.

4.2 METHODOLOGY.

4.2.1 The effect of TPA (27nM) on acid secretion in vitro.

A gastric cell preparation containing approximately 20% parietal cells was obtained as described in section 2.2.2, and the parietal cell content enriched if required using the method specified in section 2.2.3. 1.5ml of the cell preparation was incubated at 37^{0}C for 30 minutes in the presence of test reagents, and the aminopyrine accumulation ratio determined for each treatment (2.2.5). A TPA stock solution (10mg/ml), was prepared in dimethyl-sulphoxide (DMSO) (TPA is insoluble in water/saline) and stored at -20^{0}C before use. When required, TPA was added in a small volume (usually 2µl) to the cell suspension from a suitable dilution of the original stock in medium B´ . A corresponding volume of similarly diluted DMSO was added to control vials so that the concentration of DMSO was the same in all the vials (0.0002% v/v). Before the TPA was added to the cell suspension both the original stock, and the diluted TPA in medium B´ were sonicated (6 x 5 sec, 4 microns, 4^{0}C) to ensure adequate dispersion of the TPA in the solvent.

4.2.2 The effect of phorbol esters on acid secretion by parietal cells of the rat stomach in vivo.

4.2.2.1 Surgical Procedure.

Male Wistar rats weighing between 200-350g were fasted overnight and anaesthetized with sodium pentabarbitone (sagatal), 60mg/kg body weight i.m. A constant level of

anaesthesia was maintained by subsequent hourly injections of sodium pentabarbitone (20mg/kg). Surgery was performed by the method of Puurunen and Karppanen (1975) with some modifications (see below).

4.2.2.1.1 Cannulation of the jugular vein.

The rat jugular vein was identified by the regular pulsing which became visible when a small patch of skin was removed from the right side of the upper thorax. Any connective tissue covering the jugular vein was removed by scoring either side of the vein with a scalpel and careful blunt dissection. The first ligature was pulled underneath the jugular vein with curved forceps, and the vein ligated as close to the head as possible. A second loose ligature was positioned below the first to tie in the cannula. Jugular vein cannulation was carried out by making a small incision in the vein between the ligatures, followed by the insertion and tying-in of a 53cm length of Tygon flexible nylon tubing (i.d. 0.5mm, o.d. 0.63mm) bevelled at 45^0 at the tip. This tygon tubing was connected to a 1ml syringe on an injection apparatus (Palmer), and infusion begun immediately at a rate of 3.7µl/min with either pentagastrin (1.6µg/min per Kg body weight), histamine phosphate (30µg/min per Kg body weight) or carbachol (0.1µg/min per Kg body weight). To measure basal levels of acid secretion saline (9g/l NaCl) was infused intravenously.

4.2.2.1.2 Operation on the stomach.

A small mid-line incision was made through which the stomach was drawn and a loose ligature passed around the base of the oesophagus. An incision was made in the oesophagus, proximal to the ligature, and a glass cannula (i.d. 0.7mm, o.d. 1.8mm) introduced into the oesophagus as far as the cardiac region of the stomach. This cannula was secured firmly in place and connected by a length of tygon tubing (i.d. 0.5mm, o.d. 0.63mm) through a heat-exchanger and peristaltic pump to the main reservoir containing saline (9g/l NaCl) at 37^{0}C. The oesophagus and both trunks of the vagus nerve were then severed just proximal to this cannula.

A loose ligature was positioned around the top of the duodenum and a small incision made adjacent to the stomach. The residual gastric contents were removed via the duodenal incision by flushing saline at 37^{0}C through the lumen from a 20ml-syringe connected to the oesophageal cannula. Care was taken not to distend the stomach wall excessively. The syringe was removed from the cardiac cannula and the tubing reconnected. The pyloric cannula (o.d. 4.8mm, i.d. 3.4mm), made from a length of plastic tube 16mm long, was tied in position approximately 0.5cm from the pylorus. The pyloric cannula was connected to a 25.6cm length of tygon tubing (i.d. 2.4mm, o.d. 4mm) which conveyed the gastric contents to the collecting vials in position approximately 5cm below the stomach, for subsequent titration. The peristaltic pump was started at a rate of 1ml/min and saline perfused through the stomach. The stomach was then covered with moistened tissue and a piece of plastic to reduce evaporative cooling. The body temperature of the rat was maintained at 37.5^{0}C by means of a homeothermic blanket (C. F. Palmer 8185). After the operation, the animals were allowed to

stabilize until the rate of acid secretion became constant (figs 4.4 to 4.7). This time varied between animals, and with secretagogue, but was not usually longer than 1 hour.

4.2.2.2 Measurement of acid secretion

The acid secreted into each 5 minute-fraction of perfusate was measured using the method of Puurunen and Karppanen (1975) with some modifications. Fractions collected from the duodenal cannula over each 5 minute sampling period were titrated using 0.02M-NaOH and 50µl phenolphthalein (1g/100ml ethanol) as indicator. Initially 100µl aliquots of 0.02M-NaOH were used in the titration, but as the end point was approached the aliquots were reduced to 25µl. A sample of saline (5ml) was used to provide a blank titration value which was subtracted from the experimental values.

4.2.2.3 Experimental Protocol.

The stomach was perfused with saline (9g/l NaCl) at 37^{0}C from the main reservoir through the cardiac cannula, until a steady state of acid secretion was achieved. The acid secreted into each 5 minute fraction of perfusate was measured for a further 30 minutes (control period), at which point the perfusate flowing into the stomach was rapidly changed to saline containing phorbol ester (0.006% DMSO v/v), and the acid secreted into the perfusate was measured for a further 40 minutes (experimental period). The transfer from saline to saline containing phorbol ester perfusing the stomach, was made with the cardiac cannula disconnected and the flow rate increased, so that upon re-connection of the cardiac

cannula the gastric mucosa was immediately exposed to phorbol ester. The change-over between reservoirs was made by means of a 3-way valve, and was completed within 10 seconds.

4.2.2.4 Analysis of Results.

A two-way analysis of variance was used to investigate the effect of time on acid secretion. There was no effect of time on secretion during the control period whatever the secretagogue and so the mean value for the control period could be compared with individual values during the experimental period for each secretagogue by using Dunnetts test (Zar, 1974), and significant differences are indicated by asterisks *$p < 0.05$, **$p < 0.01$ (fig 4.8/table 4.4).

4.3 RESULTS AND DISCUSSION.

4.3.1 The effect of TPA (27nM) on acid secretion in vitro.

Results from 2 experiments (table 4.2) indicate that TPA (27nM) inhibited aminopyrine accumulation by gastric cell preparations stimulated by histamine (0.5mM) and IBMX (0.1mM) by over 90%. This confirms the inhibitory effect of TPA previously observed by Anderson and Hanson (1984). The gastric cell preparations used in this study contained on average 17% parietal cells. However, the specifity of the aminopyrine test enables the use of such heterogeneous cell preparations for this test.

It is possible that TPA exerts its action by causing the release of an inhibitor of acid secretion from another cell type (4.1.2). However, since TPA also significantly inhibited histamine-stimulated aminopyrine accumulation ($p < 0.01$, table 4.3) by an enriched parietal cell preparation containing $79.4 \pm 0.3\%$ parietal cells, it is more likely that TPA has its effect directly on the parietal cell. Using this enrichment procedure (section 2.2.3) the contribution of other cell types to the effect of TPA on aminopyrine accumulation is minimized, although these cell types still make up approximately 20% of the total cell population after enrichment, and the possibility of co-purification of another cell type with the parietal cells cannot be entirely discounted. Having established that TPA probably inhibits aminopyrine accumulation by a specific action on the parietal cell, it is pertinent to ask the question "Does TPA cause an inhibition of aminopyrine accumulation in isolated parietal cells because it inhibits acid secretion?" (see introduction to this chapter).

In order to determine by which mechanism TPA inhibits aminopyrine accumulation, the effect of TPA on acid secretion *in vivo* was determined.

Table 4.2

The effect of TPA (27nM) on aminopyrine accumulation by an unenriched parietal cell preparation containing on average 17% parietal cells. The results are the mean values from 2 experiments. Consequently no standard errors are given and no statistical analysis was carried out. The results agree with those obtained by Anderson and Hanson (1984, 1985).

Treatment	Aminopyrine Accumulation	
Control	3.1	(2.05, 4.05)
Control + TPA (27nM)	3.6	(1.93, 5.22)
Histamine(0.5mM) + IBMX (0.1mM)	111.6	(60.61, 162.6)
Histamine(0.5mM) + IBMX (0.1mM) + TPA (27nM)	9.9	(2.55, 17.15)

Table 4.3

The effect of TPA (27nM) on aminopyrine accumulation by control and stimulated enriched fractions of parietal cells. The results are given as mean ± S.E.M. with the number of experiments in parenthesis. The significance of the effect of TPA on the stimulated cells was determined by a paired t-test. *$p < 0.01$.

Treatment	Aminopyrine Accumulation
Control	1.4 ± 0.4 (3)
Control + TPA (27nM)	3.2 ± 1.6 (2)
Histamine(0.5mM) + IBMX (0.1mM)	36.4 ± 1.4 (3)
Histamine(0.5mM) + IBMX (0.1mM) + TPA (27nM)	6.5 ± 1.6 (3)*

4.3.2 The effect of intravenous infusion of secretagogue on acid secretion in vivo.

All the secretagogues tested caused a significant increase in acid secretion from the rat stomach *in vivo*, as determined by 1-way analysis of variance (table 4.4). Carbachol, pentagastrin (peptavlon injection) and histamine caused 4.7-fold, 7.2-fold and 10.1-fold increases in the rate of acid secretion above basal levels. Although the degree of stimulation varied with secretagogue, the rate of acid secretion in each case reached a plateau within 50 minutes (figs 4.4 to 4.7). Using the same secretagogue infusion rates Puurunen (1978) was unable to achieve the rates of acid secretion from the rat stomach obtained in this study with carbachol or histamine. In response to carbachol and histamine phosphate infusion, acid secretion reached a plateau at 2.5μmoles/5 min and 4.0μmoles/5 min in comparison with rates of 10.94 μmoles/5 min and 23.76 μmoles/5 min obtained in this study. The difference may be because Puurunen (1978) did not employ continuous perfusion of the stomach but emptied and refilled it at intervals with saline. Hedges and Parsons (1977) achieved a more dramatic increase in the acid-secretory rate (9μmoles/5 min) in response to pentagastrin infusion (1.6μg/Kg/min). This value is similar to the secretory rate obtained in this study (table 4.4). Since Hedges and Parsons (1977) also used a relatively similar perfused rat gastric lumen preparation this similarity was not unexpected.

As the infusion of each secretagogue was started as soon as the jugular vein was cannulated and not at time zero, when the first sample collection was begun, a steady increase from basal levels of secretion was not observed. The initially high levels of acid secretion in control animals (fig 4.4) was presumably due to stimulation of

Table 4.4

The effect of intravenous infusion of secretagogues on acid secretion in vivo. The results are given as mean ± S.E.M. for the average amount of acid secreted during each 5 minute fraction of the control period.

*$p < 0.05$ ** $p < 0.01$ indicate a significant difference from control animals given an intravenous infusion of saline (9g/l NaCl) as determined by 1-way analysis of variance and subsequent Dunnetts test (Zar, 1974).

Number of animals in parenthesis.

Secretagogue (infusion rate).	Rate of Acid Secretion (μmoles/5 min collection period).
Control	2.35 ± 0.35 (4)
Carbachol (0.1μg/min/Kg)	10.94 ± 1.70 (5)*
Histamine Phosphate (30μg/min/Kg)	23.76 ± 4.28 (5)**
Pentagastrin (1.6μg/min/Kg)	16.88 ± 1.92 (9)**

Figure 4.4

The effect of duration of intravenous infusion of saline (9g/l NaCl, 3.7µl/min) on acid secretion by the rat stomach in vivo. Results are means ± S.E.M. from 4 experiments.

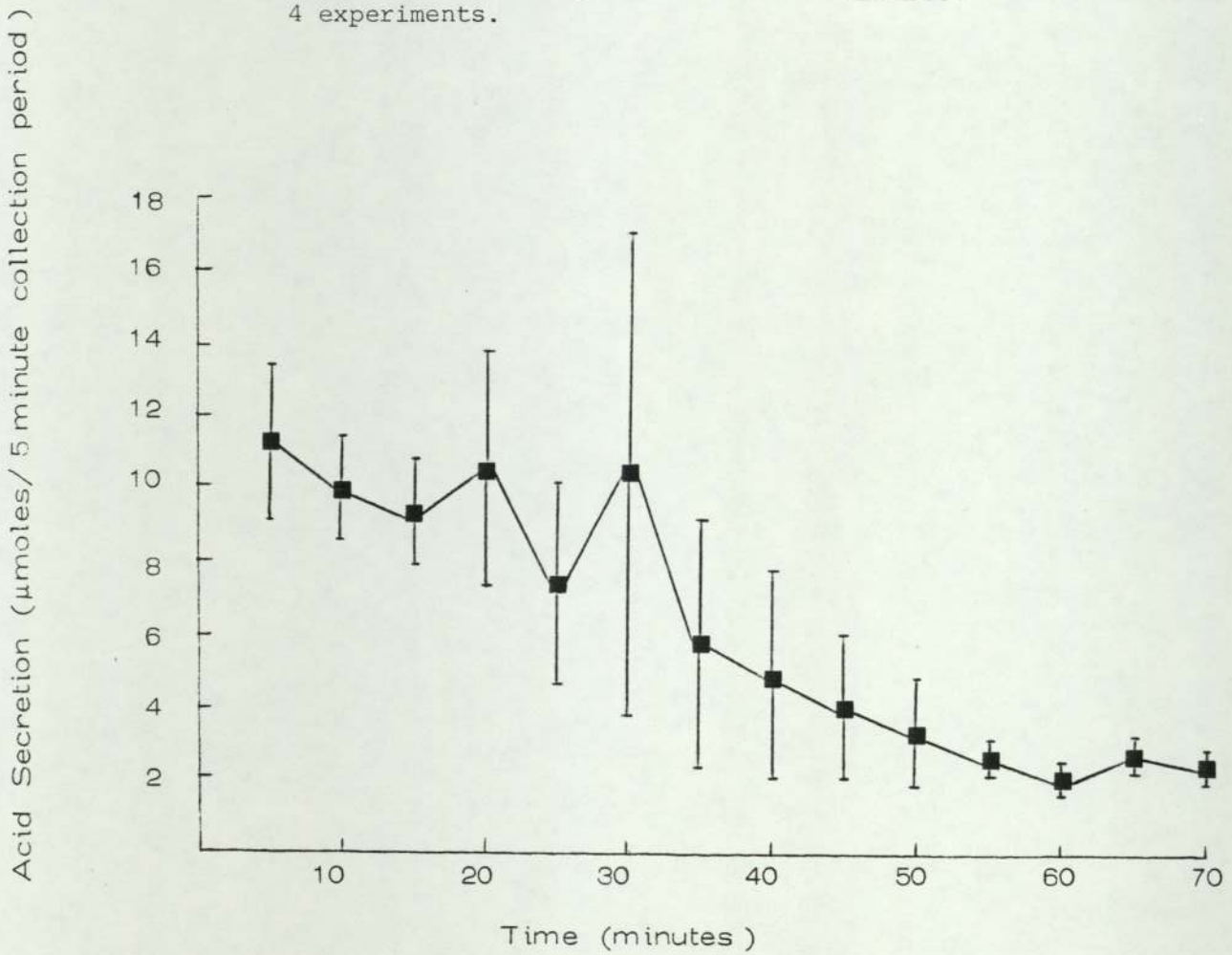


Figure 4.5

The effect of duration of intravenous infusion of histamine phosphate (30µg/min per Kg body weight) on acid secretion by the rat stomach in vivo. Results are mean ± S.E.M. with the number of experiments in parenthesis.

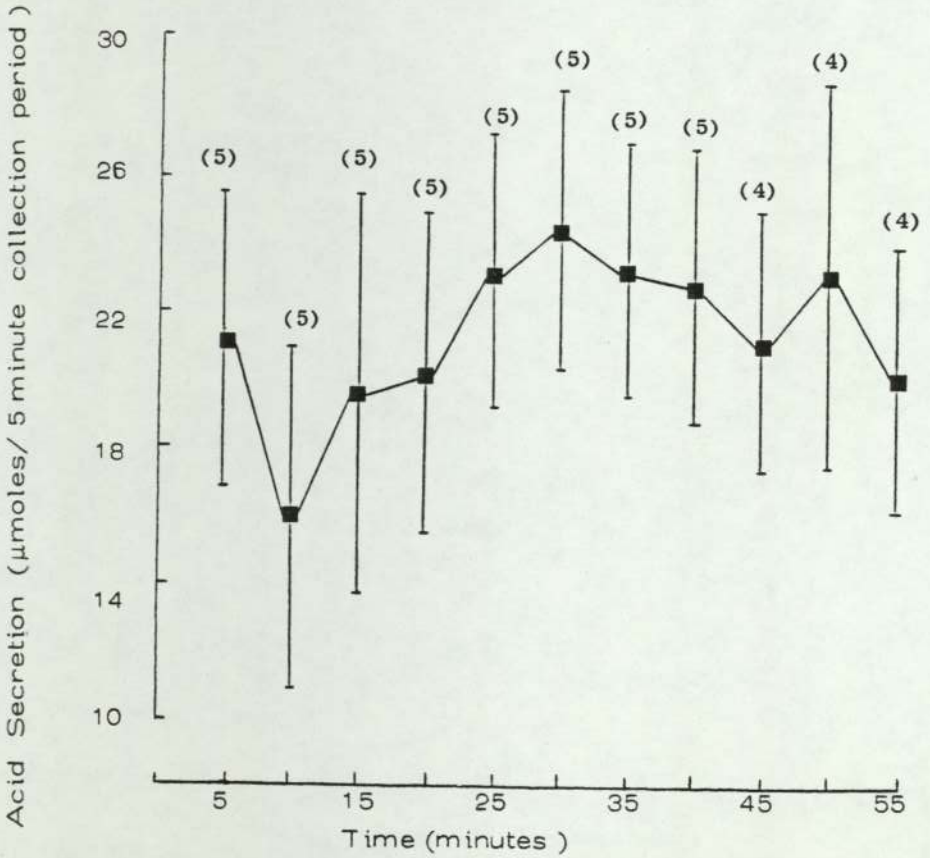


Figure 4.6

The effect of duration of intravenous infusion of pentagastrin (1.6µg/min per Kg body weight) on acid secretion by the rat stomach in vivo. Results are means ± S.E.M. with the number of experiments in parenthesis.

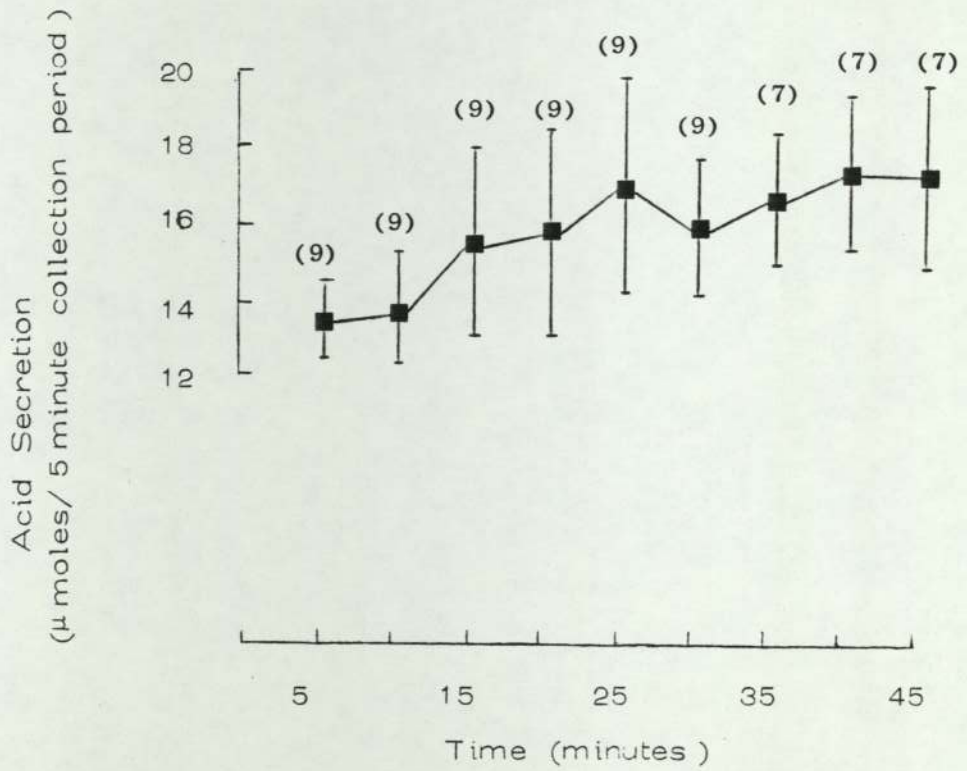
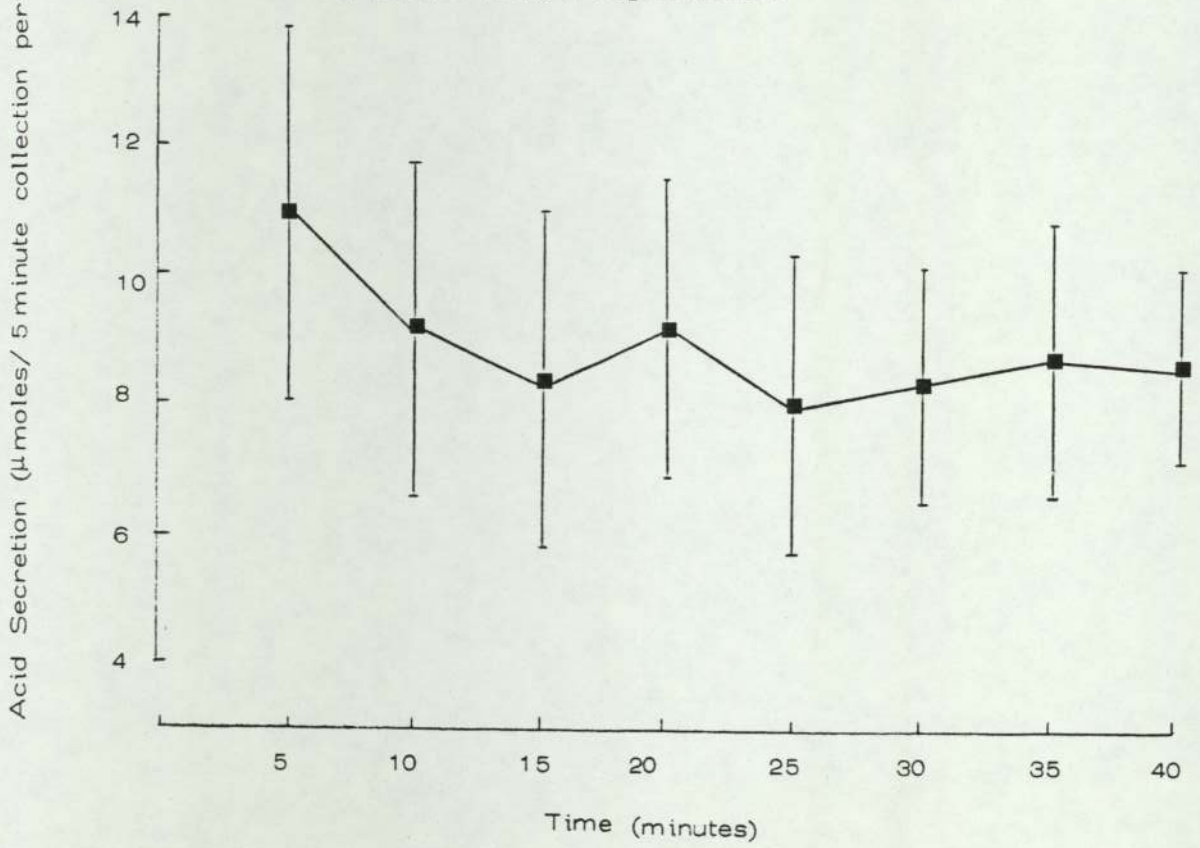


Figure 4.7

The effect of duration of intravenous infusion of carbachol (0.1µg/min per Kg body weight) on acid secretion by the rat stomach in vivo. Results are means ± S.E.M. from 5 experiments.



intramural reflexes within the stomach wall during the flushing out of the residual gastric contents. However, the amount of acid secreted levelled out at approximately 2.5μmoles/5 min collection period within 50 minutes.

4.3.3 The effect of phorbol esters on acid secretion by parietal cells of the rat stomach in vivo.

When added to the luminal perfusate, TPA (1μM) significantly inhibited the rate of acid secretion from the rat stomach in response to intravenous infusion of histamine phosphate (30μg/min/Kg) and pentagastrin (1.6μg/min/Kg) as shown in figs 4.8 and 4.9 and table 4.5. The same concentration of TPA failed to significantly reduce basal acid secretion and that stimulated by carbachol (0.1μg /min/Kg, fig 4.10). TPA reduced the rate of histamine-stimulated secretion even when the level of acid secretion during the control period was similar to that achieved during carbachol-induced secretion (fig 4.11). The action of TPA can therefore be said to be secretagogue-specific, and not to be dependent on the level of acid secretion during the control period.

The similarity between the secretagogue specificity observed both *in vivo* and *in vitro* makes it possible to suggest that TPA inhibits aminopyrine accumulation *in vitro* by directly reducing acid secretion from the parietal cell. For TPA to exert its inhibitory effect on acid secretion at the level of the parietal cell it must first gain access to the parietal cell surface. Consequently, a high concentration (1μM) of both phorbol esters were used in this study, even then, the full effect of TPA was not observed immediately but was gradual in onset. This was not surprising since the parietal cells are located towards the base of the gastric glands, and are therefore relatively inaccessible.

Table 4.5

Effect of the presence of TPA (1μM) in the luminal perfusate on gastric acid secretion *in vivo* in rats infused intravenously with pentagastin (1.6μg/min per Kg body weight), histamine phosphate (30μg/min per Kg body weight), carbachol (0.1μg/min per Kg body weight), and saline (9g/l NaCl, 3.7μl/min). TPA was present in the luminal perfusate only after a 30 minute control period. Asterisks indicate a significant difference from the mean value for secretion during the control period $*p < 0.05$, $**p < 0.01$. The results are given as mean ± S.E.M. with the number of experiments in parenthesis.

Time after reaching steady state. (min)	μmoles H^+ per 5 minute collection period			
	Pentagastrin. (5)	Histamine Phosphate.(5)	Carbachol. (5)	Saline.(4)
5	19.3 ± 3.9	26.3 ± 4.0	10.9 ± 2.1	2.2 ± 0.4
10	20.1 ± 4.2	23.9 ± 3.7	10.7 ± 1.8	2.5 ± 0.4
15	17.5 ± 2.9	24.7 ± 5.3	11.1 ± 2.0	2.2 ± 0.4
20	17.7 ± 1.9	23.5 ± 5.1	11.3 ± 1.7	2.3 ± 0.3
25	17.3 ± 2.9	22.3 ± 4.2	11.3 ± 1.5	2.6 ± 0.4
30	18.6 ± 3.2	21.9 ± 4.0	10.5 ± 1.5	2.4 ± 0.4
35	15.5 ± 1.8	16.3 ± 3.4**	9.7 ± 1.5	2.6 ± 0.4
40	14.1 ± 2.8	14.7 ± 4.0**	10.5 ± 0.8	2.3 ± 0.6
45	13.3 ± 2.5	14.3 ± 4.2**	10.1 ± 1.6	2.5 ± 0.6
50	12.7 ± 1.6*	12.6 ± 4.1**	9.5 ± 1.4	2.6 ± 0.6
55	11.7 ± 0.8*	11.7 ± 4.1**	8.9 ± 1.3	2.2 ± 0.4
60	10.9 ± 1.2**	9.8 ± 3.6**	8.9 ± 1.4	2.5 ± 0.5
65	11.1 ± 1.0**	8.8 ± 3.4**	8.7 ± 1.2	2.7 ± 0.4
70	9.9 ± 1.0**	8.0 ± 2.9**	9.3 ± 1.5	2.7 ± 0.5

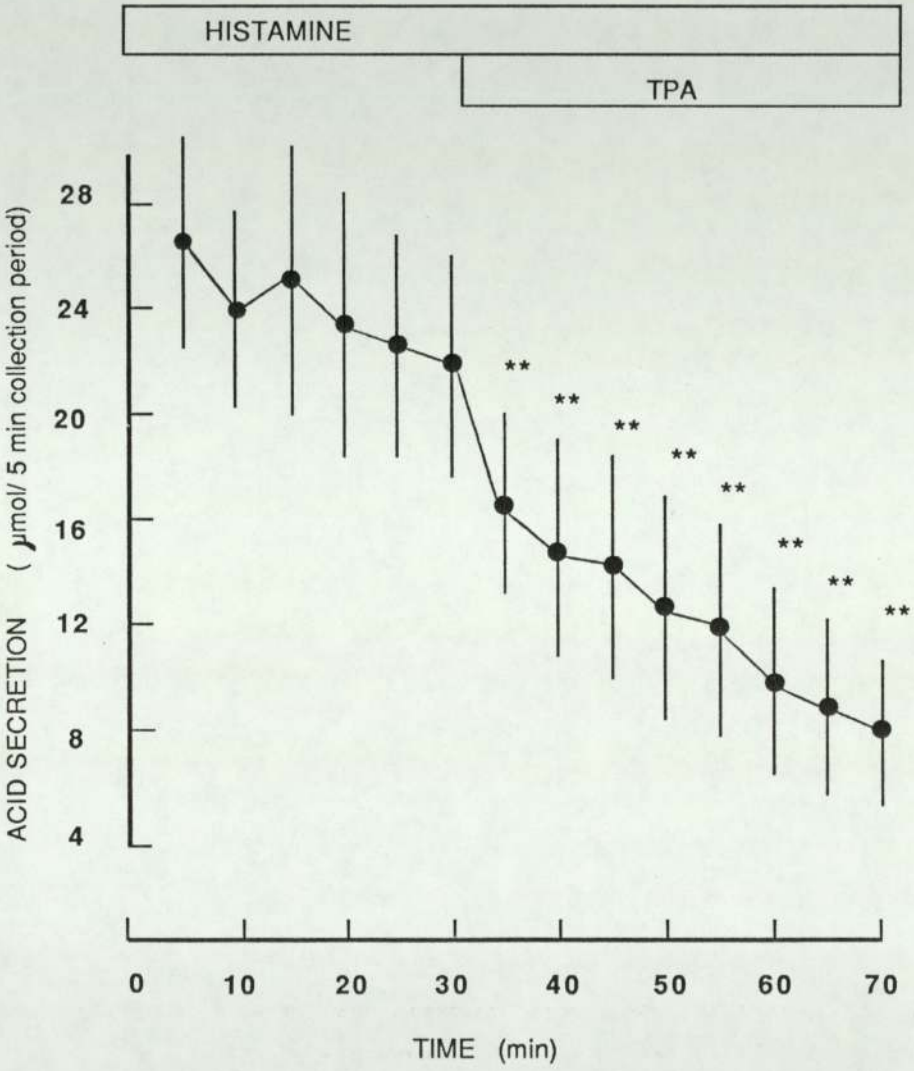


Figure 4.8

The effect of the presence of TPA (1µM) in the luminal perfusate on gastric acid secretion in vivo in rats infused intravenously with histamine phosphate (30µg/min per Kg body weight). TPA was present in the luminal perfusate only after a 30 minute control period. Asterisks indicate a significant difference from the mean value for secretion during the control period **$p<0.01$.

Conclusions concerning the significance of the effect of TPA shown above would be strengthened by further experiments showing that histamine-stimulated secretion was unaffected by time over the whole of the control and experimental period.

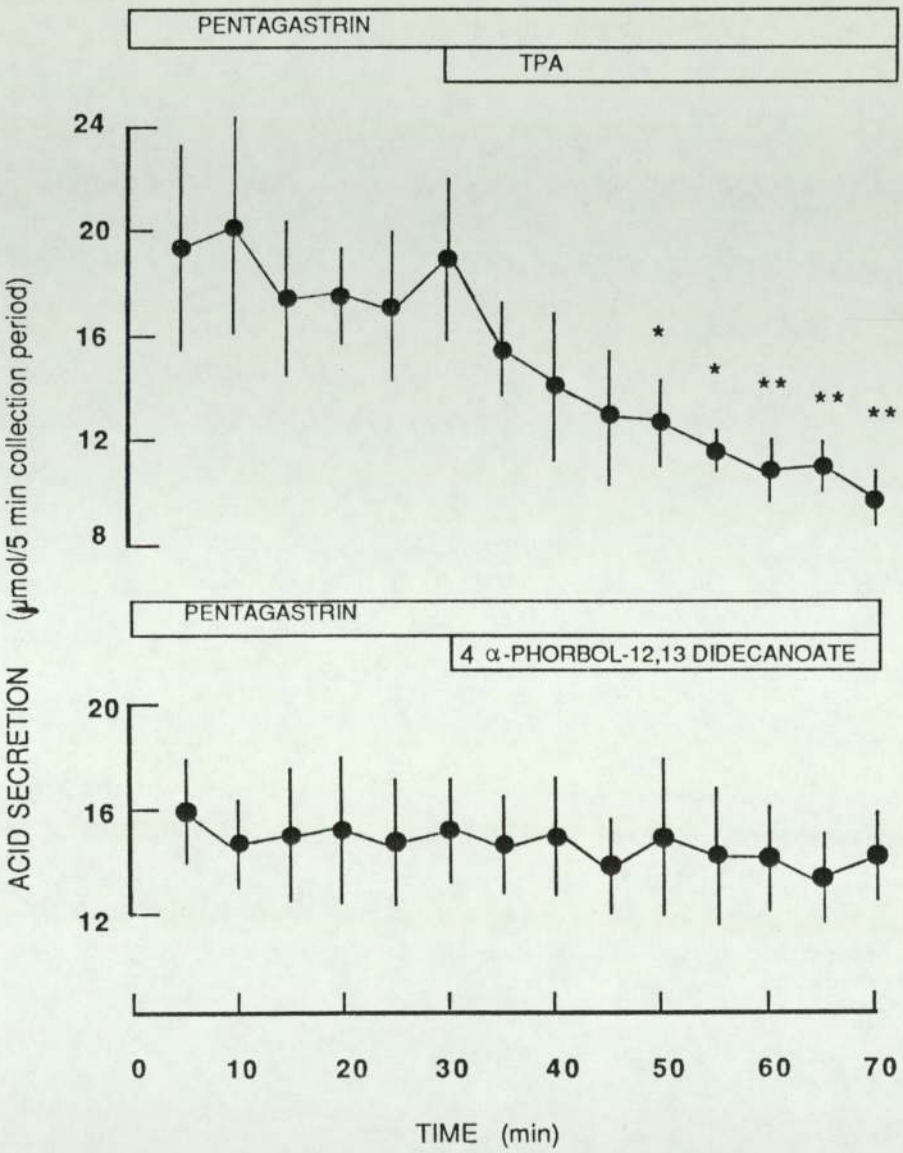


Figure 4.9

The effect of the presence of TPA (1μM) or 4α-PDD (1μM) in the luminal perfusate on gastric acid secretion in vivo in rats infused intravenously with pentagastrin (1.6μg/min per Kg body weight). The phorbol ester was present in the luminal perfusate only after a 30 minute control period. Asterisks indicate a significant difference from the mean value for secretion during the control period *$p<0.05$, **$p<0.01$.

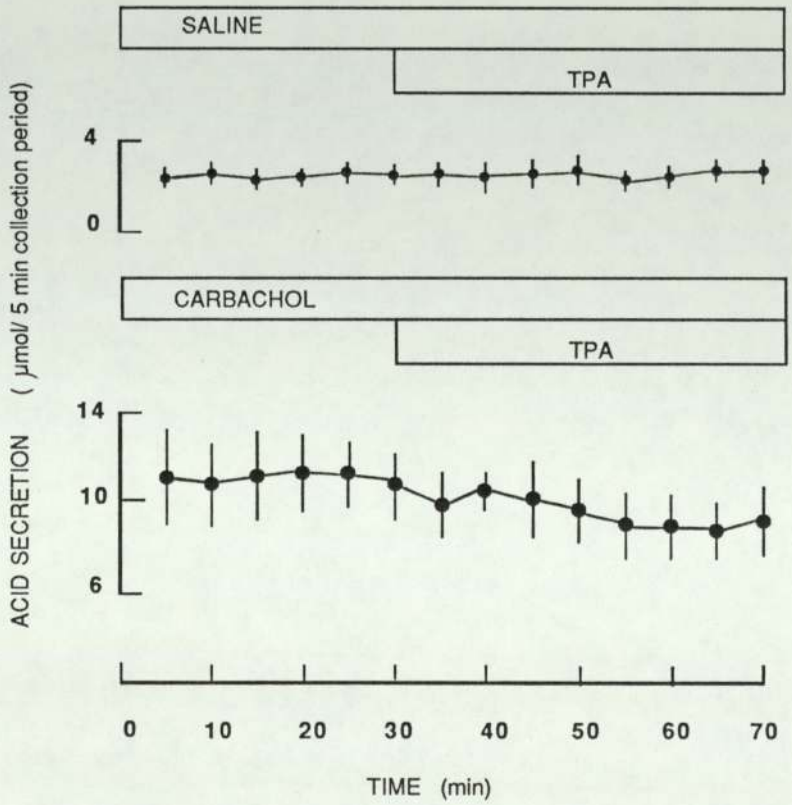
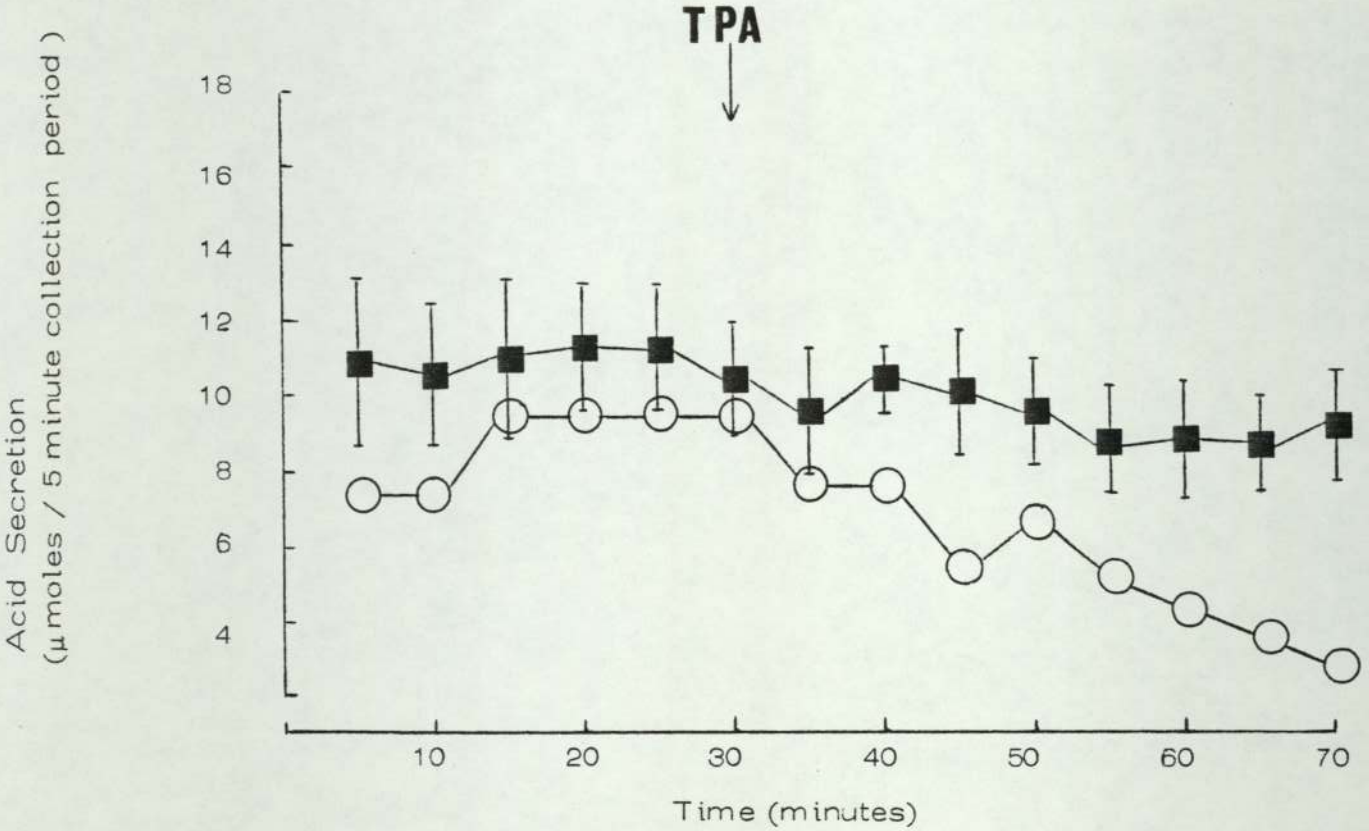


Figure 4.10

The effect of the presence of TPA (1µM) in the luminal perfusate on gastric acid secretion in vivo in rats infused intravenously with saline (9g/l NaCl,3.7µl/min) or carbachol (0.1µg/min per Kg body weight). TPA was present in the luminal perfusate only after a 30 minute control period.

Figure 4.11

The effect of the presence of TPA (1μM) in the luminal perfusate on gastric acid secretion in vivo in rats infused intravenously with carbachol (0.1μg/min/Kg body weight, ■) or histamine phosphate (30μg/min/Kg body weight, O). The carbachol data is the mean ± S.E.M. from 4 experiments and the histamine phosphate data is from a single experiment. TPA was present in the luminal perfusate only after a 30 minute control period. (Arrow indicates the point at which TPA infusion was started).



However, other inhibitors of gastric acid secretion are effective when present in the stomach e.g. a synthetic analogue of prostaglandin E_2, 16, 16-dimethyl prostaglandin E_2, significantly inhibited meal-stimulated gastric acid secretion from patients with duodenal ulcers after oral administration (Ippoliti et al., 1976). Since serum gastrin levels were also reduced, the anti-secretory effect of 16, 16-dimethyl prostaglandin E_2 may have been secondary to an effect on gastrin liberation.

Having established that TPA (1μM) does genuinely reduce acid secretion from rat parietal cells in vivo in response to histamine and pentagastrin stimulation. It is important to establish whether this effect is specific, and not due to generalized tissue damage. A drug causing non-specific tissue damage would probably induce the same degree of inhibition of acid secretion stimulated by histamine, pentagastrin or carbachol. Such a drug would also be expected to reduce basal acid secretion, and TPA did not. The phorbol ester 4αphorbol 12,13-didecanoate (4αPDD) which is structually similar to TPA but does not activate protein kinase C (Castagna et al., 1982) did not inhibit acid secretion stimulated by pentagastrin (fig 4.9, table 4.6). However, if the inhibitory action of TPA had been due to the production of non-specific tissue damage, its action may have been mimicked by the inactive phorbol ester. That DMSO itself reduces the rate of acid secretion is unlikely since the concentration of DMSO was very low (0.006%) and 4αPDD, also dissolved in DMSO, had no effect on acid secretion stimulated by pentagastrin (fig 4.9, table 4.6).

Table 4.6

Effect of the presence of 4α-PDD(1μM) in the luminal perfusate on gastric acid secretion in vivo in rats infused intravenously with pentagastrin (1.6μg/min per Kg body weight). 4α-PDD was present in the luminal perfusate only after a 30 minute control period.

The results are given for 4 experiments as the mean ± S.E.M.

Time, (minutes).	Rate of acid secretion, (μmoles of H^+ per 5 minute collection period).
5	15.9 ± 2.0
10	14.6 ± 1.7
15	14.8 ± 2.5
20	15.0 ± 2.8
25	14.4 ± 2.4
30	15.1 ± 2.0
35	14.5 ± 1.9
40	14.8 ± 2.3
45	13.8 ± 1.8
50	14.8 ± 3.0
55	14.3 ± 2.6
60	14.1 ± 2.1
65	13.4 ± 1.7
70	14.1 ± 2.1

As previously indicated, TPA may reduce the aminopyrine accumulation within the secretory canaliculus of the parietal cell by 1) causing a genuine inhibition of acid secretion, 2) enhancing the back-diffusion of acid into the cell, or 3) increasing the dissipation of acid from the secretory canaliculus (fig 4.12). Should TPA reduce aminopyrine accumulation by either mechanism 2 or 3, the degree of inhibition achieved in the presence of TPA would be expected to be similar for all secretagogues. If TPA were acting by stimulating proton back diffusion by a mechanism similar to thiocyanate, aminopyrine accumulation by resting cells would also be reduced (Wallmark et al., 1983), this is clearly not true (table 4.5). The data obtained using this in vivo model parallels previous in vitro data (Anderson and Hanson, 1984 and table 4.3), which implies that the TPA-mediated inhibition of aminopyrine accumulation stimulated by histamine plus IBMX and dbcAMP in vitro, is indeed a genuine effect on acid secretion. This inhibitory effect of TPA on acid secretion may be mediated via protein kinase C.

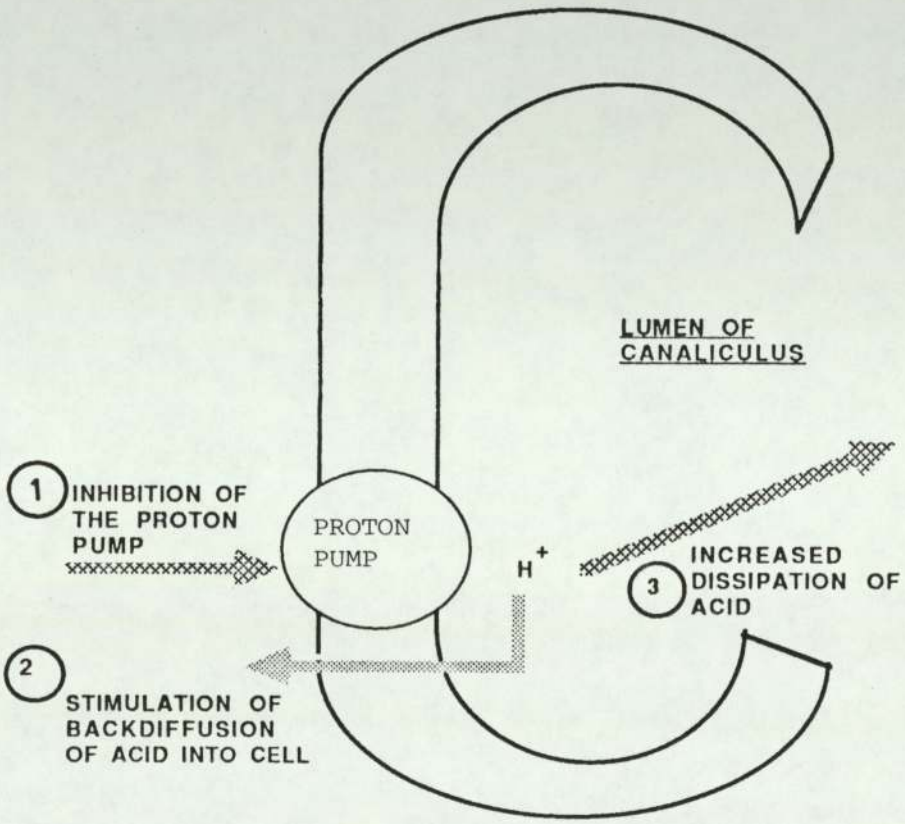


Figure 4.12

Putative mechanisms of TPA action.

CHAPTER 5.

IDENTIFICATION OF POSSIBLE ENDOGENOUS SUBSTRATES FOR THE CALCIUM-SENSITIVE, PHOSPHOLIPID-DEPENDENT PROTEIN KINASE IN RAT PARIETAL CELLS.

5.1 INTRODUCTION.

5.1.1 Protein phosphorylation.

The interaction of a given hormone with its plasma membrane receptor generates a signal which is often the alteration of the concentration of a specific chemical mediator within the cell. One function of these chemical mediators or second messengers is the regulation of the activity of intracellular protein kinases or phosphatases which govern the phosphorylation state of particular substrate proteins. In some cases these substrate proteins are enzymes which may be activated or inhibited by reversible phosphorylation. In general, enzymes involved in biodegradative pathways are stimulated by phosphorylation and enzymes involved in biosynthetic pathways are inhibited by phosphorylation (Ingebritsen and Cohen, 1983a).

The first example of enzyme regulation by reversible protein phosphorylation (the activation of glycogen phosphorylase by phosphorylase kinase) was described by Krebs and Fischer (1956). Since then, several additional protein kinases have been identified and classified according to their specific chemical mediators (Krebs and Beavo, 1979). The three principle groups of protein kinases are given in table 5.1. In addition, a protein kinase which is dependent on calcium and phospholipid for maximal activity (protein kinase C) has been identified (Takai et al., 1979). The properties of this enzyme have been more fully discussed in chapter 4.

Table 5.1

Protein kinase classification on the basis of their specific chemical mediators

Category	Protein kinase Group	Example
1	cAMP-dependent	Type I and Type II
2	cGMP-dependent	only one type
3	Calcium-dependent	Phosphorylase kinase

5.1.1.1 Cyclic AMP-dependent protein kinase.

Two distinct isoenzymes of cAMP-dependent protein kinase have been identified using DEAE-cellulose chromatography (Reimann et al., 1971). Both isoenzymes possess the subunit structure R_2C_2, and have similar kinetic properties. The inactive holoenzyme is activated by cAMP according to equation 5.1.

Equation 5.1

$$R_2C_2 \text{ (inactive)} + 4cAMP \rightleftharpoons R_2(cAMP)_4 + 2C \text{ (active)}$$

R: regulatory subunit.
C: catalytic subunit.

Although the catalytic subunits of both cAMP-dependent protein kinase isoenzymes are identical (Yamamura et al., 1983), the regulatory subunits have different molecular weights (Nimmo and Cohen, 1977).

5.1.1.2 Cyclic GMP-dependent protein kinase.

cGMP-dependent protein kinase activity has been demonstrated in a wide range of mammalian tissues (Kuo, 1974). The enzyme has a molecular weight of 165Kda, and exists as a dimer with both cGMP-binding and catalytic activities on the same polypeptide chain (Lincoln et al., 1977). Although it is clear that activation of this enzyme by low concentrations (7.5 - 30nM) of cGMP requires the presence of a stimulatory modulator protein (Kuo et al., 1976), the precise mechanism of action of cGMP is uncertain.

5.1.1.3 Calcium-dependent protein kinases.

Several calcium-dependent protein kinases have been identified, probably the best characterized of which is phosphorylase kinase (EC 2.7.1.38). A brief description of the properties of phosphorylase kinase serve as a good example of this group of enzymes. Phosphorylase kinase isolated from rabbit skeletal muscle is composed of four subunits termed α, β, γ, δ (Shenolikar et al., 1979), and the holoenzyme has a molecular weight of 133Kda (Hayakawa et al., 1973). The α and β subunits maintain the enzyme in an inactive state and modification of these subunits by phosphorylation (Cohen, 1973), limited proteolysis (Cohen, 1973), or interaction with exogenous calmodulin (Picton et al., 1980), serves to increase kinase activity by reducing the K_m for phosphorylase b, (Picton et al., 1980). The δ-subunit of phosphorylase kinase is the calcium-binding protein, calmodulin. The γ-subunit has been identified as the catalytically active domain of phosphorylase kinase (Skuster et al., 1980). Although the principle substrate for phosphorylase kinase in vivo is phosphorylase b, the purified enzyme may also phosphorylate a range of other proteins in vitro e.g. troponin T (Moir et al., 1977).

5.1.2 Protein dephosphorylation.

Four classes of protein phosphatase have been identified, termed protein phosphatase 1, and protein phosphatases 2A, 2B and 2C (Ingebritsen and Cohen, 1983b). These phosphatases account for most of the observed phosphatase activity involved in regulating metabolic pathways. Protein phosphatase 1 selectively dephosphorylates the β-subunit of phosphorylase kinase, and the three type-2 protein phosphatases preferentially

dephosphorylate the α-subunit of phosphorylase kinase. Protein phosphatases 1, 2A and 2C possess broad yet distinctive substrate specificities, unlike protein phosphatase 2B which only dephosphorylates inhibitor 1 and myosin light chains in addition to the α-subunit of phosphorylase kinase. Inhibitors 1 and 2 inhibit protein phosphatase 1 but are ineffective against the class 2 protein phosphatases (Ingebritsen and Cohen, 1983b).

5.1.3 The role of reversible protein phosphorylation in parietal cell stimulus-secretion coupling.

Changes in the phosphorylation state of intracellular proteins, as a result of altered kinase or phosphatase activity, provides the primary mechanism by which hormones and other extracellular agents regulate cellular functioning (Greengard, 1978). It is of particular interest that protein phosphorylation has been implicated in stimulus-secretion coupling in a wide range of cell types. In order to confirm the role of protein phosphorylation in stimulus-secretion coupling in a given cell type several criteria must be satisfied (Walsh and Cooper, 1979).

1. Protein kinase activity must be demonstrated within the cell.

2. Endogenous substrates for the protein kinase must also be demonstrated.

3. The state of substrate phosphorylation must alter with the onset of secretion.

These criteria have been completely satisfied for protein kinase C activity in platelets (Sano et al., 1983), and protein kinase C, calcium, calmodulin-dependent protein kinase, and cAMP-dependent protein kinase in pancreatic islets (Thams et al., 1984, and Suzuki et al., 1983). However, these criteria have only been partially satisfied for a limited number of protein kinases in parietal cells.

5.1.4 Protein kinase activity in parietal cells.

Histamine-stimulation of acid secretion is associated with an increase in the intracellular concentration of cAMP (Chew et al., 1980), while stimulation by cholinergic agonists is associated with an enhancement of calcium influx into the parietal cell (Soll, 1980a). The increase in the intracellular level of cAMP which accompanies histamine-stimulated acid secretion activates a cAMP-dependent protein kinase (Chew, 1983). Similarly, the increase in the intracellular calcium concentration associated with cholinergic stimulation may activate the calcium-dependent protein kinases, although this is by no means certain. Both isoenzymes of cAMP-dependent protein kinase have been identified in rabbit gastric glands (Jackson and Sachs, 1982), and in a rabbit enriched parietal cell preparation (Chew, 1985). However, histamine-stimulated acid secretion was shown to be associated with the specific activation of the cytosolic "type I" isoenzyme (Chew, 1985).

While a considerable amount of evidence is available to support the presence of cAMP-dependent protein kinase within the parietal cell, only a limited amount of work has been carried out to establish the existence of other

parietal cell protein kinases. Calcium and phospholipid-dependent protein kinase (protein kinase C) activity has only recently been demonstrated in an enriched parietal cell preparation (Anderson and Hanson, 1985; Chew, 1985), and a role for this enzyme in the regulation of gastric acid secretion has been proposed, such that activation of protein kinase C, may be involved in the negative modulation of histamine-stimulated acid secretion *in vivo* (Anderson and Hanson, 1985). This proposal is consistent with the data shown in chapter 4, which indicates that protein kinase C activation by TPA inhibits histamine-stimulated aminopyrine accumulation and acid secretion *in vivo*.

5.1.5 Protein kinase substrates within the parietal cell.

In order to establish a role for these protein kinases in the acid-secretory process, it is necessary to identify the specific protein substrates of these enzymes, and to demonstrate a change in their phosphorylation state with the onset of secretion (criteria 2 and 3). Endogenous protein substrates for a calcium-dependent protein kinase have been identified in a preparation of rat gastric membranes (Shaltz *et al.*, 1981). However, the parietal cell origin of these proteins cannot be certain. Modlin *et al.*, (1986), using an homogenate derived from a rabbit enriched parietal cell preparation (75 $\pm$ 5% parietal cells) showed that eight cytosolic protein substrates for cAMP-dependent protein kinase and two cytosolic protein substrates for a calcium-dependent protein kinase were most likely located in the parietal cell.

A very limited amount of information has so far been accumulated regarding the protein substrates for the parietal cell protein kinases. It is of particular importance to identify these substrate proteins since the control of cellular functioning by reversible phosphorylation is achieved through their selective distribution throughout the various cell-types (Langan, 1973). It was the aim of this work to investigate the endogenous substrate proteins for protein kinase C using a cell-free homogenate derived from a cell preparation enriched in parietal cells. This preparation was chosen in preference to an intact-cell system for the following reasons;

1) Using the cell-free system does not require long pre-incubation periods to allow equilibrium of ^{32}P between intracellular P_i , ATP, and phosphoproteins.

2) Interpretation of the protein phosphorylation data is facilitated by the use of the cell-free system, because, unlike an intact cell system, there is no prior background of ^{32}P-phosphorylation against which changes in phosphorylation have to be assessed.

5.2 METHODOLOGY

5.2.1 Phosphorylation assay of endogenous parietal cell proteins.

A cell preparation, enriched in parietal cells using the method described in section 2.2.3 , was used routinely to determine the existence of endogenous parietal cell proteins which were phosphorylated by protein kinase C. This cell preparation contained on average 74% parietal cells with approximately 89% of the aggregated cell volume being occupied by parietal cells. So as to assign specific proteins phosphorylated by protein kinase C unequivocally to the parietal cell, a modified enrichment procedure was designed (2.2.4) to produce a parietal cell fraction containing up to 97% parietal cells. By using this highly enriched fraction, the association of protein kinase C substrates with parietal cells could be definitely established.

The cell preparation was homogenized by sonication (6 x 5s, 4 watts) at 4^0C in homogenization buffer [50mM-MES(pH 7.0), 1mM-$MgCl_2$, 0.1mM-dithiothreitol, 10mM-Benzamidine, 10mM-EGTA, leupeptin (1µg/ml), and pepstatin (1µg/ml)], and the homogenate centrifuged at 100,000g for 1 hour at 4^0C to produce particulate and cytosolic fractions. Samples of the particulate or cytosolic fraction in homogenization buffer were added to the reaction mixture (final volume 50µl) containing test reagents as indicated in the legends to figures 5.1 to 5.5, and were preincubated for 2 minutes at 30^0C before the reaction was started by the addition of [γ-^{32}P]ATP (final concentration 40µM, 200µ Ci/ml). When phosphatidylserine and TPA were required in the incubation medium, 2µl was added from a combined stock solution prepared as described in appendix A6. After 3

minutes, 40µl of the reaction mixture was added to 20µl of a stop solution [0.2M-Tris/HCl (pH 7.8), 9.6% (w/v) SDS, 8% (v/v) 2-mercaptoethanol, 16% (v/v) glycerol, and 0.008% (w/v) bromophenol blue] and the mixture was heated at 95^{0}C for 5 minutes.

5.2.2 SDS/Polyacrylamide-gel electrophoresis and autoradiography

An LKB vertical electrophoresis system was used in this study. Samples in stop solution (40µl) were loaded onto SDS/polyacrylamide gels (160mm x 180mm x 0.75mm) and electrophoresed using a modification of the method of Laemmli (1970). The protein concentration of each sample was measured using the method of Lowry et al., (1951), with bovine serum albumin as standard (appendix A7).

The separating gel contained 10% (w/v) acrylamide, 0.31% (w/v) acrylaide, 0.375M-Tris/HCl pH8.8 and 0.1% SDS. The stacking gel contained 3% (w/v) acrylamide, 0.08% (w/v) bisacrylamide, 0.125M-Tris/HCl pH6.8 and 0.1% SDS. The electrophoresis buffer was 0.025M-Tris/HCl, 0.192M-glycine, and 0.1% SDS. The proteins were separated at a constant current of 25mA at 10^{0}C with the maximum voltage limited to 500V.

After fixation and staining for 1 hour with PAGE-blue 83 [0.25% (w/v) in methanol: water: acetic acid (5:5:1)] and destaining for two hours in two changes of methanol: acetic acid: water (1:4:15), gels were dried onto filter paper (LKB 2003 slab gel dryer), and subjected to autoradiography with X-OMAT AR film. The film was developed as described in appendix A8. The molecular weights of the relevant phosphorylated proteins were

determined using the following standards (molecular weight) : Myosin (200,000), β-galactosidase (116,000), phosphorylase b (97,000), bovine albumin (66,000), ovalbumin (45,000), carbonic anhydrase (29,000), and α-lactalbumin (14,200). The autoradiograms were scanned using an LKB ultrascan laser densitometer.

In experiments involving calcium, the volume of $CaCl_2$ to be added to give the required concentration of free calcium in the reaction mixture was determined by computer program (Storer and Cornish-Bowden, 1976). The validity of these calculations was determined by using an Orion 93-20 calcium electrode (appendix A9).

5.3 RESULTS AND DISCUSSION.

5.3.1 The effect of phosphatidylserine and TPA on the pattern of phosphorylation in the 100,000g supernatant of a parietal cell homogenate.

In the presence of phosphatidylserine (20µg/ml) and TPA (32nM) an increase in the phosphorylation of a band of molecular weight 89.2 ± 1.13Kda (6 preparations) in the 100,000g supernatant of a parietal cell homogenate (5.2.1) was evident (figure 5.1 and plate 5.1). It is important to note that plate 5.1 and figure 5.1 were derived from different experiments. This band will subsequently be referred to as the 89Kda band. In addition to demonstrating the increased phosphorylation of this band in the presence of phosphatidylserine and TPA, plate 5.1 shows the ability of calcium (100µM) to enhance the dephosphorylation of a number of proteins present in the 100,000g supernatant derived from a parietal cell homogenate. This effect of calcium will be discussed in section 5.3.5. Although in individual experiments other bands with approximate molecular weights of 15Kda, 100Kda and 170Kda were also phosphorylated in the presence of phosphatidylserine and TPA, this phosphorylation was not consistent, and therefore this discussion will concentrate only on the phosphorylation of the 89Kda band.

5.3.2 The nature of the 89Kda band

The possibility that this 89Kda band may correspond to phosphorylated lipid residues can be excluded, since extraction of the gel with chloroform : methanol (2:1, v/v) and ether : ethanol (1:1, v/v) prior to drying and

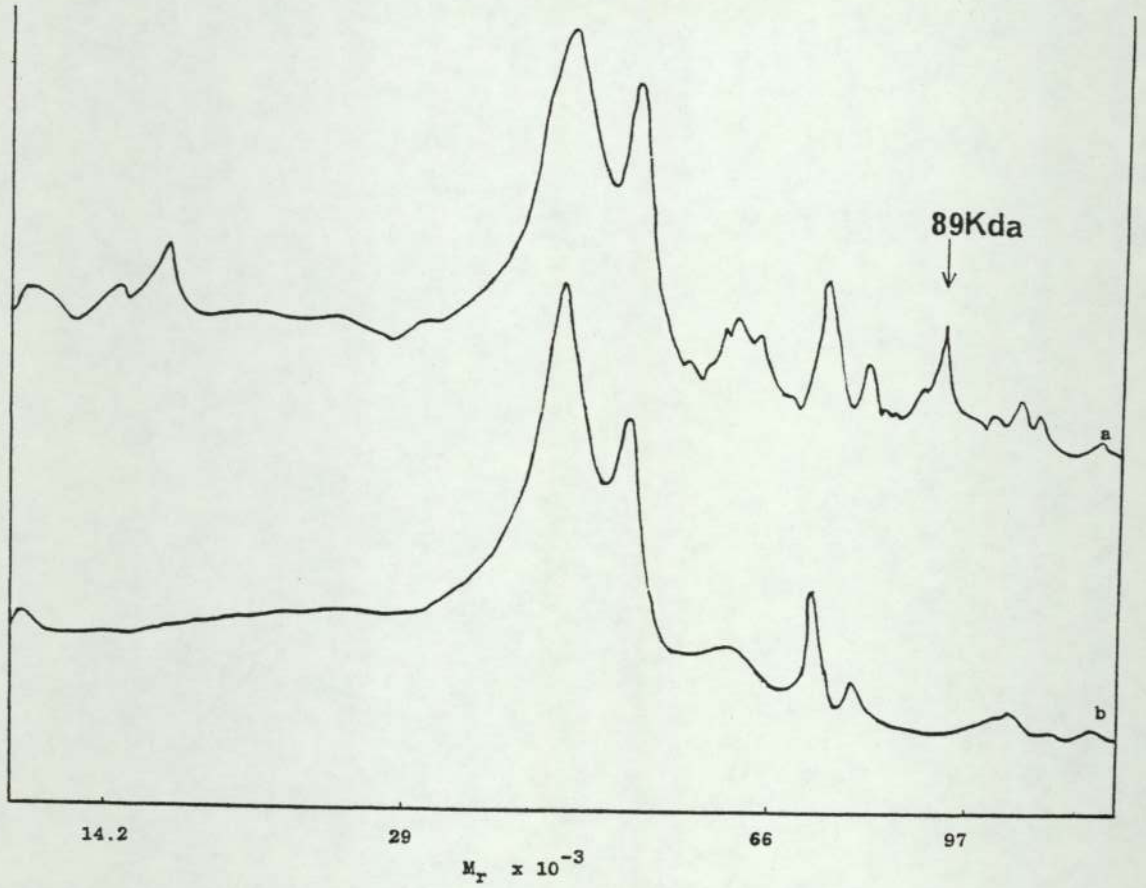


Figure 5.1

Densitometer traces of autoradiograms showing the effect of a 3 minute incubation in the presence of phosphatidylserine (20µg/ml) and TPA (32nM) on the phosphorylation of proteins in a 100,000g supernatant derived from a cell preparation containing 73% parietal cells (2.2.4).

Lane a: phosphatidylserine and TPA. Lane b: no additions.

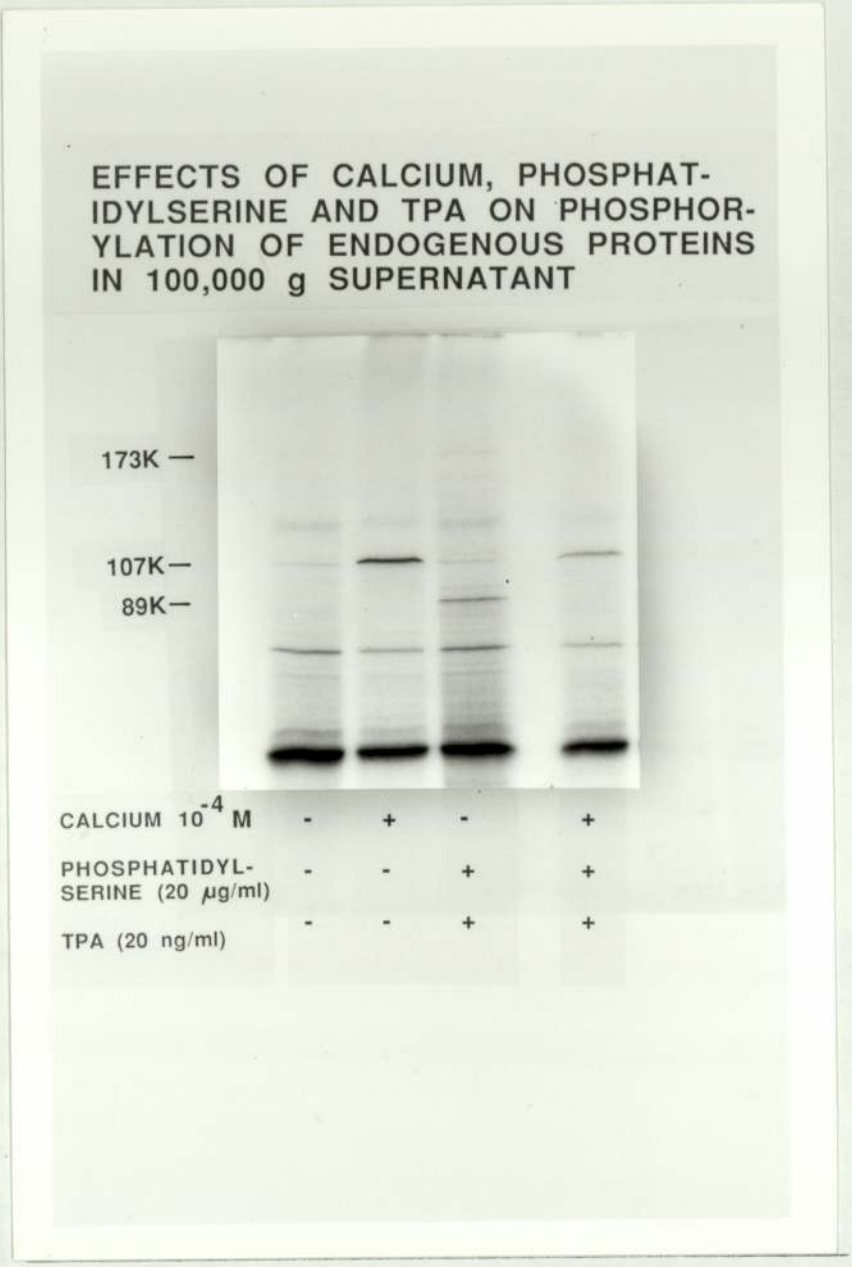


Plate 5.1

Autoradiogram showing the effect of a 3 minute incubation in the presence of phosphatidylserine (20µg/ml) plus TPA (32nM) and/or calcium (100µM), on the phosphorylation of proteins in a 100,000g supernatant derived from a cell preparation containing 63% parietal cells.

autoradiography did not diminish the intensity of this band. This extraction procedure was used previously to ensure that the phosphorylated bands in a solubilized preparation of C-6 glioma cells were in fact phosphorylated polypeptides and not phosphorylated lipids (Groppi and Browning, 1980). In addition, the possibility that the 89Kda band present in the 100,000g supernatant was an acyl-phosphate can be excluded, since these compounds are removed by the elevated temperature and pH used to stop the reaction (Rudolph and Krueger, 1979). The 89Kda band is therefore neither a phospholipid nor an acyl-phosphate. In fact, since the 89Kda band on the polyacrylamide gel could be soaked in ice-cold 0.5M-NaOH for 1 hour without any loss of label, but was susceptible to treatment with 0.5M-NaOH at 90^0C, it is most likely that the $^{32}P_i$ is bonded to a serine or threonine residue (Garrison, 1983).

5.3.3 Is the 89Kda band an endogenous substrate for protein kinase C?

The 89Kda band was present in the 100,000g cytosolic fraction derived from the parietal cell homogenate but was absent from the particulate fraction. For this reason all the experiments in this section were performed using only the 100,000g cytosolic fraction.

An increase in the degree of phosphorylation of this 89Kda protein was observed in the presence of phosphatidyl-serine and TPA without exogenous calcium (figure 5.1). This result may be explained if the 89Kda protein were a substrate for the parietal cell protein kinase C, and this enzyme could be activated in the presence of exogenous phosphatidylserine and TPA alone. The following evidence supports this hypothesis. Firstly, activation of protein kinase C by TPA occurs via a reduction in the calcium

requirement of the enzyme (Castagna et al., 1982), and maximal activation of protein kinase C by TPA occurs at calcium concentrations in the μM range (Couturier et al., 1984). The total (bound plus free) intracellular calcium concentration of the parietal cell is likely to be between 1 and 10mM (Campbell, 1983), and the free calcium concentration in the parietal cell homogenate used for these experiments in the presence of 10mM-EGTA, can be estimated to be between 0.61nM and 6.3nM in the absence of added calcium (appendix A9.2). However, even at these very low levels of intracellular calcium, TPA may still substantially activate protein kinase C in vitro (Couturier, et al., 1984). Secondly, TPA alone is able to stimulate protein kinase C activity and exocytosis in neutrophils when the intracellular calcium concentration is within the nM range (Di Virgilio et al., 1984). Thus, it seems likely that the activity of protein kinase C may be stimulated in a parietal cell homogenate by phospholipids and TPA in the absence of added calcium.

The increase in the degree of phosphorylation of the 89Kda protein observed in the presence of phosphatidylserine and TPA, may alternatively be explained by the existence of a calcium-independent but TPA-activated and phosphatidylserine-dependent enzyme, distinct from protein kinase C. Such an enzyme has been termed protein kinase L by Malviya et al., (1986). Although these two enzymes are physically distinct, both activities are influenced by phorbol ester or diacylglycerol, phosphatidylserine is required for maximal activity, and histone H1 is the preferred substrate for both enzymes. If the enzyme responsible for the phosphorylation of the 89Kda protein is indeed protein kinase L then this enzyme exhibits similar properties to protein kinase C, with respect to translocation to the plasma membrane upon activation and inhibition by trifluoperazine and polymyxin B.

Thus pre-incubation of the parietal cell preparation (30 min) in the presence of 100nM-TPA caused a reduction in the phosphorylation of the 89Kda protein when the subsequently isolated 100,000g cytosolic fraction was incubated with phospholipid and TPA (figure 5.2, plate 5.2). This can be explained by the translocation of protein kinase activity to the plasma membrane as suggested by Kraft and Anderson (1983), for protein kinase C, thereby reducing the cytosolic kinase activity. In the presence of TPA, protein kinase C becomes so tightly associated with the plasma membrane that it cannot be disssociated with high salt (0.3M-NaCl) buffer or with buffer containing 1mM-EDTA (Kraft and Anderson, 1983).

The polypeptide antibiotic polymyxin B, and the anti-psychotic drug trifluoperazine, both reduced the degree of phosphorylation of the 89Kda protein (figure 5.3). This finding is consistent with the 89Kda protein being a substrate for protein kinase C. Although trifluoperazine can inhibit protein kinase C without affecting protein kinase G activity (Schatzman et al., 1981), it does function as a calmodulin antagonist (Levin and Weiss, 1976) and cannot therefore be considered a specific inhibitor of protein kinase C. However, at the concentration used in this study (10^{-5}M) polymyxin B may be considered a potent and selective inhibitor of protein kinase C (Wrenn and Wooten, 1984).

Finally, the possibility exists that the 89Kda protein may be autophosphorylated protein kinase C. This has previously been shown in a number of preparations. Wolf et al., (1984), demonstrated the existence of a 77Kda polypeptide which co-purified with protein kinase C activity. These workers concluded that this polypeptide was in fact protein kinase C which was autophosphorylated in a calcium, phospholipid-dependent manner. Hansson et al., (1986) also demonstrated significant autophosphorylation of partially

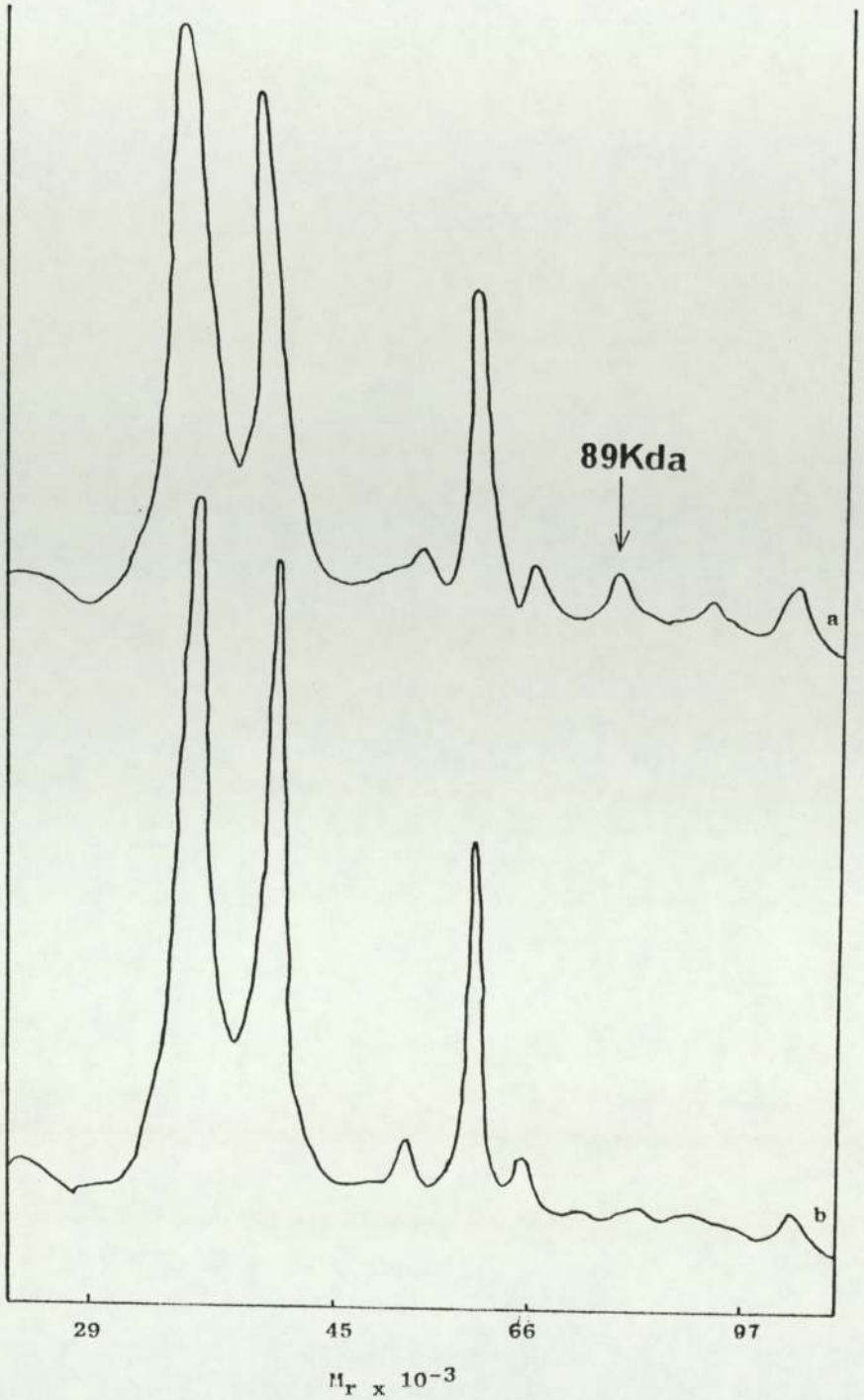


Figure 5.2

Densitometer traces of autoradiograms showing the effect a 30 minute pre-incubation of intact cells with TPA (100nM) at 37°C on the subsequent phosphorylation of proteins in the 100,000g supernatant incubated for 3 minutes in the presence of phosphatidylserine (20µg/ml) and TPA (32nM).

Lane a; cells pre-incubated in the presence of DMSO before homogenization.

Lane b; cells pre-incubated in the presence of TPA before homogenization.

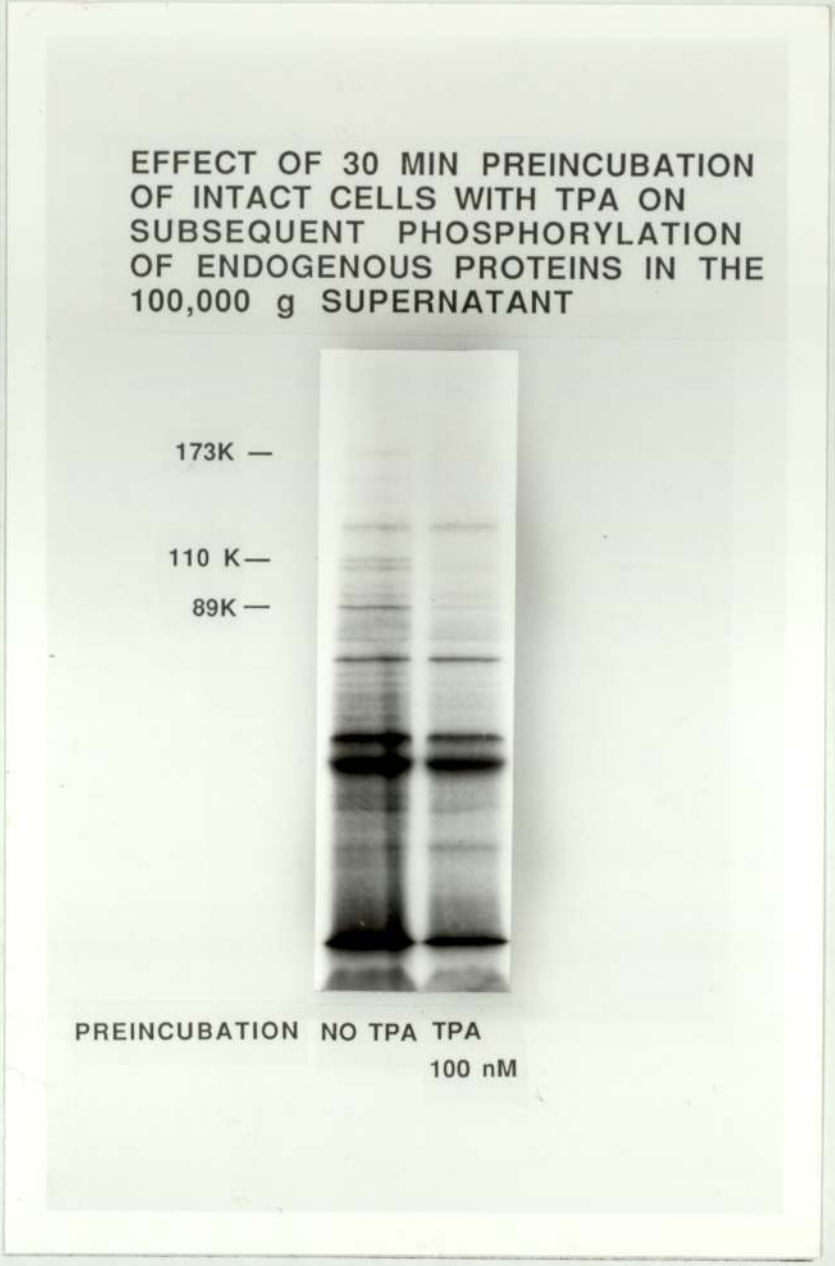


Plate 5.2

Autoradiogram showing the effect of a 30 minute pre-incubation of intact cells with 100nM-TPA at 37°C on the subsequent phosphorylation of proteins in the 100,000g supernatant incubated for 3 minutes in the presence of phosphatidylserine (20µg/ml) and TPA (32nM). The 100,000g supernatant was derived from a cell preparation containing 70% parietal cells (2.2.4).

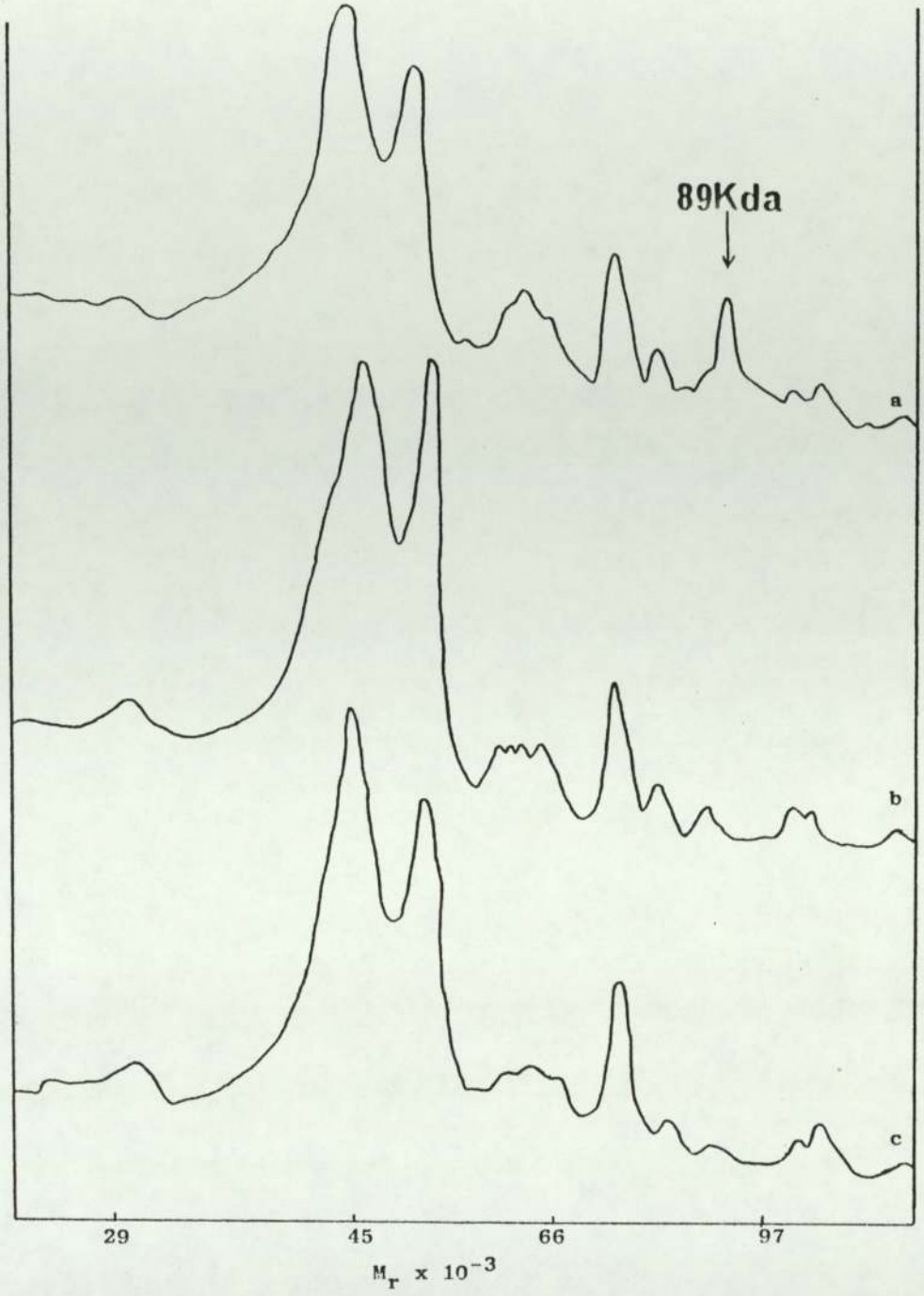


Figure 5.3

Densitometer traces of autoradiograms showing the effect of a 3 minute incubation in the presence of polymyxin B (10μM) or trifluoperazine (100μM) on the phosphorylation of certain proteins in a 100,000g supernatant derived from a cell preparation containing 73% parietal cells (2.2.4).

Lanes a-c: phosphatidylserine (20μg/ml) and TPA (32nM), in addition, Lane b: polymyxin B and Lane c: trifluoperazine.

purified protein kinase C (mwt 80Kda) from human placental cytosol in the presence of calcium and phosphatidylserine. Autophosphorylation of protein kinase C in the presence of calcium, phospholipid and diacylglycerol has also been demonstrated in macrophage-depleted human lymphocytes (Kaibuchi et al., 1985). While it is possible that the phosphorylated 89Kda protein may be autophosphorylated protein kinase C, this is by no means certain. In fact, other workers (Albert et al., 1986), have demonstrated the widespread occurrence of an 87Kda protein kinase C substrate which is distinct from protein kinase C. In addition, Blackshear et al., (1986) have presented evidence based on several physical criteria that an 80Kda protein kinase C substrate [thought to be similar or identical to the 87Kda protein of Albert et al., (1986)], present in 3T3-L1 fibroblasts and the protein kinase itself are distinct proteins.

5.3.4 The cellular origin of the 89Kda protein

Having established that the 89Kda protein present in the 100,000g cytosolic fraction is indeed a substrate for protein kinase C, the presence of this protein within the parietal cell must be established. This was achieved using homogenates derived from cell preparations containing varying proportions of parietal cells. The degree of phosphorylation of the 89Kda protein in the presence of phosphatidylserine and TPA was shown to increase with the proportion of parietal cells in the starting homogenate (figure 5.4). This suggests that the 89Kda protein is associated with the parietal cell. This was further confirmed by the observation than an 89Kda cytosolic protein derived from an homogenate containing 97% parietal cells was phosphorylated in the presence of phosphatidylserine and TPA (figure 5.5), making a non-parietal cell origin of the protein most unlikely.

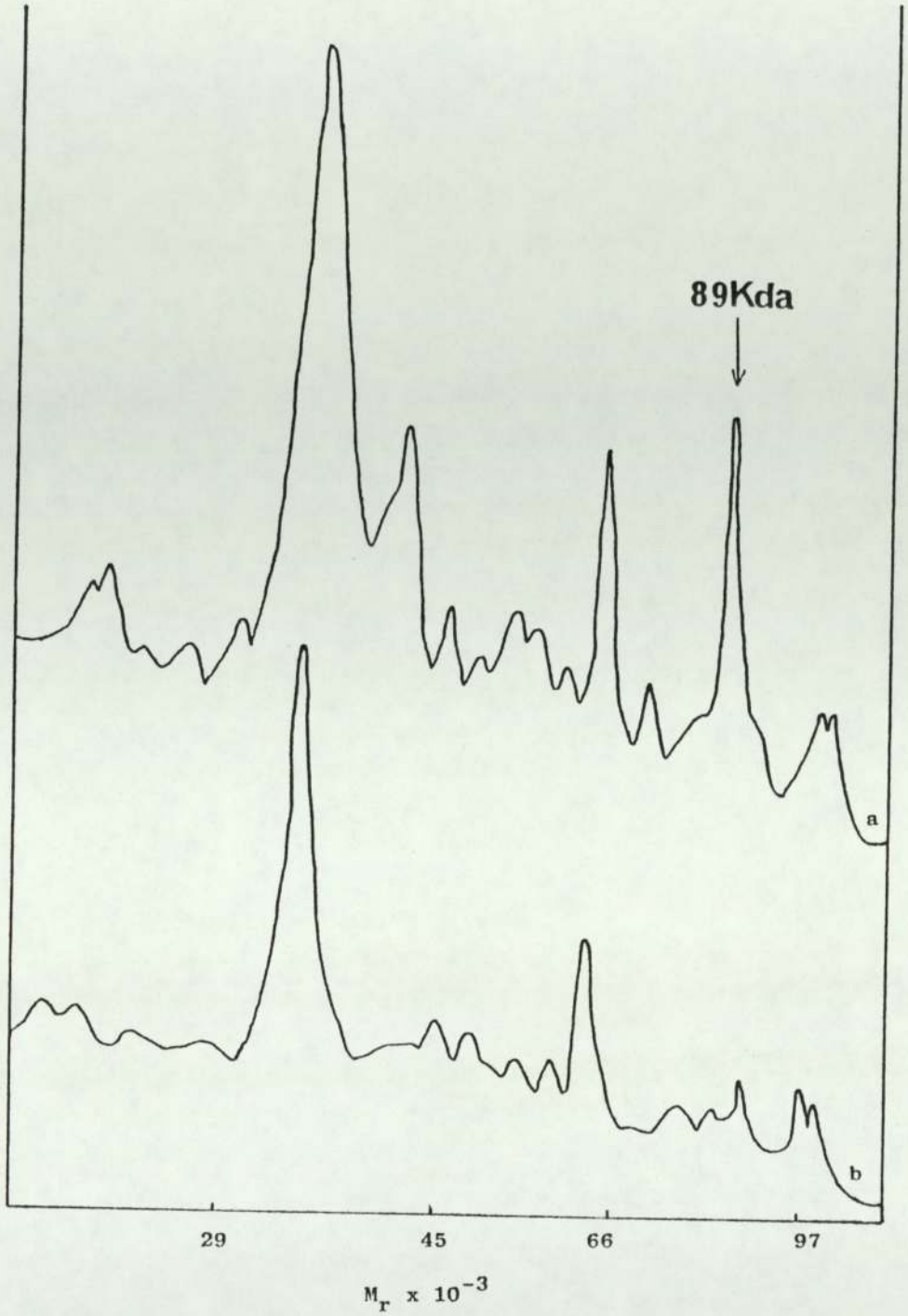


Figure 5.4

Densitometer traces of autoradiograms showing the pattern of protein phosphorylation in two 100,000g supernatants derived from cell preparations containing high and low proportions of parietal cells.

Both cell preparations were incubated for 3 minutes in the presence of phosphatidylserine (20µg/ml) and TPA (32nM). 47µg of protein was applied to each lane.

Lane a: 80% parietal cells, Lane b: 24% parietal cells.

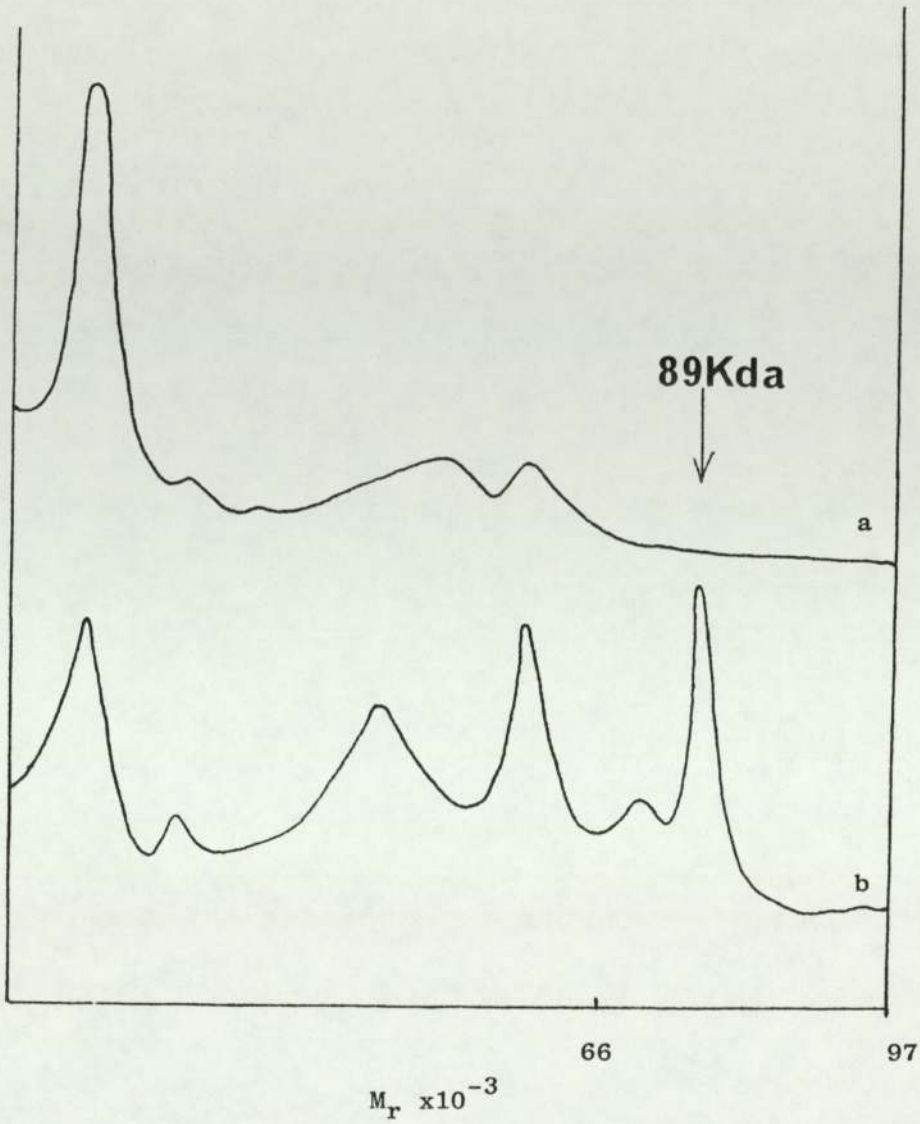


Figure 5.5

Densitometer traces of autoradiograms showing the effect of a 3 minute incubation in the presence of phosphatidylserine (20µg/ml) and TPA (32nM), on the phosphorylation of certain proteins in a 100,000g supernatant derived from a cell preparation containing 97% parietal cells (2.2.4).

Lane a: no additions,

Lane b: phosphatidylserine and TPA.

5.3.5 The effect of calcium on the phosphorylation of the 89Kda protein induced by phosphatidylserine and TPA.

Phosphatidylserine and TPA enhanced the phosphorylation of an 89Kda protein present in the 100,000g supernatant derived from a parietal cell homogenate. Phosphorylation of this protein was reduced in a concentration-dependent manner by calcium (figure 5.6). In the presence of 100nM-calcium no reduction in the degree of phosphorylation of the 89Kda protein was observed, however, when the calcium concentration was increased to 500nM and 100μM (plate 5.1), phosphorylation of the 89Kda band was completely suppressed. Since the assay system measured the net phosphorylation of parietal cell proteins, this effect of calcium could be explained by either an inhibition of phosphorylation or a stimulation of dephosphorylation. As calcium, at the concentrations used in this study, is more commonly associated with the stimulation of endogenous protein phosphorylation (Thams et al., 1984), the first alternative was considered unlikely. However, the discovery of a calcium and calmodulin-dependent protein phosphatase (protein phosphatase 2B) by Stewart et al., (1982), makes the second alternative plausible. In the presence of calcium alone, protein phosphatase 2B can dephosphorylate phosphorylase kinase, inhibitor-1, and myosin P-light chain from rabbit skeletal muscle although calmodulin is required for maximal activity (Stewart et al., 1983). Other evidence also supports this second alternative. Using histone H1 phosphorylated by protein kinase C and protein kinase A, Le Vine et al., (1984), demonstrated the existence of a group of rat liver phosphatases which selectively dephosphorylated the histone at the sites initially phosphorylated by protein kinase C. The presence of a phosphatase of this type within the parietal cell homogenate which is activated by calcium would explain the calcium effect (figure 5.6).

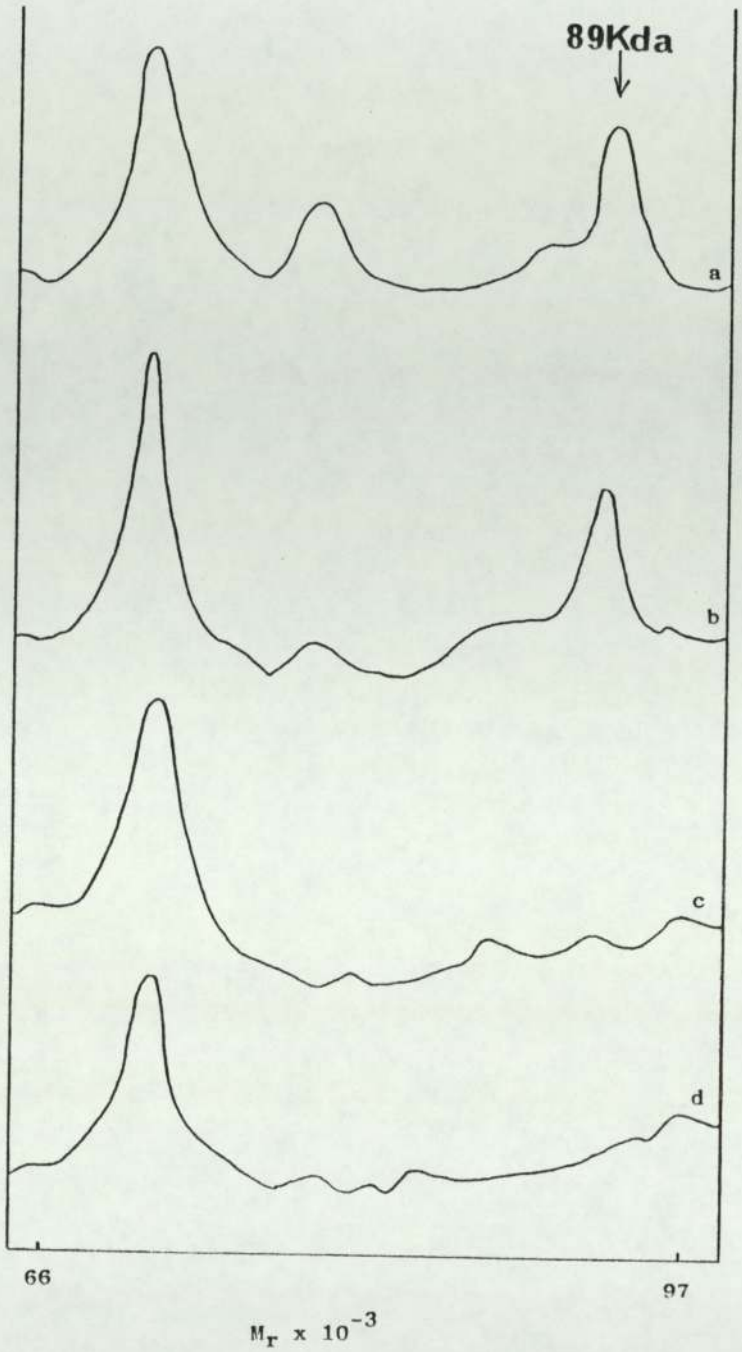


Figure 5.6

Densitometer traces of autoradiograms showing the effect of a 3 minute incubation in the presence of various calcium concentrations, on the phosphorylation of certain proteins in a 100,000g supernatant derived from a cell preparation containing 73% parietal cells (2.2.4).

Lanes a-d: phosphatidylserine (20µg/ml) and TPA (32nM), in addition, Lane b: 100nM-Calcium, Lane c: 500nM-Calcium. Lane d: 100µM-calcium.

A significant reduction by calcium in the degree of phosphorylation induced by phospholipids and TPA was achieved within 30 seconds of the start of the reaction (figure 5.7, trace c), and even when calcium was added at the same time as the [γ-^{32}P] ATP used to start the reaction, a considerably reduced phosphorylation was observed (figure 5.7, trace d). These results indicate that calcium may be promoting rapid dephosphorylation during the incubation period, and that the reduction in the phosphorylation of the 89Kda band is not a consequence of any action of calcium during the preincubation period. While the existence of a calcium-dependent phosphatase, as previously suggested, remains the most likely explanation of the calcium effect, calcium may promote dephosphorylation by several other mechanisms as follows:

i) Stimulation of a parietal cell ATPase by calcium would result in a reduction in the concentration of [γ-^{32}P] ATP, and therefore a general reduction in protein phosphorylation. This explanation can be discounted since calcium-stimulated phosphorylation was observed (110Kda band, plate 5.1) coincident with a reduction in net phosphorylation of the 89Kda band.

ii) Stimulation of a parietal cell phospholipase by calcium, as proposed by Moskowitz et al., (1984), for the phospholipase A_2 of brain synaptic plasma membranes, might reduce the concentration of phosphatidylserine. However, it seems somewhat unlikely that this action would be so severe as to affect the phosphorylation of the 89Kda band within 30 seconds after the addition of calcium.

iii) The interaction of calcium with the substrate proteins may cause precipitation of these proteins thereby providing a further possible explanation of the calcium effect. This alternative is unlikely since precipitation is only likely to occur at calcium concentrations in the mM-range, whereas calcium reduced the phosphorylation of the 89Kda band at a concentration of 500nM

iv) The presence of a calcium-dependent protease, e.g. Calpain (EC 3.2.22.17) in the cytosolic fraction of the parietal cell homogenate could also explain the calcium effect. This is not unlikely since calpain is widely distributed in mammalian tissues. However, this alternative may be discounted since protease inhibitors are present in the homogenization buffer, and calpain has previously been shown to activate protein kinase C (Kishimoto et al., 1983), converting it to a phospholipid and calcium-independent form.

Finally, the protein kinase responsible for the phosphorylation of the 89Kda protein in the presence of phosphatidylserine and TPA is slightly more active than the putative phosphatase during this early period, since a significant degree of phosphorylation was observed after 30 seconds (figure 5.7, trace b). Such rapid changes in the phosphorylation state of proteins are necessary for efficient stimulus-secretion coupling.

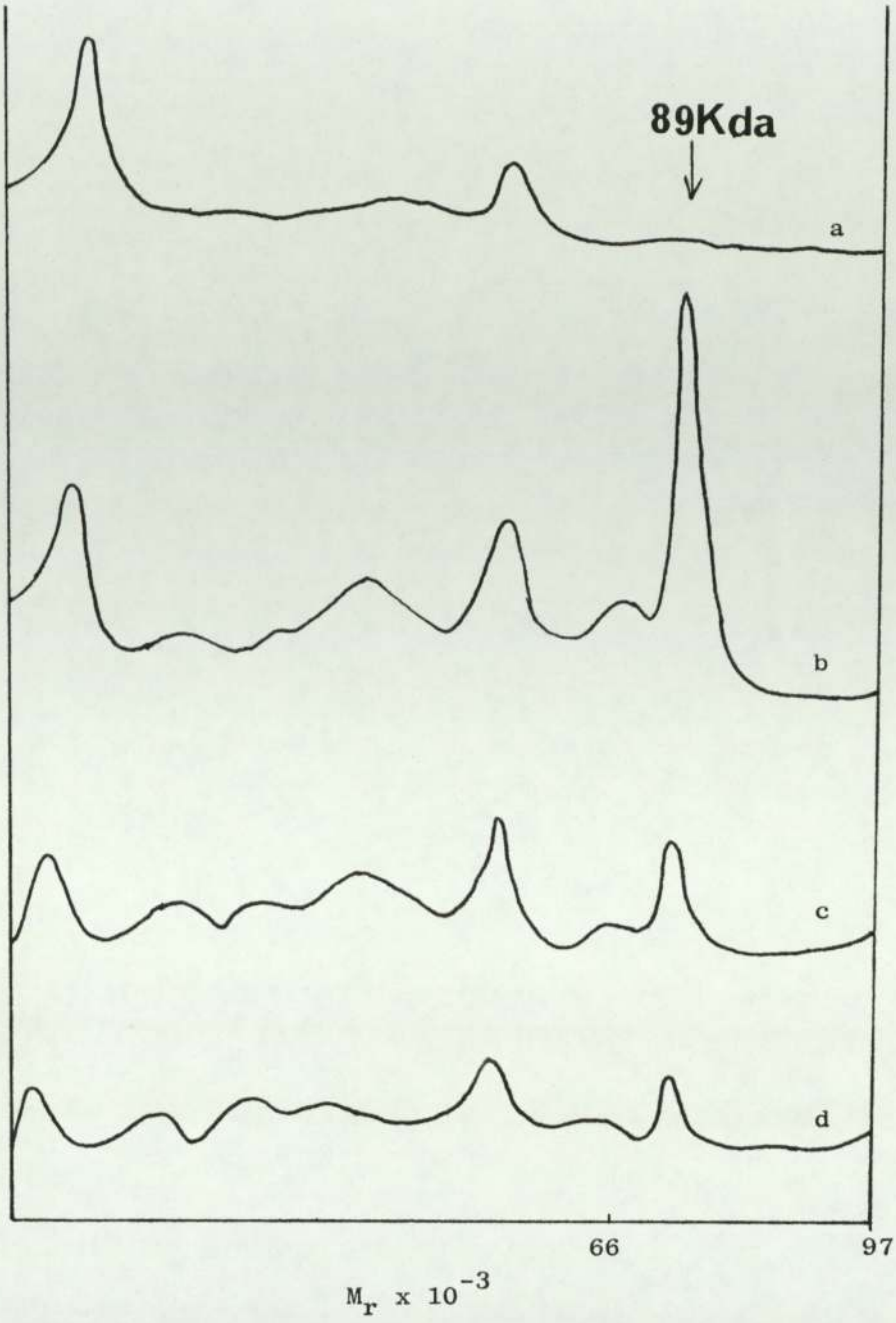


Figure 5.7

Densitometer traces of autoradiograms showing the effect of a 30 second incubation in the presence of phosphatidylserine (20µg/ml) plus TPA (32nM) and/or calcium (1µM) on the phosphorylation of proteins in a 100,000g supernatant derived from a cell preparation containing 97% parietal cells (2.2.4).

Lane a: no additions, Lane b: phosphatidylserine plus TPA,
Lane c: calcium and phosphatidylserine plus TPA,
Lane d: phosphatidylserine plus TPA, and calcium (added at start of 30 second incubation).

5.4 CONCLUSIONS

Two general conclusions can be drawn from the work in this section:

1. Phosphatidylserine and TPA promote the phosphorylation of an 89Kda parietal cell protein present in the 100,000g cytosolic fraction of a parietal cell homogenate. The enzyme responsible for the phosphorylation of this protein may be a) Protein kinase C which is active at the low levels of endogenous calcium present in the homogenate, or b) Protein kinase L, a derivative of protein kinase C which has lost the ability to bind calcium (Malviya *et al.*, 1986).

2. Calcium reduces the net phosphorylation of the 89Kda protein in a concentration-dependent manner. The mechanism by which calcium exerts this effect is most likely to be via a calcium-dependent phosphatase, and not by some non-specific action.

By considering the above conclusions in conjunction with the known ability of protein kinase C to inhibit acid secretion (chapter 4), it is possible to tentatively suggest a role for the 89Kda protein in the regulation of acid secretion.

CHAPTER 6.

THE EFFECT OF EPIDERMAL GROWTH FACTOR ON ACID SECRETION BY RAT PARIETAL CELLS.

6.1 INTRODUCTION.

6.1.1 Physiological effects of epidermal growth factor (EGF).

EGF is a single chain polypeptide (molecular weight 6045) of fifty-three amino acid residues, originally isolated from the submaxillary gland of male mice (Cohen, 1962). A remarkable structural similarity exists between mouse EGF and the β-urogastrone extracted from human urine. In fact, thirty-seven of the fifty-three amino acid residues are common to both molecules (Gregory, 1975). In view of this structural homology it is not entirely surprising that mouse EGF and β-urogastrone exhibit identical biological properties, which include the ability to induce precocious eye-lid opening and precocious eruption of teeth in mice, and the ability to inhibit gastric acid secretion (Gregory, 1975). In fact, urogastrone and mouse EGF share a common receptor site in cultured human fibroblasts (Hollenberg and Gregory, 1976). EGF promotes cell division in epithelial cells (Dembinski et al., 1982), and exerts a wide range of effects on cultured cell lines (table 6.1) which are probably associated with its growth promoting activity.

6.1.2 Effect of EGF on gastric acid secretion.

EGF administered subcutaneously inhibits histamine-stimulated acid secretion from rats and cats surgically provided with gastric fistulas (Konturek et al., 1981). However, EGF is ineffective when administered intra-gastrically (Dembinski et al., 1982). Parenteral

Table 6.1

Some of the effects of EGF on cultured cell lines.

Author	Effect of EGF
Cohen & Stastny, (1968)	Increased rate of RNA and protein synthesis in chick embryo epidermis.
Hollenberg & Cuatrecasas,(1973)	Increased rate of RNA and DNA synthesis in contact inhibited human fibroblasts.
Barnes & Colowick, (1976)	Rapid increase (x2) of 2-deoxyglucose uptake by density-inhibited mouse 3T3 cells.
Diamond et al., (1978)	Increased rate of glycolysis in quiescent 3T3 cells.
Macara, (1986)	Enhanced Na^+/H^+ exchange in A431 cells.

administration of EGF (0.1 - 1.0μg/kg) to dogs with Heidenhain pouches completely inhibits histamine, pentagastrin and cholinergic stimulation of acid secretion within 15 minutes (Gregory et al., 1977). More recent work has confirmed this observation, demonstrating that EGF can inhibit acid secretion from the innervated and vagally denervated canine stomach stimulated by histamine, pentagastrin or cholinergic agonists in vivo (Konturek et al., 1984).

Some in vitro work has also been carried out, although conflicting results have been obtained as to the secretagogues against which EGF is effective. Using a rabbit gastric gland preparation, Reichstein et al., (1984) demonstrated that the inhibitory action of EGF on acid secretion was secretagogue-specific. Thus, EGF significantly reduced aminopyrine accumulation by parietal cells stimulated with histamine ($P < 0.01$ paired t-test), but was without effect on dbcAMP-stimulated aminopyrine accumulation. Further work by Chen et al., (1984) using canine isolated parietal cells confirmed the selectivity of the anti-secretory effect of EGF for histamine. Thus, Chen et al., (1984) demonstrated that EGF did not inhibit aminopyrine accumulation stimulated by cholinergic agonists or gastrin, and was only minimally effective against dbcAMP, but significantly reduced aminopyrine accumulation stimulated by histamine. EGF also reduced histamine-stimulated acid secretion by a guinea pig gastric mucosal preparation when present on the serosal side of the tissue (Finke et al., 1985). However, in contrast to the results obtained by Reichstein et al., (1984), Konturek et al., (1984) demonstrated that EGF caused indiscriminate inhibition of aminopyrine accumulation by resting rabbit gastric glands and in those stimulated by histamine, carbachol and dbcAMP.

6.1.3 Events linked with activation of the EGF receptor.

It is likely that EGF interacts with a specific plasma membrane receptor, and that this EGF-receptor complex is subsequently internalized leading to the degradation of EGF in the lysosomes and possibly recycling of the receptor back to the plasma membrane. The interaction of EGF with the plasma membrane receptor of A431 cells leads to an increase in the phosphorylation state of numerous endogenous protein substrates (Carpenter et al., 1978), primarily on tyrosine residues (Ushiro and Cohen, 1980). Indeed, subsequent work using A431 cells by Cohen et al., (1982) has indicated that the principle substrate for the EGF receptor tyrosine kinase was a 170Kda protein thought to be the EGF receptor itself. Interaction of EGF with its plasma membrane receptor also causes the phosphorylation of the receptor on a threonine residue (654) (King and Cooper, 1986). Autophosphorylation of the receptor at threonine 654 as a result of EGF binding causes a reduction in the tyrosine kinase activity of the EGF receptor, and a reduction in the high-affinity binding of EGF. Both effects can be mimicked by the addition of tumour promoters to the cell system, suggesting a possible role for protein kinase C in the phosphorylation of threonine 654 (Friedman et al., 1984). Since EGF may induce calcium influx and enhance phosphatidylinositol turnover in A431 cells (Sawyer and Cohen, 1981), and therefore activate protein kinase C (Sahai et al., 1982) the action of EGF may be subject to feedback inhibition via the phosphorylation of threonine 654 by protein kinase C.

6.1.4 Mechanism of action of EGF in parietal cells.

It is possible to suggest three mechanisms by which EGF-receptor interactions at the cell surface may be coupled to the inhibition of gastric acid secretion:-

1. In addition to mediating down regulation of the EGF receptor via phosphorylation of threonine 654, protein kinase C activated upon EGF binding to its plasma membrane receptor, may inhibit gastric acid secretion as proposed by Anderson and Hanson (1984).

2. The anti-secretory effect of EGF may be mediated via a reduction in the intracellular levels of cAMP. This may be achieved by an inhibition of the adenylate cyclase associated with the histamine receptor on the parietal cell or by activation of a cAMP-phosphodiesterase.

3. EGF may alternatively reduce acid secretion by inducing prostaglandin production (Levine and Hassid, 1977) either in parietal cells or other gastric mucosal cells. Indeed, Chiba et al., (1982) have shown that EGF causes prostaglandin production from the isolated perfused rat stomach. It is even possible to suggest a mechanism by which EGF increases the level of endogenous prostaglandins. The interaction of EGF with its plasma membrane receptor activates the receptor linked tyrosine kinase which can phosphorylate lipomodulin (Pepinsky and Sinclair, 1986) thereby reducing the inhibition of phospholipase A_2 by this protein (Hirata, 1981). In this way the liberation of arachidonic acid may be enhanced and prostaglandin production increased.

Since the mechanism of inhibition of gastric acid secretion by EGF is uncertain at present, it is the primary objective of this chapter to establish which of these three alternatives, if any, is operating *in vivo*. This study may also help to reconcile the differences regarding the secretagogue-specificity of the action of EGF in inhibiting acid secretion. In addition, no previous investigation of the effects of EGF on rat parietal cells has been undertaken.

6.2 METHODOLOGY.

6.2.1 The effect of EGF on aminopyrine accumulation.

A parietal cell suspension was prepared in medium B´ containing bovine serum albumin (1mg/ml) and 20mM-HEPES as described in section 2.2.2. 1.5ml of this cell suspension was added to siliconized incubation vials containing [^{14}C] aminopyrine (0.1μCi/ml), [^{3}H] polyethyleneglycol (0.4μCi/ml), suitable secretagogues, and where appropriate EGF (200nM). This was a near-maximally effective concentration of EGF since increasing the concentration to 500nM did not increase the inhibitory effect of EGF against histamine-stimulated aminopyrine accumulation. Indeed, the half-maximally effective concentration of EGF for inhibition of histamine-stimulated secretion has subsequently been shown to be 19nM (J. F. Hatt, unpublished work).

Pre-incubation of the cell preparation for 3 hours at 37^{0}C in the presence of 100nM-EGF was followed by the subsequent addition of more EGF, giving a concentration of 200nM-EGF for the last 30 minutes of incubation (assuming no degradation of previously added EGF). During this final 30 minute period both control and EGF-treated cells were challenged with histamine. Pre-incubation with EGF did not change the percentage inhibition of histamine-stimulated aminopyrine accumulation effected by EGF by comparison with cells exposed to EGF only for the period with histamine. In both cases the percentage inhibition effected by EGF was approximately 40%. Thus, experimental vials were routinely incubated for 30 minutes at 37^{0}C without pre-incubation, and the aminopyrine accumulation ratios determined (2.2.5). When required, indomethacin, IBMX and Ro 20-1724 were dissolved in ethanol, TPA was dissolved in DMSO, and small

volumes from these stock solutions were added to the experimental vials. In control vials containing no agents, the same final concentrations of solvents were present (0.1% v/v ethanol and 0.0002% v/v DMSO).

6.2.2 Analysis and presentation of data.

In order to overcome the variation in aminopyrine accumulation between cell batches, the data obtained in this section have been normalized as described in the legends to figures 6.1, 6.3 and 6.4, and analysed using a paired t-test or analysis of variance followed by a Newman-Keuls test. The relative efficiency of TPA and EGF to inhibit aminopyrine accumulation was determined by means of an unpaired t-test.

6.3 RESULTS AND DISCUSSION.

6.3.1 The effect of 200nM-EGF on aminopyrine accumulation by rat parietal cells stimulated with various secretagogues.

EGF (200nM) had no effect on basal aminopyrine accumulation or on that in the presence of IBMX. 200nM-EGF significantly inhibited aminopyrine accumulation by parietal cells stimulated by 0.5mM-histamine alone ($P < 0.001$, paired t-test) as shown in table 6.2, but was without effect on aminopyrine accumulation stimulated by carbachol (0.1mM), dbcAMP (1mM) or 0.5mM-histamine plus 0.1mM-IBMX (table 6.2). The secretagogues employed in these experiments were used at their near-maximally effective concentrations.

The inability of 200nM-EGF to reduce basal aminopyrine accumulation observed in this study is consistent with previous work on guinea pig fundic mucosa by Finke *et al.*, (1985). The overall pattern of inhibition shown in table 6.2 correlates with that observed in canine isolated parietal cells (Chen *et al.*, 1984). In this study histamine-stimulated aminopyrine accumulation by rat isolated parietal cells was inhibited by approximately 40% in the presence of 200nM-EGF. A similar degree of inhibition was observed using isolated gastric glands from rabbit [Konturek *et al.*, (1984) and Reichstein *et al.*, (1984)].

However, contradictory evidence has been obtained from studies *in vivo* by Konturek *et al.*, (1984) using dogs with a gastric fistula. Such discrepancies between the effect of EGF *in vivo* and *in vitro* can be explained by the potentiating interactions of secretagogues *in vivo* (Chapter

One). Acetylcholine may stimulate acid secretion *in vivo* by causing the release of histamine from histamine-containing cells of the gastric mucosa (Nylander *et al.*, 1986), in addition to having a direct effect on the parietal cell. Therefore, *in vivo*, the inhibitory effect of EGF on carbachol-stimulated secretion may occur at the level of the histamine-containing cell or alternatively, by inhibiting the action of the subsequently released histamine. However, in the rat isolated parietal cell preparation used in this study, the level of endogenous histamine is low since IBMX (0.1mM) alone had no effect on basal aminopyrine accumulation (table 6.2). In addition, cimetidine had only a minimal effect on carbachol-induced acid secretion (Anderson and Hanson, 1984), indicating that the primary site of action of carbachol is directly on the parietal cell itself, and that interaction between endogenous histamine and exogenous carbachol was largely absent.

Table 6.2

The effect of EGF on aminopyrine accumulation by rat parietal cells in the presence of various secretagogues

Aminopyrine accumulation ratios are presented as means ± SEM, and a significant effect of EGF was determined using the paired t-test (***, $P < 0.001$).

Secretagogues (mM)	Number of cell-batches	Aminopyrine Accumulation Ratio	
		Control	200nM EGF
No addition	4	3.7 ± 0.6	3.9 ± 0.8
IBMX (0.1)	4	2.6 ± 0.4	2.4 ± 0.3
Histamine (0.5)	17	11.5 ± 1.7	6.3 ± 1.0***
Histamine (0.5) + IBMX (0.1)	11	146 ± 21	136 ± 21
Histamine (0.5) + Ro 20-1724 (0.1)	6	105 ± 15	95 ± 17
Dibutyryl cyclic AMP (1)	7	201 ± 38	201 ± 41
Carbachol (0.1)	4	10.3 ± 1.1	10.5 ± 1.7

6.3.2 Effect of inhibitors of cyclic AMP-phosphodiesterase on EGF action.

EGF (200nM) did not effect any part of the dose-response curve for histamine-stimulated aminopyrine accumulation in the presence of the cAMP-phosphodiesterase inhibitor IBMX (0.1mM). The non-transformed results are shown in table 6.3, while a plot of the normalized data is presented in figure 6.1. Therefore the inability of 200nM-EGF to inhibit aminopyrine accumulation stimulated by histamine (0.5mM) plus IBMX (0.1mM), was not simply due to the greater aminopyrine accumulation ratios achieved in the presence of IBMX (table 6.2). In addition, when comparable aminopyrine accumulation ratios were obtained in the presence (□ , fig 6.2), and absence of IBMX (■ , fig 6.2), the degree of inhibition effected by 200nM-EGF was consistently greater in the absence of IBMX (fig 6.2).

Although histamine-stimulation of acid secretion is mediated by the elevation of intracellular cAMP (Soll and Wollin, 1979), it is unlikely that EGF inhibits secretion by acting at a site distal to cAMP production since EGF was ineffective against acid secretion stimulated by a range of concentrations of the stable analogue of cAMP, dibutyryl cAMP (table 6.4). EGF is more likely to act by inhibiting parietal cell adenylate cyclase or alternatively by increasing cAMP-phosphodiesterase activity. Since EGF is ineffective against histamine-stimulated aminopyrine accumulation in the presence of the cAMP-phosphodiesterase inhibitor, IBMX (0.1mM) (table 6.3), activation of this enzyme is indicated as a possible site of action of EGF. However, this is only one possible explanation of the data shown in table 6.3, since IBMX may also inhibit cGMP-phosphodiesterases thereby elevating the levels of intracellular cGMP (Wells and Kramer, 1981).

In order to more accurately determine the site of action of EGF, the ability of EGF to inhibit histamine-stimulated aminopyrine accumulation was investigated in the presence of a non-methylxanthine cAMP-phosphodiesterase inhibitor, Ro 20-1724, which has no effect on either basal or stimulated cGMP levels in rat cerebellar slices (Schwabe et al., 1976). Under these conditions EGF failed to reduce aminopyrine accumulation (table 6.2) to the same extent as observed in the presence of histamine alone, indicating that the EGF-effect is most likely due to activation of a parietal cell cAMP-phosphodiesterase and not due to the alternative mechanisms suggested above.

The mechanism of action of EGF has, in general, been poorly characterized. While considerable evidence exists in favour of EGF promoting an increase in cAMP levels (e.g. Greene and Lloyd, 1985), only this work, and that of Anderson et al., (1979), provide any evidence for an action of EGF mediated by a reduction in the intracellular levels of cAMP.

Table 6.3

The effect of 200nM-EGF on the aminopyrine accumulation by rat parietal cells stimulated by various concentrations of histamine and IBMX (0.1mM).

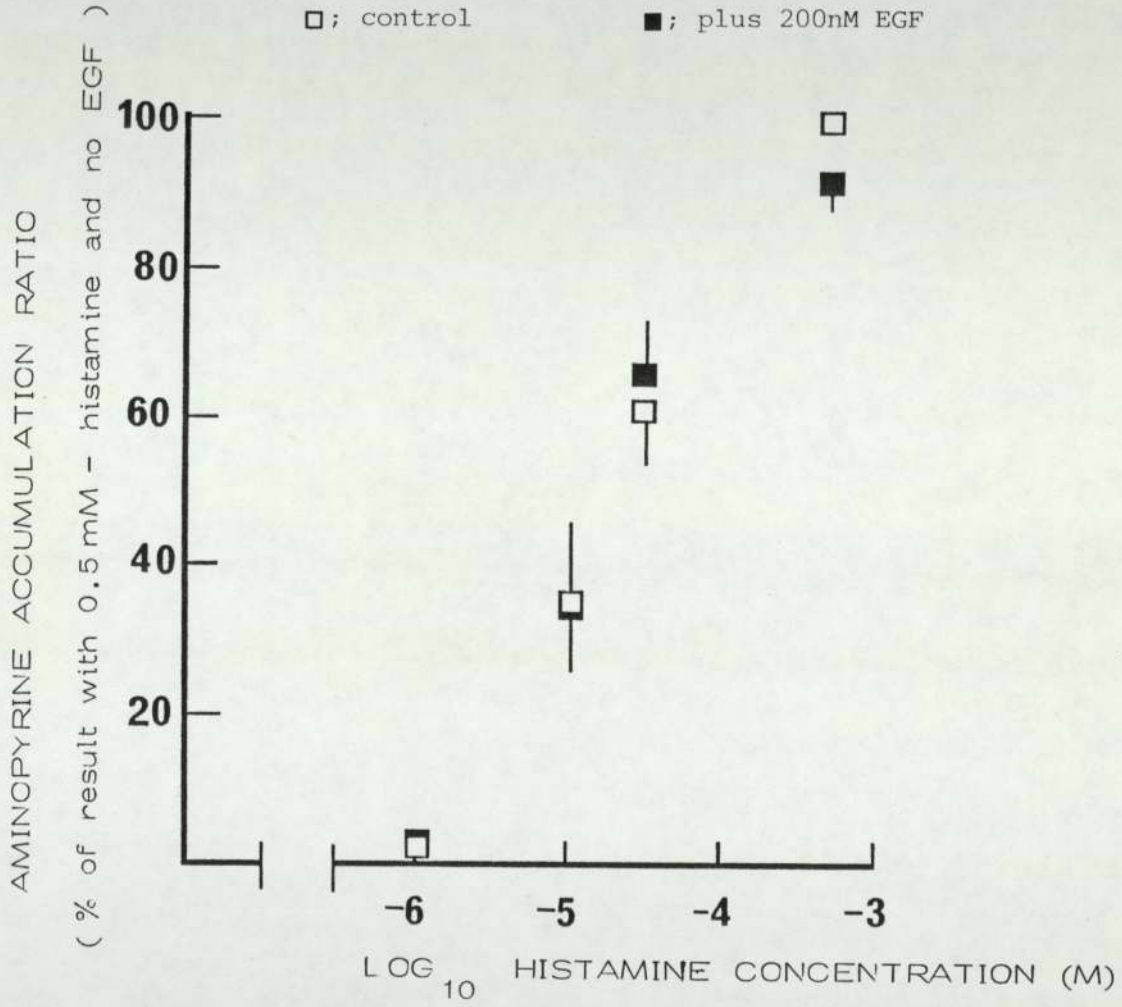
Aminopyrine accumulation ratios are presented as means ± SEM with the number of cell batches in parentheses.

Concentration of histamine (M) plus 0.1mM-IBMX	Aminopyrine accumulation ratio	
	control	+ 200nM-EGF
1×10^{-6}	3.9 ± 0.7 (5)	4.1 ± 0.9 (5)
1×10^{-5}	44.3 ± 14.0 (3)	58.7 ± 11.9 (5)
3×10^{-5}	101.6 ± 18.2 (5)	115.8 ± 27.8 (5)
5×10^{-4}	172.1 ± 37.1 (5)	158.6 ± 38.1 (5)

Figure 6.1

The effect of EGF (200nM) on the aminopyrine accumulation by rat parietal cells stimulated by various concentrations of histamine, and IBMX (0.1mM).

The results from 3-5 experiments have been normalized by expressing the aminopyrine accumulation ratios as a percentage of the value obtained with 0.5mM-histamine + 0.1mM-IBMX and no EGF which was 172.1 ± 37.1 (5).



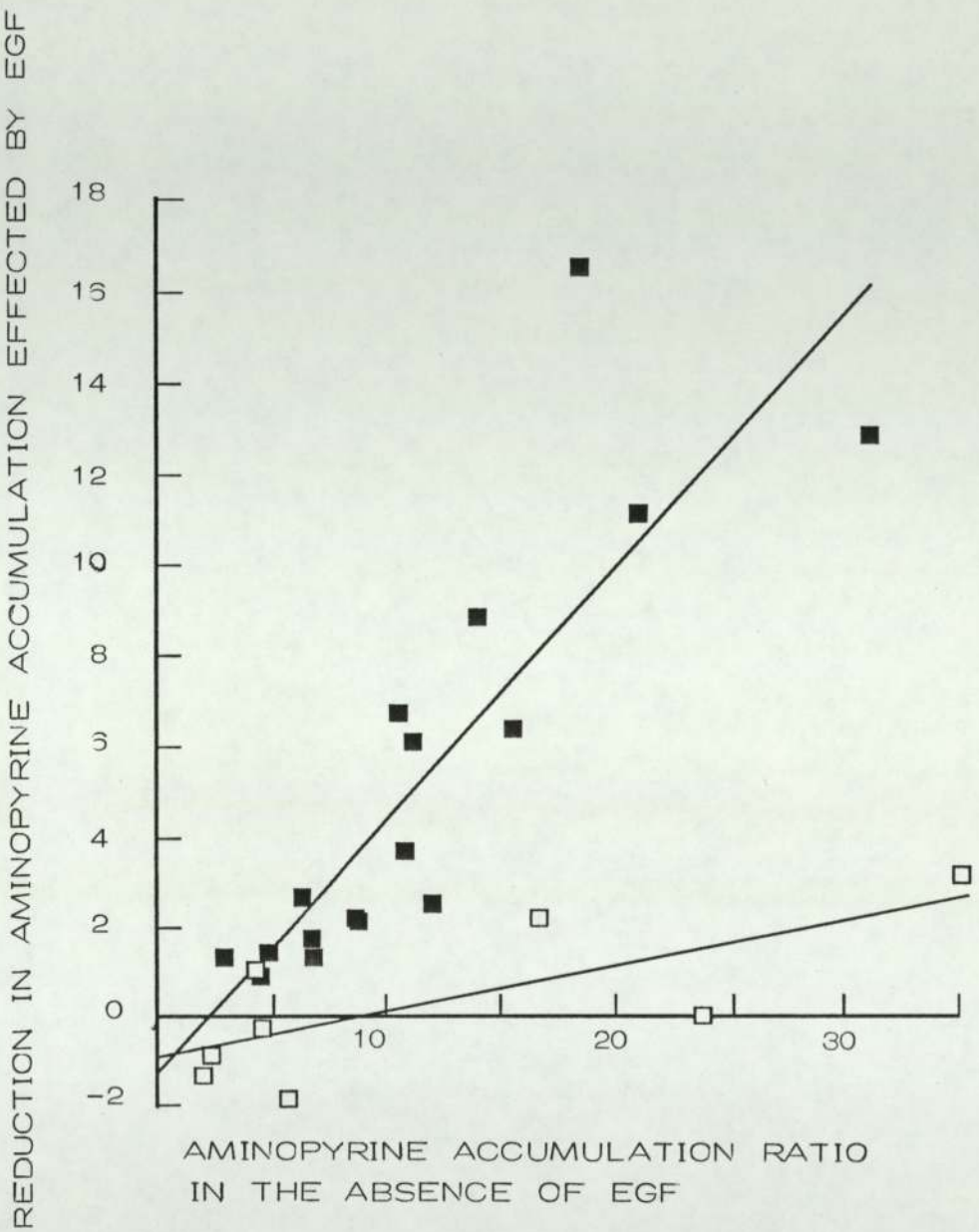


Figure 6.2

Relationship between the absolute reduction in aminopyrine accumulation produced by EGF (200nM), and the level of aminopyrine accumulation in the absence of EGF.

□, values obtained in the presence of IBMX (0.1mM) and 1-30μM-histamine (equation of the regression line: $y = -0.97 + 0.11x$, correlation coefficient = 0.74).

■, values obtained in the presence of 0.5mM-histamine alone (equation of the regression line: $y = -1.23 + 0.56x$, correlation coefficient = 0.85). Analysis of covariance (Snedecor and Cochran, 1967) demonstrated a significant difference ($P < 0.001$) between the slopes of the two lines.

Table 6.4

The effect of 200nM-EGF on dbcAMP-stimulated aminopyrine accumulation.

Aminopyrine accumulation ratios are presented as means ± SEM. No significant effect of EGF was observed at any concentration of dbcAMP.

Concentration of dbcAMP (μM)	No of cell batches	Aminopyrine Accumulation	
		Control	+ EGF (200nM)
30	4	5.5 ± 1.7	5.0 ± 1.8
56	6	49.3 ± 15.3	46.3 ± 16.8
100	5	139.0 ± 47.7	125.0 ± 42.5
1000	7	201.0 ± 38.0	201.0 ± 41.0

6.3.3 A comparison of the effect of 200nM-EGF and TPA on aminopyrine accumulation stimulated by histamine (0.5mM) plus IBMX (0.1mM), dbcAMP (1mM) and carbachol (0.1mM).

For this comparison a TPA concentration of 32nM was chosen as this gives near-maximal inhibition of aminopyrine accumulation, except when carbachol is the secretagogue when a higher concentration (800nM) is required (Anderson and Hanson, 1984). TPA was a significantly more effective inhibitor of aminopyrine accumulation stimulated by the secretagogues tested than 200nM-EGF ($P < 0.01$, unpaired t-test). TPA (32nM) effected a 50% reduction in dbcAMP-stimulated aminopyrine accumulation, whereas EGF (200nM) caused only a 3% inhibition (fig 6.3). Considering histamine (0.5mM) plus IBMX (0.1mM)-stimulated aminopyrine accumulation, TPA (32nM) caused a 75% reduction compared with only 7% inhibition effected by EGF (200nM). Carbachol-stimulated aminopyrine accumulation is more resistant to inhibition by TPA but at a concentration of 800nM, TPA effected a 49% reduction in aminopyrine accumulation which was significantly higher than the negligible effect of 200nM-EGF ($P < 0.01$, unpaired t-test). A comparison between the effects of EGF and TPA on aminopyrine accumulation stimulated by histamine alone was made by J.Hatt, and the data (personal communication) have been included in figure 6.3. Although both EGF and TPA were inhibitory, TPA was significantly more effective ($P < 0.01$, unpaired t-test). Since there were considerable differences in the relative abilities of TPA and EGF to inhibit aminopyrine accumulation stimulated by a range of secretagogues (fig 6.3), it is improbable that the observed inhibitory effect of these agents is mediated via the same mechanism. Therefore, the mechanism by which EGF causes an inhibition of aminopyrine accumulation by rat parietal cells is unlikely to involve protein kinase C. This is not surprising since although EGF activates protein kinase C in

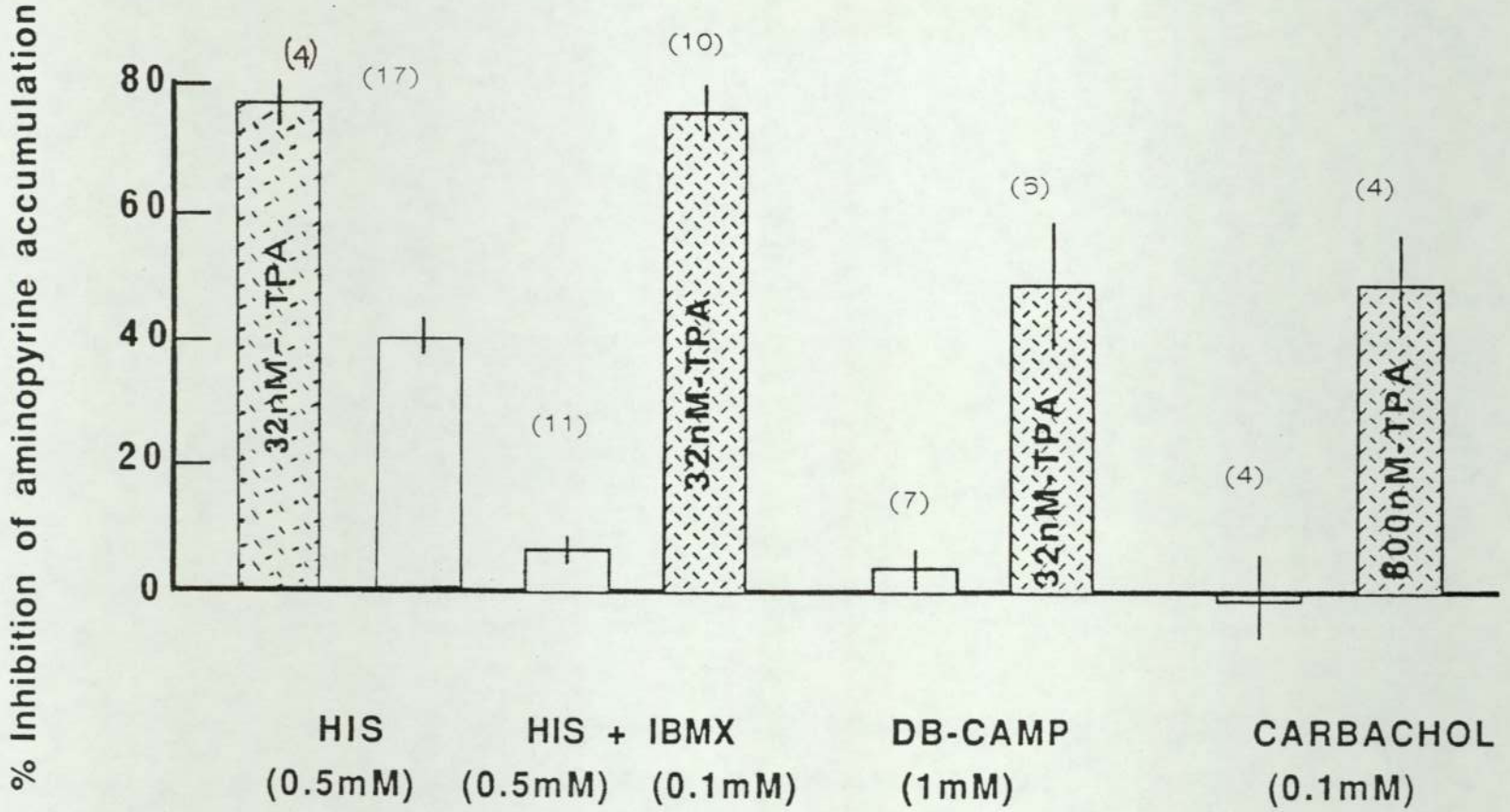


Figure 6.3

The effect of TPA and EGF on aminopyrine accumulation stimulated by histamine (0.5mM), histamine (0.5mM) and IBMX (0.1mM), dbcAMP (1mM), and carbachol (0.1mM).

The concentration of EGF used was 200nM, and that of TPA was 32nM when histamine or dbcAMP were the secretagogues or 800nM when carbachol was the secretagogue. The effect of both TPA and EGF on secretagogue activity is expressed as a percentage inhibition of the aminopyrine accumulation in the absence of TPA and EGF, with the number of cell batches in parenthesis.

A431 cells, (Sahai et al., 1982), it does not enhance protein kinase C activity in other cell types e.g. 3T3 cells (Vara and Rozengurt, 1985).

6.3.4 The effect of indomethacin (10μM), ibuprofen (100μM), mefenamic acid (10μM) and flurbiprofen (10μM) on the inhibitory action of 200nM-EGF on histamine-stimulated aminopyrine accumulation.

Since prostaglandins are known inhibitors of acid secretion in the rat (e.g. Schepp et al., 1983b), and EGF may stimulate prostaglandin production from the isolated perfused rat stomach (Chiba et al., 1982), it is possible that the inhibitory action of EGF on acid secretion could be mediated via prostaglandin production. This possibility was considered even more likely since both prostaglandin E_2, (Soll, 1980b) and EGF (table 6.2) inhibit histamine-stimulated aminopyrine accumulation, and both are ineffective against aminopyrine accumulation stimulated by dbcAMP and carbachol.

The ability of EGF to inhibit aminopyrine accumulation in the presence of inhibitors of prostaglandin synthesis was investigated to determine the involvement of prostaglandins in the inhibitory action of EGF. The previously observed ability of EGF to significantly inhibit aminopyrine accumulation by parietal cells stimulated with 0.5mM-histamine alone (table 6.2), was reduced by the presence of indomethacin (10μM), (fig 6.4, table 6.5).

Indomethacin, like IBMX and Ro 20-1724, also inhibits cAMP-phosphodiesterase (Newcombe et al., 1974), and therefore these results might be explained by indomethacin

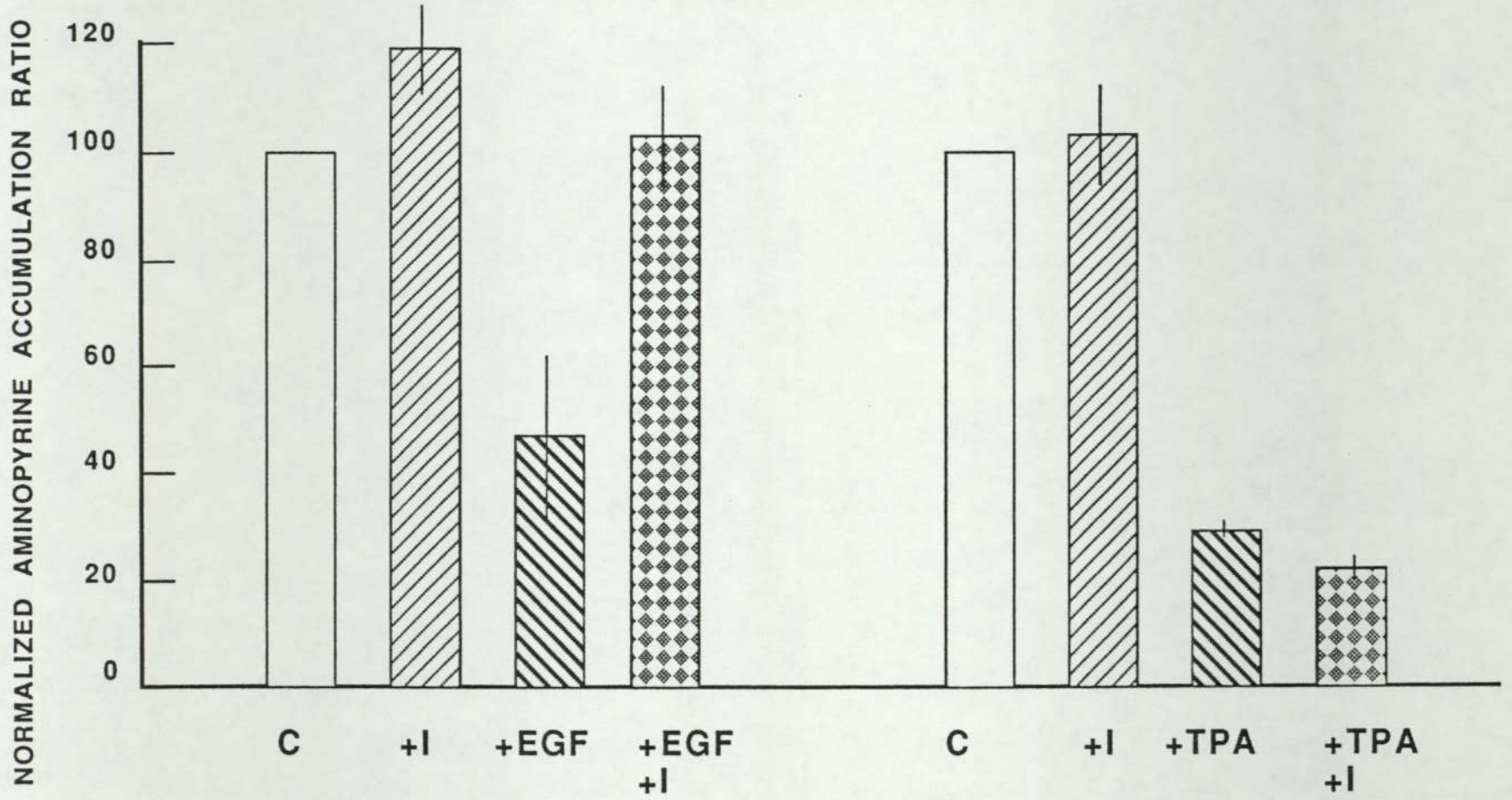


Figure 6.4

The effect of indomethacin (10μM) on the inhibitory action of EGF (200nM) and of TPA (32nM) on histamine stimulated aminopyrine accumulation.

The results have been normalized by expressing values as a percentage of the aminopyrine accumulation ratio obtained with 0.5mM-histamine alone [EGF data, control aminopyrine accumulation, 12.3 ± 2 (4)] or 0.5mM histamine + 0.1mM - IBMX [TPA data, control aminopyrine accumulation, 61 ± 7 (4)]. The normalized results are presented as means ± S.E.M.

Table 6.5

The effect of prostaglandin synthesis inhibitors on the inhibition of aminopyrine accumulation caused by 200nM-EGF.

Treatment	Aminopyrine Accumulation Ratio	
	No EGF	200nM-EGF
Histamine (0.5mM)	11.8 ± 1.3 (7)	5.3 ± 0.7 (7)**
Histamine (0.5mM) + Indomethacin (10μM)	13.1 ± 2.0 (7)	10.4 ± 1.9 (7)
Histamine (0.5mM) + Mefenamic acid (10μM)	11.5 (1)	7.8 (1)
Histamine (0.5mM) + Ibuprofen (100μM)	7.3 ± 2.4 (3)	5.2 ± 2.0 (3)
▲ Histamine (0.5mM)+ Flurbiprofen (10μM)	13.5 ± 2.4 (4)	8.2 ± 1.3 (4)**

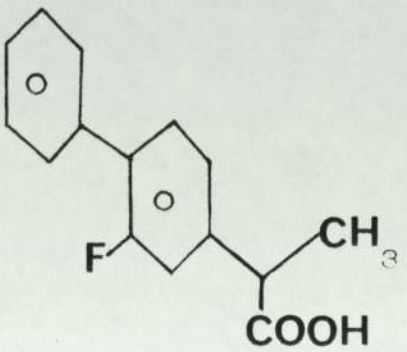
Aminopyrine accumulation ratios are given as mean ± SEM with the number of experiments in parentheses. A significant effect of EGF was determined by analysis of variance and Newman-Keuls multiple range test, ** $P < 0.01$. No statistical procedures were employed on rows 3 and 4 as insufficient data were obtained. ▲ data of P. J. Hanson and J. Hatt (personal communication).

preventing EGF enhancement of the breakdown of cAMP. This mode of action of indomethacin is unlikely since the presence of 10μM-indomethacin alone had no effect on aminopyrine accumulation stimulated by histamine (table 6.5), and did not inhibit cAMP-phosphodiesterase activity in parietal cell homogenates (Gerber et al., 1981). Indomethacin has been shown to inhibit protein kinase A activity (Kanter and Hampton, 1978), and the transcellular transport of calcium (Northover, 1978), which further complicates the interpretation of any data obtained using this compound.

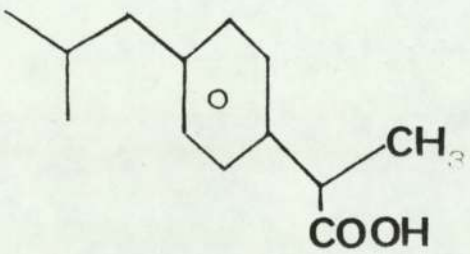
In an attempt to clarify the putative involvement of prostaglandins in the mechanism of action of EGF, the ability of EGF to inhibit histamine-stimulated aminopyrine accumulation in the presence of other cyclo-oxygenase inhibitors structurally unrelated to indomethacin (fig 6.5) was investigated. Preliminary data indicates that ibuprofen (100μM) and mefenamic acid (10μM) do not prevent the inhibitory action of EGF on histamine-stimulated aminopyrine accumulation (table 6.5). Although only a limited number of experiments were performed it is unlikely that enhanced prostaglandin production does contribute to the anti-secretory action of EGF. This assumption has subsequently been confirmed by Hanson and Hatt (personal communication), who demonstrated that flurbiprofen, a potent and selective cyclo-oxygenase inhibitor (MacAdams et al., 1984), at concentrations of 1, 10 and 100μM did not prevent the anti-secretory effect of EGF (an example of results obtained with 10μM flurbiprofen is shown in table 6.5). In view of this observation, indomethacin must be assumed to be acting by some mechanism other than an inhibition of prostaglandin synthesis. A further reason for dismissing the hypothesis that the action of EGF might be mediated by prostaglandins is that the inhibitory action of EGF is prevented by IBMX, but that of prostaglandin E_2 is not (Soll, 1980b).

Figure 6.5

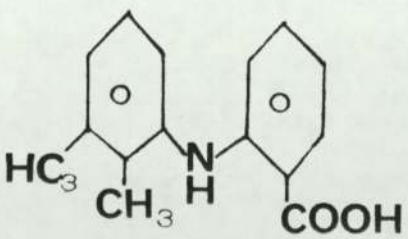
The structures of the cyclo-oxygenase inhibitors used in this study.



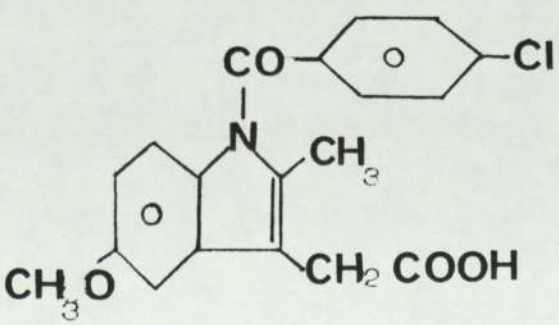
Flurbiprofen



Ibuprofen



Mefenamic Acid



Indomethacin

Finally, since EGF protects gastric mucosal cells in monolayer culture against indomethacin-induced damage (Hiraishi et al., 1984), it is likely that EGF may serve an important cytoprotective function in vivo. This gastric cytoprotective function of EGF has been observed in vivo (Konturek et al., 1981), but it is not mediated by mucosal prostaglandins. This observation, by Konturek et al., (1981), therefore parallels that described in this work on the anti-secretory action of EGF.

6.3.5 The effect of indomethacin on the inhibitory action of TPA.

Although indomethacin (10μM) reduced the ability of EGF (200nM) to inhibit histamine-stimulated aminopyrine accumulation, the ability of 32nM-TPA to significantly inhibit aminopyrine accumulation stimulated by histamine (0.5mM) plus IBMX (0.1mM), ($P < 0.02$, Anderson and Hanson, 1984), was not significantly diminished by indomethacin (fig 6.4). This data also suggests that the mechanisms by which EGF and TPA inhibit aminopyrine accumulation are quite distinct.

6.4 CONCLUSIONS.

The secretagogue-specificity of the inhibition of acid secretion by EGF suggested a site of action close to the generation or hydrolysis of the second messenger cAMP. Further work indicated that the inhibitory action of EGF was possibly mediated by the stimulation of a cAMP-phosphodiesterase. Prostaglandins and protein kinase C do not appear to be involved in the inhibition of histamine-stimulated acid secretion by EGF. In the general discussion a possible physiological role for the inhibition of acid secretion by EGF will be discussed.

CHAPTER 7.

GENERAL DISCUSSION.

7 GENERAL DISCUSSION.

In order to match the three principle areas of research outlined in the introduction to this work, (chapter one), this discussion has been divided into three sections. The primary objective of each section is to outline the major findings of the work, and to discuss their implications and physiological significance.

7.1 SUBSTRATE-DEPENDENCY OF ACID SECRETION.

This work established that of all the potential metabolic substrates tested at physiological concentrations, glucose was best able to support acid secretion (3.3.1). However, glucose alone was shown to be unable to support maximal rates of acid secretion, and possible explanations for this have been given (3.4.1.1). For maximal rates of acid secretion to be achieved, supraphysiological concentrations of either lactate or L-isoleucine were required in the incubation medium (3.3.4). Alternatively, physiological concentrations of certain substrates (3.3.5), in addition to glucose, were required as supplements to the incubation medium.

In the light of these observations it is apparent that any future work on the control of acid secretion must involve a careful assessment of the constituents of the incubation medium. In order for the control of acid secretion to be studied accurately, it is desirable to obtain a maximal secretory-rate. From this work it is apparent that this maximal secretory-rate can best be achieved if the incubation medium contains, for example, L-isoleucine and lactate in addition to glucose. To conclude, the current work indicates that acid secretion can be

regulated by the supply of metabolic substrates to the parietal cell *in vitro*. Whether this might also occur *in vivo* is as yet speculation.

7.2 THE INVOLVEMENT OF PROTEIN KINASE C IN THE ACID-SECRETORY PROCESS.

This work strongly indicated that TPA inhibited acid secretion by a specific, direct action on the parietal cell, most likely by activation of protein Kinase C (Castagna *et al.*, 1982). Once activated, protein Kinase C phosphorylates an 89Kda protein present in the 100,000g cytosolic fraction of a parietal cell homogenate which may mediate the effects of this enzyme in the parietal cell. Under physiological conditions, this enzyme is activated by diacylglycerol, which is transiently produced by the receptor-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate. Activation of protein Kinase C by diacylglycerol is therefore reversible, since diacyglycerol is rapidly removed either by phosphorylation, or by hydrolysis with the production of arachidonic acid (Berridge, 1984). In contrast, the phorbol ester, TPA, used in this study, probably cannot be metabolized by the parietal cell, it therefore causes prolonged activation of the enzyme (Castagna *et al.*, 1982).

In view of the secretagogue-specificity of the inhibitory action of TPA *in vitro* (Anderson and Hanson, 1984), and *in vivo* (table 4.5), it is possible to suggest a physiological role for protein Kinase C in the negative modulation of gastric acid secretion. This physiological role of protein Kinase C most likely occurs distal to the production of cAMP, since TPA inhibited aminopyrine accumulation by rat isolated parietal cells stimulated by both histamine and dbcAMP (Anderson and Hanson, 1984). Two

possible mechanisms for the involvement of protein kinase C in the negative modulation of gastric acid secretion are shown in figure 7.1. Thus, just as there is potentiation between histamine and carbachol in the stimulation of acid secretion (Soll, 1978b), it is possible that negative modulation between these two secretagogues also exists. Stimulation of the muscarinic receptor on the parietal cell surface is likely to increase the diacylglycerol concentration thereby activating protein kinase C (Berridge, 1984), although this has yet to be proven in parietal cells. This enzyme may form part of a negative-feedback system preventing the over-production of acid in response to histamine stimulation of the parietal cell.

An alternative possibility is that protein kinase C may be activated *in vivo* by another receptor-ligand interaction (fig 7.1). However, the nature of this ligand is at present unknown and one potential candidate, EGF, (see below), does not seem to inhibit acid secretion by activating protein kinase C. Although protein kinase C may be involved in the negative modulation of acid secretion, recent work suggests it may have an additional, possibly stimulatory, role within the parietal cell, (this effect can only be detected in cells incubated with high extracellular K^+ , J. F. Hatt, personal communication) and this has also been indicated (fig 7.1).

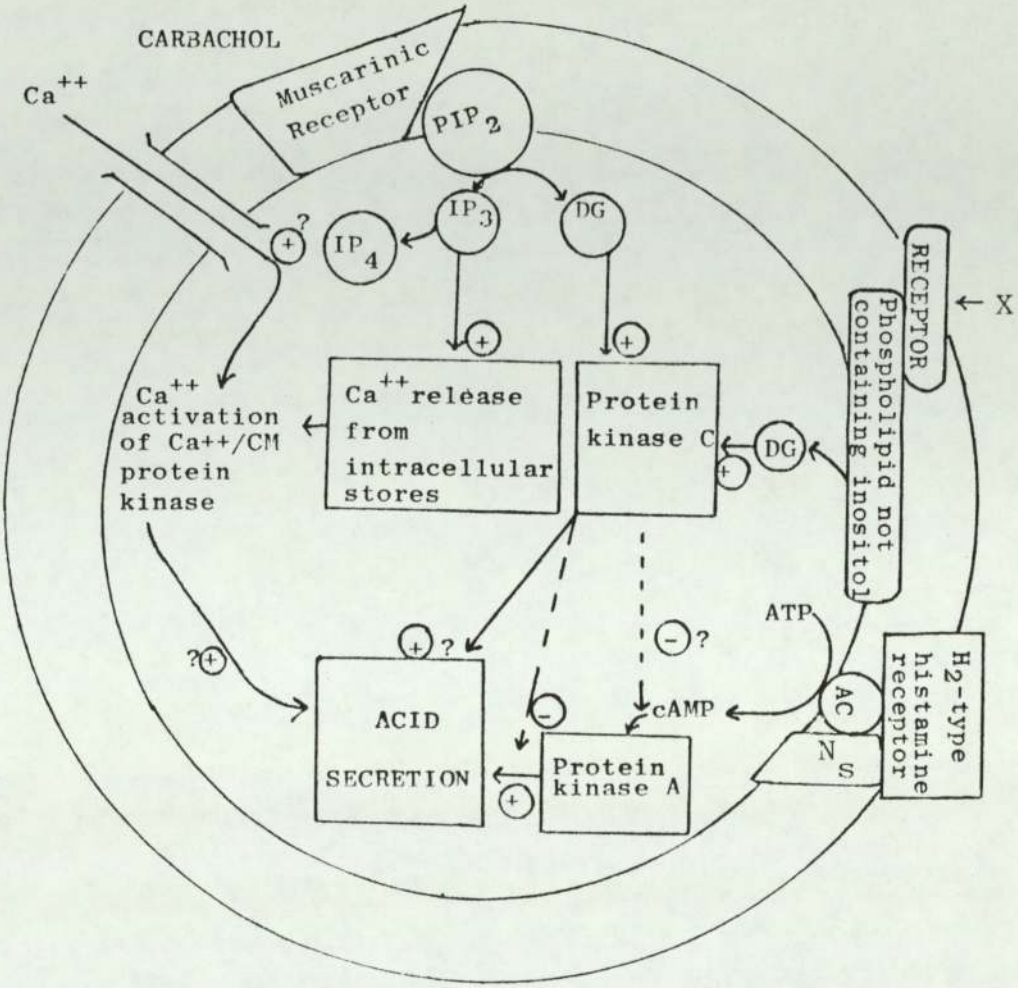
7.3 THE ANTI-SECRETORY ROLE OF EGF

EGF was shown to significantly inhibit histamine-stimulated aminopyrine accumulation *in vitro*, but was ineffective against basal aminopyrine accumulation and that stimulated by carbachol, dbcAMP and histamine plus IBMX. The mechanism of action of EGF was tentatively postulated to be via the activation of a cAMP-phosphodiesterase, and

not due to activation of protein kinase C or to increased prostaglandin production.

In vivo, EGF may function as a paracrine modulator of acid secretion, since it has no effect on acid secretion when added to the lumen of the rat stomach (Konturek et al., 1981), even though it is present in saliva. In addition, the fact that the concentration of EGF in mouse plasma [0.16nM, Carpenter and Cohen (1979)] is considerably less than the concentration of EGF required to produce half-maximal inhibition of acid secretion from rat parietal cells in vitro (19nM, J. F. Hatt, personal communication), also implies a paracrine mode of action for EGF. In man, although EGF is noticably absent from the antral and fundic regions of the stomach (Elder et al., 1978), it is present in the blood where it is contained in platelets (Oka and Orth, 1983). EGF may be released from platelets upon their activation, as may well occur during acute damage to the gastric epithelium. The inhibitory action of this EGF on acid secretion may prevent the accumulation of acid under the protective cap of mucus and fibrin (Ito and Lacey, 1985) which forms over the damaged area. Should this acid be allowed to accumulate then re-epithelialization may be unable to occur.

Fig. 7.1 A speculative scheme suggesting the involvement of protein kinase C in the negative modulation of gastric acid secretion.



The interaction of carbachol with the muscarinic receptor on the parietal cell surface enhances calcium influx and possibly calcium release from intracellular stores, thereby activating a calcium, calmodulin-dependent protein kinase (Ca^{++}/CM-protein kinase). The breakdown of phosphatidylinositol 4,5-bisphosphate (PIP_2) produces two second messengers in the parietal cell, namely inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DG). IP_3 may promote the release of calcium from intracellular stores, and when further phosphorylated by a specific kinase to inositol 1,3,4,5-tetrakisphosphate (IP_4) it may also stimulate calcium influx into the parietal cell in a manner similar to that proposed by Irvine and Moor (1986) for sea urchin eggs. DG may promote protein kinase C activity.

Both Ca^{++}/CM-protein kinase and protein kinase C are responsible for the phosphorylation of cytosolic proteins. In addition, protein kinase C may inhibit histamine-stimulated acid secretion at a point distal to cAMP production. cAMP is generated from ATP by the adenylate cyclase (AC) associated with the histamine, H_2-class, receptor on the parietal cell, and is responsible for protein kinase A activation. A GTP-binding subunit (Ns) also forms an integral part of this receptor complex. It is also possible that an exogenous ligand (X) may activate protein kinase C as a consequence of interaction with its receptor on the parietal surface.

PUBLICATIONS RESULTING FROM THIS WORK.

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Shaw, G. P., Hatt, J. F., Anderson, N. G. and Hanson, P. J. Action of epidermal growth factor on acid secretion by rat isolated parietal cells. Biochem. J. (in press 1987).

APPENDICES.

A1 SOURCE OF REAGENTS.

Reagent	Supplier
A. General Chemicals and biochemicals.	
Acrylaide	BDH
Acrylamide	BDH
Adenosine-5′-diphosphate, disodium salt (ADP)	Boehringer
Adenosine-5′-triphosphate, disodium salt (ATP)	Boehringer
Benzamidine hydrochloride	BDH
Bovine serum albumin (BSA), fraction V	Miles Labs
Bromophenol blue	Bio-Rad Labs
Dextran, mol. wt. 40,000da	Sigma
4-dimethylaminoantipyrine (aminopyrine)	Sigma
Dimethyldichlorosilane	BDH
Dimethylsulphoxide (DMSO) Sp. gr. 1.1g/ml	Sigma
DL-dithiothreitol	Sigma
Ethylenediaminetetraacetic acid (EDTA)	Hopkins & Williams
Ethyleneglycol-bis-(β-aminoethylether) N, N′- tetraacetic acid (EGTA)	Sigma
Fatty acid-free bovine serum albumin	Sigma
Folin and Ciocalteu's phenol reagent	Sigma
Glucose	BDH
Glutamate	Sigma
L-glutamine	Sigma
Glycerol	BDH
Glycine	Sigma
High molecular weight standard mixture	Sigma
Hydrazine hydrate Sp. gr. 1.03g/ml	BDH
DL-3-Hydroxybutyrate, disodium salt	Boehringer
N-2-hydroxyethylpiperazine- N′-2-ethane sulphonic acid (HEPES)	Sigma

Inorganic salts, analytical grade	BDH
L-isoleucine	Sigma
L-(+)-lactic acid L-1 grade	Sigma
L-leucine	Sigma
Leupeptin	Sigma
Lithium acetoacetate	Sigma
2-mercaptoethanol Sp. gr. 1.12g/ml	BDH
2[N-Morpholino] ethanesulfonic acid (MES)	Sigma
NAD^+, grade II	Boehringer
NADH disodium salt	Boehringer
$NADP^+$, disodium salt	Boehringer
2-oxoglutarate, disodium salt	Boehringer
Pepstatin	Sigma
Perchloric acid	Hopkin & Williams
Percoll, for density gradient centrifugation Sp. gr. 1.13g/ml	Pharmacia
Phenolphthalein	BDH
L-α-Phosphatidyl-L-serine	Sigma
Sodium acetate trihydrate	BDH
Sodium-n-butyrate	BDH
Sodium dodecyl sulphate	BDH
Sodium oleate	Sigma
Sodium pyruvate	Sigma
Sucrose	BDH
Tris (hydroxymethyl) methylamine	BDH
Trypan blue, mwt 961da	BDH
Trypsin inhibitor, lyophilised from soybean	Sigma
L-valine	Sigma

B. Enzymes.

Glutamate dehydrogenase (1200U/ml) from beef liver	Boehringer
Pronase, 70,000 PUK/g	BDH

C. Scintillation counting.

"Econofluor"	New England Nuclear
"Protosol"	New England Nuclear

D. Radiochemicals.

Adenosine -5′-[γ-^{32}P] triphosphate triethylammonium salt	Amersham Int.
Aminopyrine,dimethylamine-^{14}C	Amersham Int.
[1,2-^{3}H]- polyethylene glycol, av.mwt. 4000da.	Amersham Int.

E. Drugs and Secretagogues.

Carbamylcholine chloride (carbachol)	Sigma
Dibutyryl cyclic AMP, sodium salt	Sigma
Epidermal growth factor	ICI
Flurbiprofen	Boot Co PLC
Histamine dihydrochloride	Sigma
Ibuprofen	Sigma
Indomethacin	Sigma
3-isobutyl-1-methylxanthine (IBMX)	Sigma
Mefenamic Acid	Sigma
Sodium pentabarbitone	May & Baker
Polymyxin B sulphate	Sigma
Ro 20-1724	Hoffmann-La Roche
12-0-tetradecanoylphorbol-13-acetate (TPA)	Sigma
Trifluoperazine dihydrochloride	Sigma

A2 COMPOSITION OF MEDIA.

A2.1 Preparation of Krebs-Ringer bicarbonate medium containing fatty acid-free bovine serum albumin.

Krebs-Ringer bicarbonate medium (1 litre) was prepared containing NaCl (120mM), $NaHCO_3$(25mM), KCl (4.5mM), $MgSO_4$(1.0mM), Na_2HPO_4(1.8mM), NaH_2PO_4 (0.2mM), and $CaCl_2$ (1.25mM). The medium was warmed to 37°C gassed with 95% O_2: 5% CO_2 for 1 hour and the pH adjusted to 7.4. The bovine serum albumin was dialysed to ensure that it was free from potential substrates as follows: 3g of fatty acid-free bovine serum albumin was dissolved in 30ml of freshly prepared (as above) Krebs-Ringer bicarbonate medium, and poured into a 3/4" diameter dialysis bag. The dialysis bag was suspended in a measuring cylinder at 4^0C, and the albumin dialysed against 3 changes of Krebs-Ringer bicarbonate medium (200ml) with continual stirring over 48 hours. After dialysis the albumin solution was made up to 100ml with Krebs-Ringer bicarbonate medium, filtered through sterile disposable filters (0.45µm: Schleicher and Schull, Dassel, W. Germany) and stored in 20ml aliquots at -20^0C.

A 2.2 Composition of Minimum Essential Medium Eagle (modified) with Earles salts.

The medium was purchased from Flow Laboratories, Ayr, U.K. and contained the following constituents: Arginine (0.73mM), Cystine (0.23mM), Histidine (0.27mM), Isoleucine (0.4mM), Leucine (0.4mM), Lysine (0.5mM), Methionine (0.09mM), Phenylalanine (0.2mM), Threonine (0.4mM), Tryptophan (0.05mM), Tyrosine (0.25mM), Valine (0.4mM), $CaCl_2$ (1.8mM), KCl (5.37mM), $MgSO_4$ (0.81mM), NaCl (116.4mM), NaH Co_3 (23.8mM), $NaH2PO4$ (1.01mM) and glucose (5.56mM). A range of vitamins and cofactors were also present in small quantities as well as 17g phenol red/l.

A 3 SILICONIZATION OF GLASS VIALS.

25ml plastic scintillation vials were cleaned thoroughly using distilled water, and dried in an oven at 90°C. When dry, the interior of each vial was siliconized using dimethyldichlorosilane and again dried in an oven at 90°C. Finally, each vial was rinsed in distilled water and allowed to dry before use. This siliconization procedure was carried out after the vials had been used for five or six experiments.

A4 LIQUID SCINTILLATION COUNTING.

In order to determine the aminopyrine accumulation ratio of a given sample, it was necessary to detemine the amount of ^{3}H and ^{14}C in each sample. Therefore, two counting channels were necessary to detect and differentiate the pulses from each radionuclide. The upper and lower limits of both are computed in the TRI-CARB 2660 system. These channel settings are such that the lower energy radionuclide, ^{3}H, does not contribute to the total count in the high energy channel, and the high energy radionuclide, ^{14}C, contributes minimally to the total count in the low energy channel. The activity (dpm) of the high energy radionuclide present in the sample was determined from equation A4.1.

Equation A4.1: $$Y = \frac{B}{E_4}$$

were Y = dpm of high energy radionuclide
B = Total cpm in high energy channel
E_4 = Efficiency of high energy radionuclide in high energy channel.

The counting efficiency of the high energy radionuclide in the high energy channel (E_4) was determined by means of a quench correction curve relating the external standard channels ratio (ESCR) and the % efficiency. This general curve was produced from a set of scintillation vials containing the same amount of ^{14}C- or ^{3}H- labelled material and varying degrees of quencher (chloroform). The activity of the low energy radionuclide was determined from equation A4.2.

Equation A4.2: $$X = \frac{A - E_3 Y}{E_1}$$

were X = dpm of low energy radionuclide.

A = Total cpm of low energy channel.

E_3 = Efficiency of high energy radionuclide in low energy channel.

E_1 = Efficiency of low energy radionuclide in low energy channel.

A5 PREPARATION OF SUBSTRATE STOCK SOLUTIONS.

A5.1 Preparation of oleate-albumin complex.

Sodium oleate is relatively insoluble in water and at high concentrations is cytotoxic. Thus, it was complexed with albumin present in the incubation medium before addition to parietal cell suspensions. The procedure was as follows; an aliquot (0.5ml) of sodium oleate stock (65.3mM) warmed to 50°C, was added via a warmed pipette tip to a rapidly stirring solution of incubation medium (0.5ml) containing 3% fatty acid-free albumin. In this way the concentration of oleate in the final oleate-albumin stock solution was 32.65mM. 30µl of this stock was added to 1.5ml of cell suspension.

A 5.2 Preparation of sodium acetoacetate.

Sodium acetoacetate was synthesized using the method of Krebs and Eggleston (1945). Ethyl acetoacetate (2M) was hydrolysed with sodium hydroxide (2M) in a volume of 10ml at 40°C for 60 minutes with occasional agitation. The solution was then freeze-dried and a sample taken for assay of acetoacetate (appendix A5.3).

A 5.3 Assay of acetoacetate.

The purity of the acetoacetate solution (see A5.2) was determined using the method of Mellanby and Williamson (1974). A 0.2ml sample of acetoacetate was added to the assay mixture containing phosphate buffer (33mM) and NADH (0.2mM) in a total volume of 3.1ml. After noting the initial absorbance (time O), 10µl of D-3-hydroxybutyrate dehydrogenase (EC 1.1.1.30) was added to give an activity of 50mU/ml, and the absorbance was noted at 340nm until the experimental cuvette was changing at the same rate as the blank. In addition to the sample assay, the end point was accurately determined for control and blank solutions. The purity of the sample acetoacetate solution was determined using the Beer-Lambert law, equation A5.1.

Beer-Lambert law, equation A5.1: $\Delta A = \varepsilon cd$

were ε = The extinction coefficient of NADH ($6.3\ l\ \mu mole^{-1}\ cm^{-1}$).

ΔA = The change in absorbance.

d = The pathlength of the cuvette.

c = The concentration of the sample (unknown).

A6 PREPARATION OF A COMBINED PHOSPHATIDYLSERINE AND 12-O-TETRADECANOYLPHORBOL-13-ACETATE (TPA) STOCK SOLUTION.

A 50µl aliquot was taken from a phosphatidylserine stock (10mg/ml in chloroform: methanol, 95:5), and evaporated under nitrogen. 1ml of homogenization buffer was added to the phosphatidylserine and sonicated for 4 x 1 minute at 40 watts at 0°C.

A 10µl aliquot was taken from a TPA stock (10mg/ml in DMSO) and added to 1ml of homogenization buffer. 5µl of this mixture was added to the phosphatidylserine solution prepared as above to give a combined stock solution of phosphatidylserine (500µg/ml) and TPA (500ng/ml) which was used in the protein phosphorylation experiments.

A7 PROTEIN ASSAY.

A7.1 Sample preparation.

The protein concentration of each sample (cytosolic or particulate in homogenization buffer) was assayed before electrophoresis using a modification of the method of Lowry et al., (1951). The following stock solutions were prepared: A (10g Na_2CO_3/500ml, 0.1M NaOH), B_1 (1g $CuSO_4$ $5H_2O$/100ml), B_2 (2g sodium potassium tartrate/100ml), and just prior to the start of the assay, solution C was prepared by mixing 0.5ml of B_1, 0.5ml of B_2 and 50ml of A.

Each sample (0.2ml) was diluted with an equal volume of water. To this diluted sample 2ml of C was added, mixed and the solution allowed to stand at room temperature for 10 minutes. To this solution, 0.2ml of Folin reagent (diluted 1:1 with water) was added, mixed rapidly and then allowed to stand for a further 30 minutes at room temperature. After 30 minutes the absorbance of the sample was measured at 750nm, and the protein concentration determined using the standard curve (A7.2). On each occasion a reagent blank was prepared as above, except 0.2ml of water was used instead of sample.

A7.2 Standard curve.

A standard curve was constructed for each assay using a stock solution of bovine serum albumin (1mg/ml) as follows; a range of protein concentrations were prepared in distilled water (tot.vol. 0.2ml), to which an equal volume of suitably diluted homogenization buffer was added. These standards were then treated as for the sample (A7.1), and the absorbance measured at 750nm.

A8 DEVELOPMENT OF X-OMAT AR FILM AFTER AUTORADIOGRAPHY.

Fresh stock solutions of developer (172ml made up to 1 litre with tap water) and fixer (200ml made up to 1 litre with tap water) were prepared on each occasion, and the developing process was performed in a dark room. The X-OMAT AR film, which had been in contact with the gel inside a cassette (Kodak X-Omatic cassette) for 60 hours, was immersed in developer for 3 minutes. The film was removed from the developer, drained and rinsed in tap water before it was added to the solution of fixer. After 3 minutes in fixer, with occasional agitation, the film was rinsed in tap water and hung up to dry.

A9 ESTIMATION OF CALCIUM CONCENTRATIONS USING THE CALCIUM ELECTRODE.

A9.1 The use of the Orion 93-20 calcium electrode.

A calibration buffer was prepared of identical composition to that of the phosphorylation reaction buffer but without EDTA or ATP. Aliquots from a 10mM-$CaCl_2$ solution were added to 100ml of this buffer, and the change in electrode potential (mv) was monitored using the calcium electrode. In this way a calibration curve (Fig A9.1) was constructed on graph paper, from which the calcium concentration of unknown samples was determined.

A9.2 Calculation of the calcium concentration in a parietal cell homogenate.

Three major sites of calcium accumulation exist within the cell (Rasmussen et al., 1984):

Cytosol	0.1pmoles/µl
Endoplasmic reticulum	100pmoles/µl
Mitochondria	100pmoles/µl

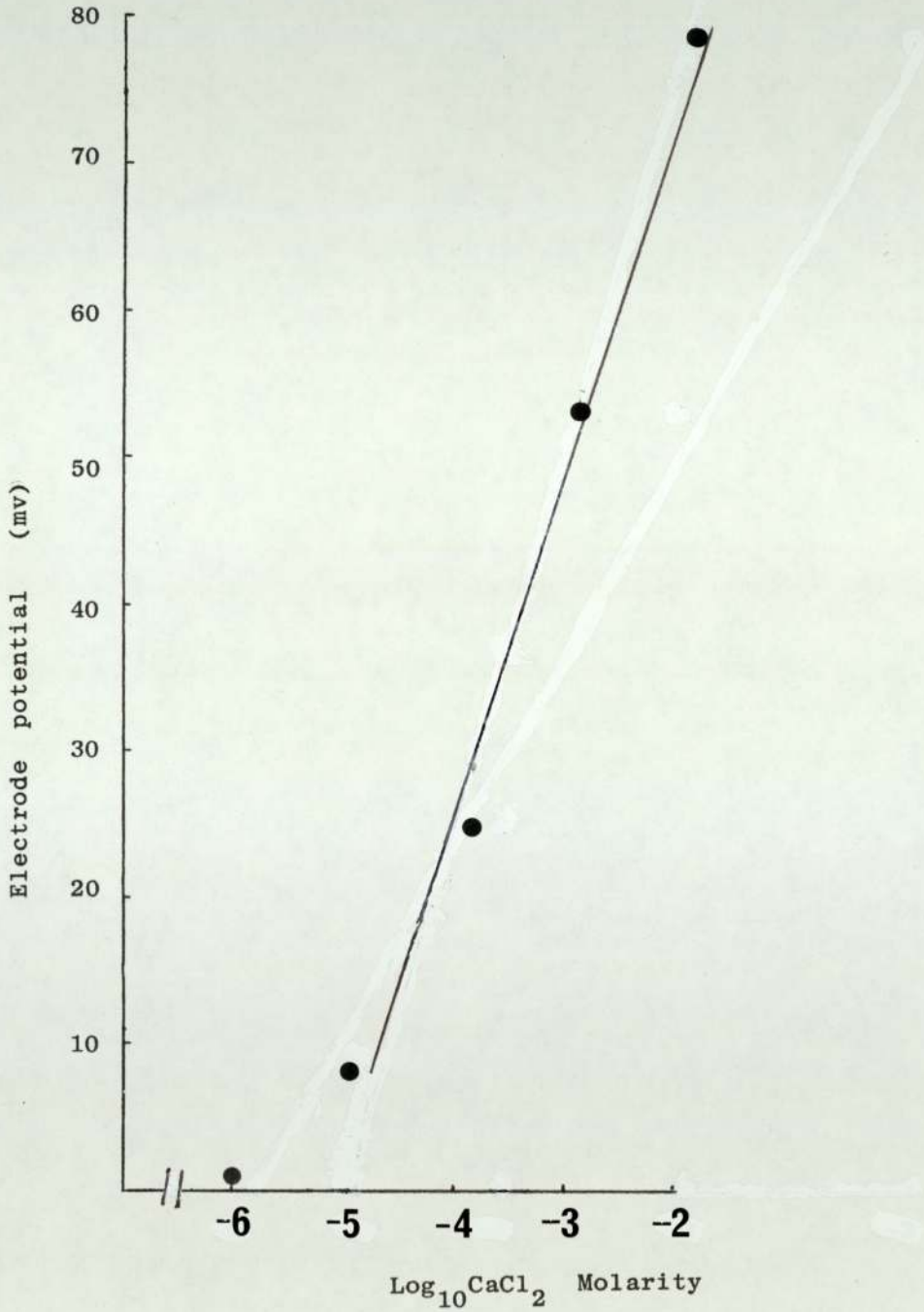
Assuming a parietal cell diameter of 14µm then the parietal cell volume can be calculated to be 1436.75μm^3.

As 1µl = $1mm^3$ = $10^9 \mu m^3$ then 1.4367µl/10^6 cells.

Assuming a cell concentration of 2 x 10^7 cells/ml then 28.73µl of cell water per ml of homogenate.

Figure A9.1

Orion 93.20 calcium electrode calibration curve. The equation of the straight line was determined by a linear regression computer programme to be $y = 124.6 + 23.85x$.



Campbell (1983) suggested that the total calcium concentration in most cells was between 1 and 10mmoles/l of cell water (1-10 nmoles/µl), which corresponds to a range of between 28.73 and 287.3nmoles/ml homogenate.

Using a computer program (Storer and Cornish-Bowden, 1976) the concentration of free calcium in the homogenate at the upper and lower limits were found to be 6.3nM and 0.61nM respectively.

A 10 ANIMALS

Male Wistar strain rats were obtained from Banting and Kingman, Hull, U.K. and were fed on Heygates breeding diet (modified 41b diet) supplied by Pilsbury, Edgbaston, Birmingham, U.K.

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