SUBSTRATE METABOLISM AND ACID SECRETION IN THE RAT STOMACH.

by

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A thesis submitted for the degree of

Doctor of Philosophy

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SUMMARY

The aim of this work was, firstly, to identify which substrates could support acid secretion by rat parietal cells and secondly, to investigate the intracellular events involved in stimulus transduction in rat parietal cells.

A technique was developed which enabled satisfactory measurements of arteriovenous differences across the rat stomach *in vivo*. Glucose, D-3-hydroxybutyrate and branched-chain amino acids were taken up by the stomach wall under control and acid-secreting (pentagastrin-stimulated) conditions. The pattern of substrate metabolism appeared unaffected by stimulation of acid secretion.

Fractions of viable gastric cells containing ~20% parietal cells were isolated and were responsive to stimulation by secretagogues as judged by an increase in accumulation of the weak base aminopyrine, in acidic spaces within the cells. The parietal cell content could be enriched to ~77% by density gradient centrifugation in Percoll with retention of responsiveness to secretagogues.

Parietal cells appeared to possess stores of endogenous substrate which could be utilised to provide energy for acid secretion. Alone, and at physiological concentrations, glucose, oleate, lactate, D-3-hydroxybutyrate, isoleucine, acetoacetate and valine supported acid secretion by parietal cells, as judged by their stimulation of aminopyrine accumulation. At higher concentrations, acetate, butyrate and leucine, but not glutamine, could also support acid secretion. The maximally effective concentration of glucose did not support maximal rates of acid secretion, probably because, *in vivo*, parietal cells derive energy from more than one substrate. Data from studies *in vivo* and *in vitro* suggested that metabolism of glucose, D-3-hydroxybutyrate and isoleucine is important in supporting acid secretion *in vivo*.

The tumour-promoting phorbol ester, 12-O-tetradecanoyl-phorbol-13-acetate (TPA), inhibited acid secretion by parietal cells stimulated with histamine and isobutylmethylxanthine or dibutyrylcyclic AMP. Action of TPA did not involve production of prostaglandins nor release of somatostatin but could have been mediated by activation of calcium-sensitive, phospholipid-dependent protein kinase (protein kinase C).

Key words: stomach. acid secretion. parietal cell. metabolism. phorbol esters.

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ABBREVIATIONS

The following represents a list of non-standard abbreviations used throughout this work.

cAMP	cyclic AMP
concn.	concentration
dbcAMP	dibutyryl cyclic AMP
i.d.	internal diameter
IMX	3-isobutyl-1-methylxanthine
o.d.	outside diameter
S.D.	standard deviation
S.E.M.	standard error of mean
V-A	venous-arterial

CONVENTIONS

The following convention was used throughout this work,

Where values are quoted as a $\pm$ b (c), this refers to the mean $\pm$ SEM, with the number of experiments in brackets.

CHAPTER 1.

INTRODUCTION

1. INTRODUCTION

This work is concerned with two aspects of the biochemistry and physiology of the parietal cell. Firstly, studies have been performed with both the stomach *in vivo* and isolated parietal cells *in vitro* in an attempt to eludicate the substrate-dependency of acid secretion. Thus, the aim of the first part of this work was to identify which substrates were best able to provide, via their metabolism, the quantities of energy required by the acid-secreting parietal cell.

The second section is concerned with an investigation of an aspect of the intracellular events mediating acid secretion in the parietal cells. In particular, experiments have been performed to investigate the putative role of the calcium-sensitive, phospholipid-dependent protein kinase (protein kinase C) in this process.

1.1 BASIC PHYSIOLOGY OF THE RAT GASTRIC PARIETAL CELL.

1.1.1 Location in the Gastric Mucosa.

The stomach of most non-ruminant mammals, including the rat, is a single-chambered organ broadly consisting of a non-glandular region involved primarily in the secretion of mucus, and a glandular portion which is divided by an ill-defined line into the lower third proximal to the duodenum, the antrum, and the upper two-thirds, referred to here as the fundus. The antrum is predominantly populated with G-cells which secrete gastrin while the fundus is the region where most of the acid-secreting parietal cells and pepsinogen-secreting chief cells are located. The epithelium of the gastric fundus

is a single layer of various cell types which is highly involuted forming glands buried in the stomach wall. The glands are connected with the gastric lumen through gastric pits, and more than one gland may be associated with one pit. These infoldings effectively increase the surface of the gastric mucosa 20-fold. A typical gland of the fundic mucosa is shown diagramatically in Fig. 1.1. Both parietal cells and chief cells tend to be located towards the lower half of the glands. There are several other cell types which may be found in the glands of the gastric fundus (Table 1.1) although there are some species variations, the major difference being in the nature of the cells associated with storage of histamine (Soll et al., 1981), and the site of production of intrinsic factor (Donaldson, 1981).

1.1.2. Morphology.

The mammalian parietal cell is oval to pyramidal in form with a diameter, at its widest point, up to 25μm. It is inserted into the gastric gland so that the basolateral surface extends out from the wall of the cylindrical gland. Parietal cells are probably the most distinctive cell type in the stomach, in that they possess large concentric nuclei and a large number of mitochondria which account for about 34% of the cell volume (Helander & Hirschowitz, 1972). Perhaps the most striking features of the cell are the presence of intracellular canaliculi, when the cell has been stimulated to secrete acid by secretagogues. These are a network of canals generally situated near the apical side but which may extend into the basal cytoplasm and encircle the nucleus (Fig 1.2). Under the electron microscope the

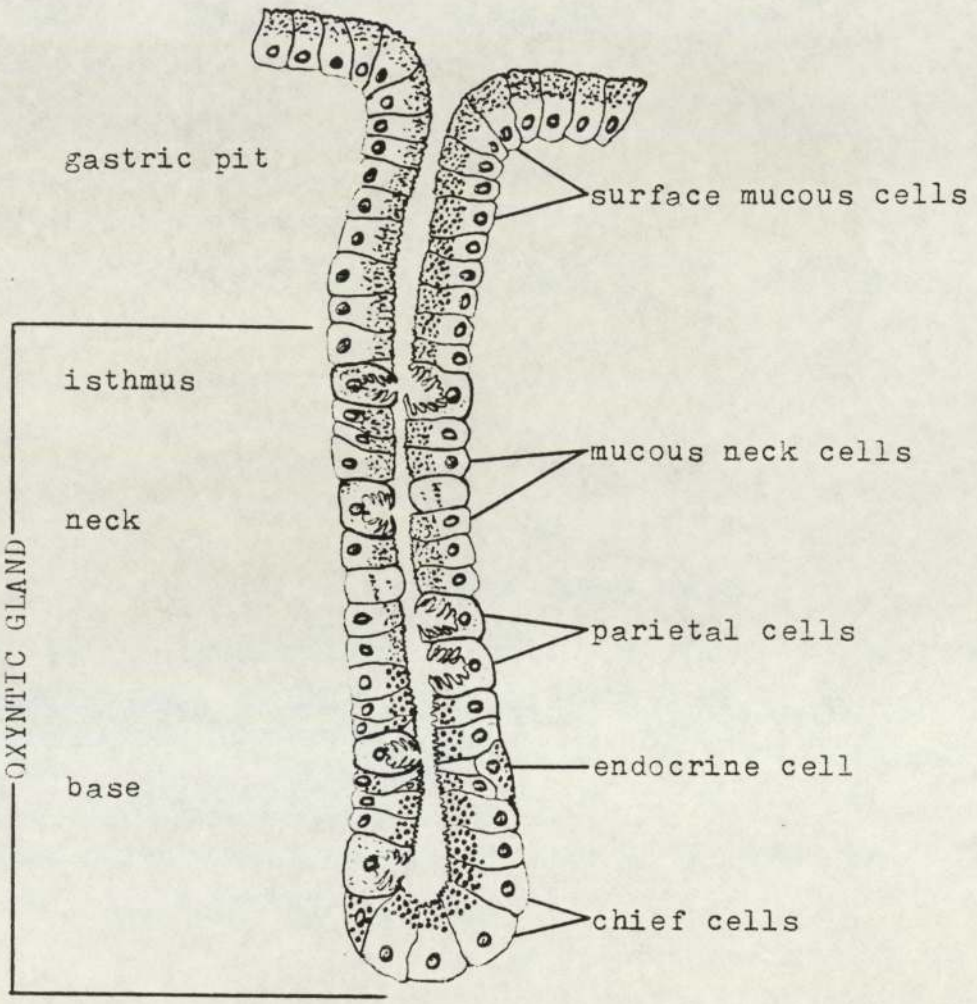


Fig 1.1 Diagramatic representation of an oxyntic gland from the fundus of a mammalian stomach.

Table 1.1 Cell Types in regions of the mammalian stomach.

Region	Cell-type	Function/secretory product
non-glandular	surface mucous	mucus, HCO_3^-
	undifferentiated mucous	cell renewal
fundus	Surface mucous	mucus, HCO_3^-
	mucous neck	cell renewal
	parietal	HCl, intrinsic factor*
	chief	pepsinogen
	A-cell	enteroglucagon
	G-cell	gastrin
	mast ‡	histamine, serotonin
	argyrophil	secretin (?)
	D-cell	somatostatin
antrum	surface mucous	mucus, HCO_3^-
	parietal	HCl
	G-cell	gastrin
	D-cell	somatostatin

* In the rat, intrinsic factor, but not HCl, is secreted by chief cells.

‡ In the rat, histamine, but not serotonin, is stored and released by enterochromaffin-like cells.

canaliculi are seen to possess microvilli, structures important in parietal cell function (see below). The other functionally important structures are the tubulovesicles, present in large numbers towards the apical pole of the resting parietal cell.

1.1.3. Stimulation of Acid Secretion.

Stimulation of acid secretion, *in vivo*, is under tri-phasic control. The cephalic phase is that gastric secretion evoked by messages acting on the central nervous system and is mediated via the vagus nerves. Initiators of the cephalic phase normally include the sight or smell of food and the presence of food in the mouth. The gastric phase of acid secretion results from stimuli acting in the stomach. Distention of the stomach wall results in stimulation of parietal cells and G-cells via vagovagal and intramural reflexes (Grossman, 1981). The promotion of gastrin release from G-cells by calcium ions and peptides, or by certain aromatic or long-chain aliphatic amino acids (Lichtenberger et al., 1982), also appear to initiate acid secretion. The intestinal phase occurs via distention, and the presence of amino acids and peptides in the small intestine and may be mediated by the stimulation of the release of an as yet unidentified hormone which may act directly or indirectly on the parietal cell (Grossman, 1981). The mechanism by which these stimulatory pathways finally mediate parietal cell function is discussed in 1.1.3.2.

1.1.3.1. Ultrastructural changes.

The ultrastructural appearance of the parietal cell

undergoes large changes from rest to active acid secretion (Forte et al., 1981). Upon stimulation, the numerous tubulovesicles are greatly reduced in number and are replaced by an expansion of the intracellular canaliculi from the surface of which project long microvilli (Fig 1.2). The apical membrane, as a result of this change, is amplified, in terms of surface area, by a factor of 10. Membrane freeze-fracture studies (Forte et al., 1981) suggest that the expanded surface is derived from fusion of the cytoplasmic tubulovesicular membranes with the existing limited apical surface. Also, studies using monoclonal antibodies to the $H^+ + K^+$ ATPase (which is involved in the unidirectional pumping of protons across the secretory membranes of parietal cells; see 1.2.1) suggest tubulovesicles are precursors of the secretory canaliculi for such antibodies selectively label the tubulovesicles of resting parietal cells and the microvilli of the secretory canaliculi of secreting cells (Smolka et al., 1983). An alternative hypothesis has been proposed to explain the transformation process (Berglindh, 1984). This suggests that the tubulovesicles, seen in resting cells, are really collapsed secretory channels which expand osmotically upon stimulation. Accumulation of HCl within the tubulovesicles and the pumping of K^+ into the cytoplasm leads to an increased osmolarity in the acid-containing space which in turn draws in excess water and increases the volume of the space.

Whatever the mechanism, the ultrastructural changes take place fairly rapidly after stimulation of the cell by acid secretagogues (Forte et al., 1981). Within 3 minutes a

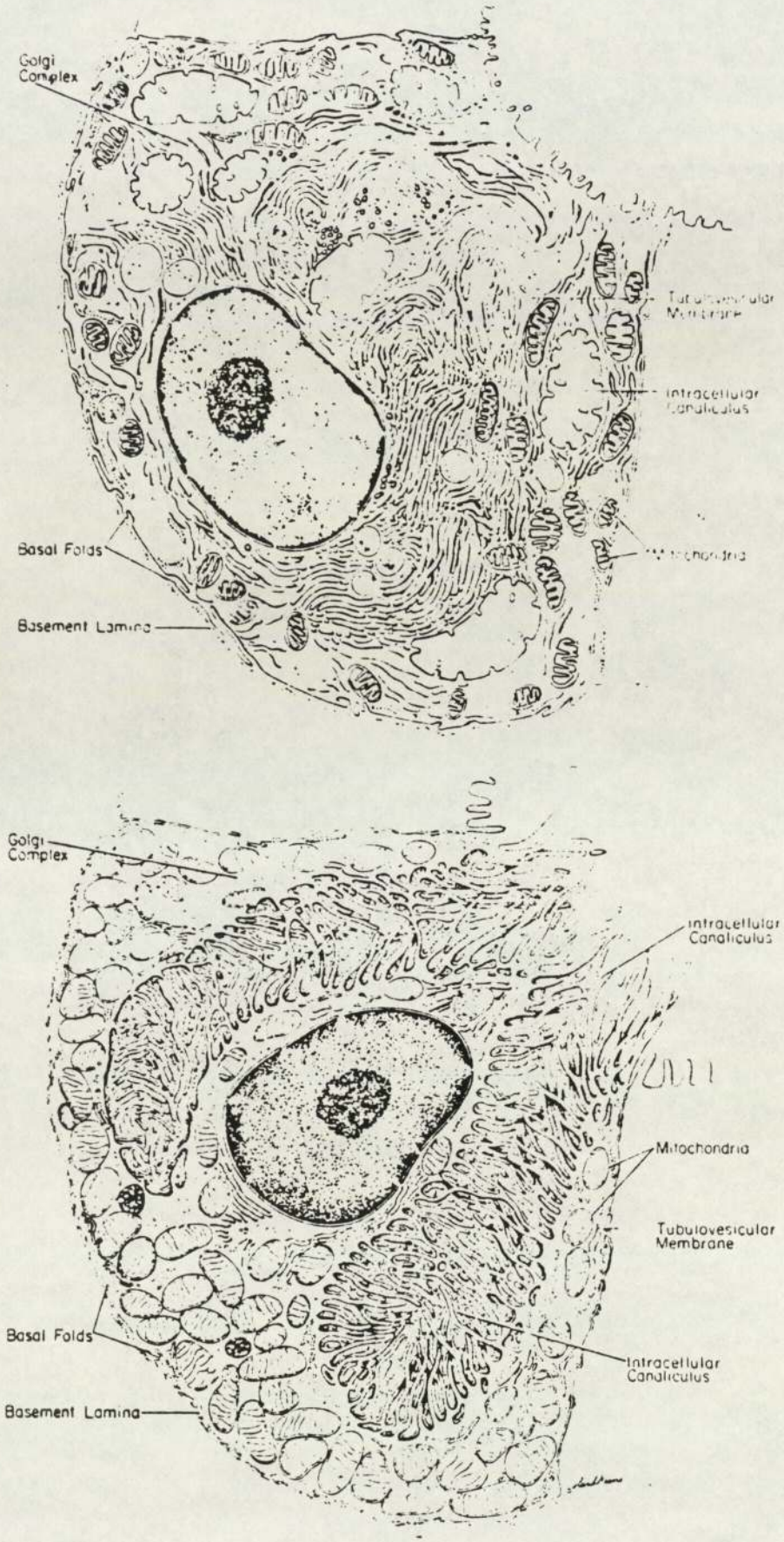


Fig 1.2 Top:-ultrastructural appearance of a non-secreting parietal cell. The cytoplasm is replete with tubulovesicles and the internalised canaliculus is devoid of microvilli. Bottom:-ultrastructural appearance of an acid-secreting parietal cell containing long microvilli and few tubulovesicles.

distinct increase in the length of microvilli is observed, concomitant with a reduction in the number of tubulovesicles. Within 30 minutes, when acid secretion has reached a steady-state the membrane expansion process is complete - the canaliculi are replete with numerous long processes and few tubulovesicles are present within the cytoplasm.

1.1.3.2. Secretory response to stimuli.

Acid secretion is influenced by endocrine, neurocrine, and paracrine effectors. Three substances found in the body are capable of acting directly or indirectly to induce acid secretion by the parietal cell. These are gastrin, acetylcholine and histamine and all use one of the three modes to deliver chemical messages to the parietal cell. The hormone gastrin is released from the G-cells of the antral mucosa and the first part of the duodenum into the blood and directly activates the parietal cell (dog) or causes the release of histamine from endocrine cells (rabbit) (Berglindh, 1984). In the rat, gastrin appears to cause release of histamine from enterochromaffin - like cells (Hakanson et al., 1974), but whether gastrin receptors exist on the parietal cells is not known. Neurocrine stimulation of acid secretion occurs via acetylcholine which is released at or near the parietal cell by postganglionic neurons. Histamine (paracrine) is released from mast-like cells, or in the rat, enterochromaffin-like cells (Soll et al., 1981) in the lamina propria of the fundic mucosa into the extracellular fluid through which it diffuses to the adjacent parietal cells.

Evidence that the three substances participate in the

physiological regulation of gastric acid secretion has been derived from the use of specific antagonists, at least for acetylcholine and histamine. Thus, muscarinic antagonists such as atropine and histamine H_2-antagonists such as cimetidine both strongly inhibit all the physiological features of acid secretion including basal secretion and the response to a meal (Sachs & Berglindh, 1981). The physiological role of gastrin has been demonstrated by showing that gastric acid secretion takes place as a result of the infusion of gastrin into the bloodstream to a concentration similar to that observed during a meal (Feldman et al., 1977).

Potentiation among parietal cell stimulants appears to occur in vivo i.e. when two stimulants act simultaneously, the response is greater than the sum of the individual responses (Grossman, 1967). Much of the information on the way in which secretagogues cause stimulation and interact with each other has been derived from studies with isolated parietal cells from various species, and this will be reviewed briefly in section 1.3.

1.1.4. Inhibition of acid secretion.

A variety of agents are known to inhibit acid secretion including somatostatin, prostaglandins, gastric inhibitory polypeptide, secretin, glucagon, vasoactive intestinal polypeptide and cholecystokinin (Schepp et al., 1983a; Gespach et al., 1982). Physiological roles for some of these substances are unlikely since, for example, secretin and cholecystokinin do not cause inhibition at concentrations known to be physiological (Schepp et al., 1983a). However, convincing evidence has been produced suggesting that

inhibition caused by somatostatin and prostaglandins are physiological effects. Somatostatin is released from D-cells in gastric glands and these cells possess projections which often end on parietal cells (Larsson et al., 1979), and somatostatin, at physiological concentrations, inhibits acid secretion by rabbit parietal cells stimulated by histamine (Chew, 1983).

Acid secretion by isolated canine parietal cells was inhibited by both endogenously synthesised and exogenously added prostaglandins (Skoglund et al., 1982), indicating a direct action on parietal cells, but prostaglandins may also act indirectly. Thus, although infusion of prostaglandins, into the canine gastric artery potently inhibits gastric acid secretion (Gerkens et al., 1978), the effect could be related to the influence by prostaglandins on mucosal blood flow on which gastric acid secretion is dependent.

1.2. ENERGY SOURCE FOR ACID SECRETION

1.2.1. Primary Source of Energy.

Parietal cells are capable of generating a 10^6-fold H^+ gradient across their secretory membrane with luminal pH values of around 0.8 achieved during maximal acid secretion (Berglindh et al., 1980a). The primary source of energy for the active transport of protons has been considered to be derived from one of two mechanisms. The 'redox' hypothesis (Hersey, 1974), states that energy is provided directly in response to exogenous stimulation and cellular metabolism is associated with a reduction of respiratory chain components. Hydrogen ions are then delivered to the secretory surface via reduced pyridine

nucleotide (Fig 1.3). Data showing the absolute O_2 dependence of acid secretion in amphibian mucosa (Hersey, 1974) and a maximum H^+ : O ratio of 2 in dogs (Moody, 1968) provide some support for this mechanism .

The alternative hypothesis, now more widely accepted, cites ATP as the sole primary source of energy for acid secretion. Hydrolysis of ATP by an $H^+ + K^+$ ATPase located on the membranes of secretory components of the parietal cell (Smolka et al., 1983) results in the vectorial transport of protons across the membrane in exchange for K^+ (Fig 1.4). Exogenous ATP restores the acid–secretory response of permeabilised rabbit gastric glands to secretagogues in the presence of a high K^+ concentration (Berglindh et al., 1980) and vesicles isolated from parietal cells (which are mainly orientated with cytosolic face on the outside) accumulate H^+ by means of a $K^+ - H^+$ exchange pump mechanism requiring intravesicular K^+ (Saccomani et al., 1977). Thus, although it seems likely that proton transport occurs as a result of ATPase activity, the possibility that a form of redox component exists distal to the H^+ pumping site has not been discarded. Indeed there have been proposals (Sachs et al., 1978) suggesting that cell metabolism may provide some H^+ from reduced pyridine nucleotides, the H^+ then being transported by energy derived from ATP hydrolysis.

1.2.2. Substrate Metabolism

If, as seems likely, the acid-secreting parietal cell hydrolyses large amounts of ATP during transport of protons

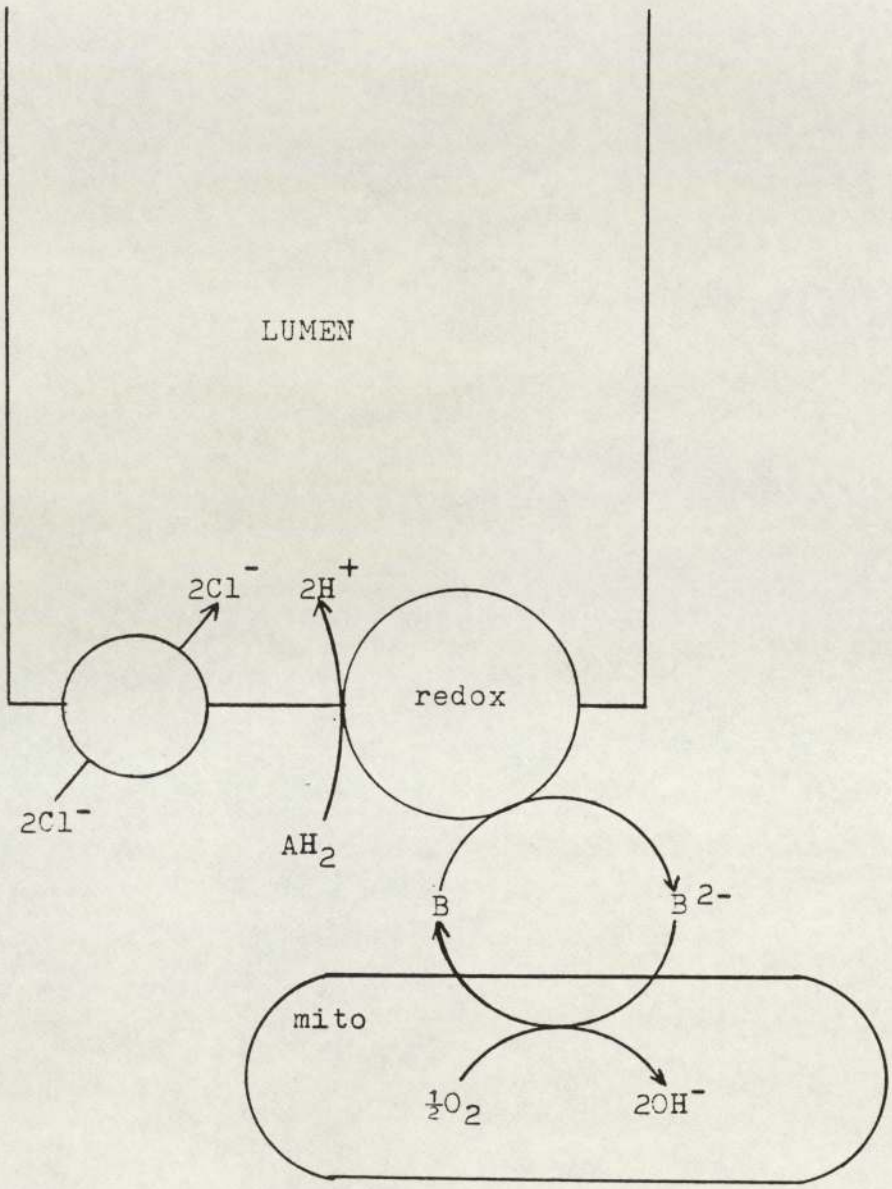


Fig 1.3 Possible mechanism by which electrons and protons are separated by an oriented redox reaction across the canalicular membrane.

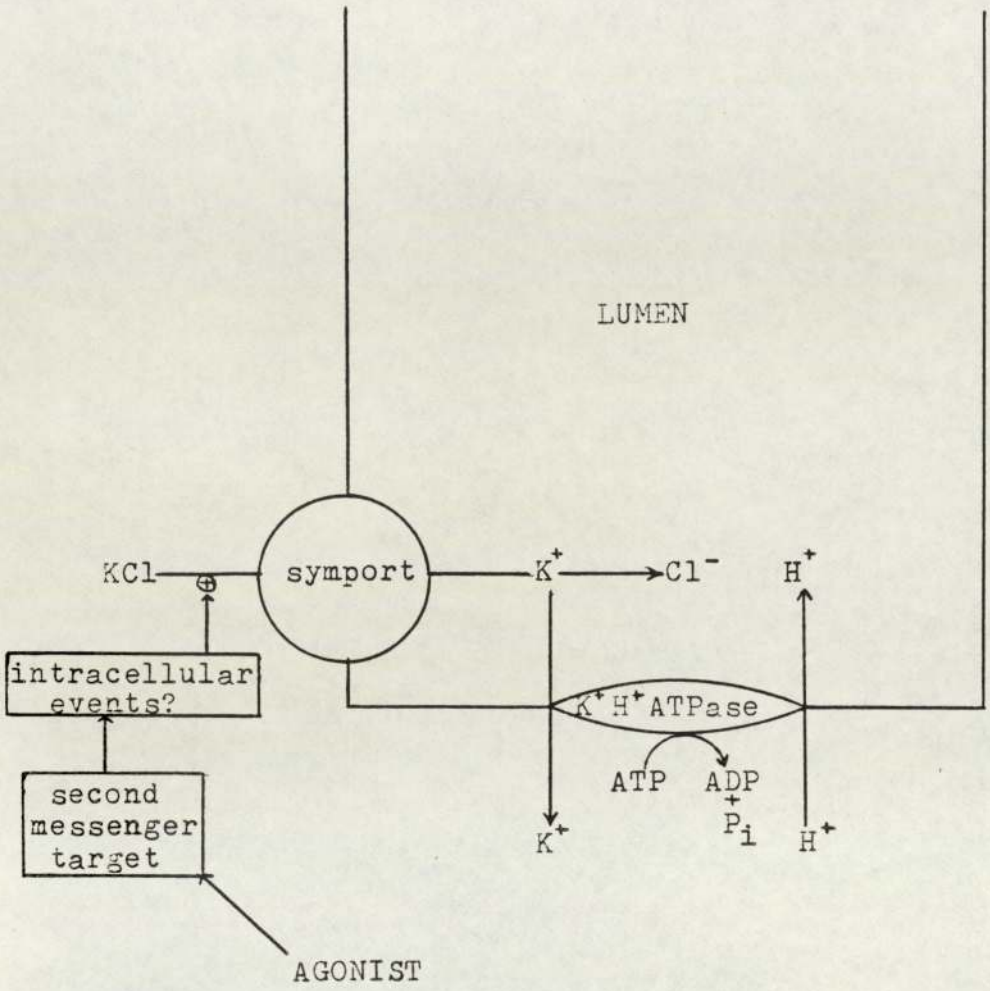


Fig 1.4 Transport of protons across the canalicular membrane of the parietal cell via a K^+/H^+ATPase. K^+ leaks from the cytosol to the canalicular lumen and is then exchanged for H^+ by the ATPase. Stimulation of K^+ transport may activate the process (Wolosin & Forte, 1982).

across its secretory membranes, a continuous supply of ATP must be maintained. Endogenous ATP and creatine phosphate levels could not support maximal acid secretion for longer than a few seconds without replenishment of the high energy phosphate, and moreover, the phosphorylation potential (ATP/ADP + P_i) in frog gastric mucosa *in vitro* (Durbin et al., 1974) and in parietal cell-enriched biopsies from dog gastric mucosa *in vivo* (Sarau et al., 1975) shows little change at the onset of acid secretion. The high mitochondrial content of parietal cells, and the fact that acid secretion is absolutely dependent on oxygen (Davenport & Chavre, 1950), suggests much energy is derived from oxidative phosphorylation, but there is still controversy as to which substrates are metabolised by parietal cells to provide energy.

It is important to identify substrates supporting acid secretion and to assess their metabolism in parietal cells since the metabolism of particular substrates may be intrinsically associated with the acid-secretory process (Sernka & Harris, 1972; Hersey, 1977). If the onset of acid secretion is associated with increased parietal cell metabolism, then it is also possible that the pattern of metabolism may change. This could be due to direct action of secretagogues on the metabolism of certain substrates, by increasing their transport into the cell or by activation of key catabolic enzymes. In amphibian gastric mucosa, secretagogues appeared to cause reduction of cytochromes by mobilisation of endogenous substrate, before secretion of acid (Hersey, 1974). In dog

gastric mucosa *in vivo*, increased substrate metabolism accompanies acid secretion (Sarau et al., 1977) and maximal acid secretion by piglet gastric mucosa is dependent on a supply of exogenous substrate (Forte et al., 1980), as is that by rabbit gastric glands (Hersey, 1981). However, if metabolism of particular substrates is related to the acid-secretory process, then there is still controversy as to which substrates these are.

A detailed review of the substrate-dependency of acid secretion is described in 5.1.2, but may be summarised. Thus, there appears to be differences between the amphibian and mammalian stomach and this is perhaps expected given the differences in the dietary habits of amphibians and mammals. In the amphibian there is a general concensus that lipid-derived substrates, such as butyrate, are better able to support acid secretion than carbohydrate fuels (Alonso et al., 1967; Hersey, 1977). In mammals there have been conflicting results, and this may be caused by several factors. Comparisons have been made between rabbits (herbivorous), dogs (carnivorous) and piglets (omnivorous). Developmental status may also be a factor in the substrate preference of the acid-secreting stomach. Therefore a comparison between the substrate-dependency of acid secretion in neonatal piglet gastric mucosa (Forte et al., 1980) with that in, for example, mature rabbit gastric glands (Hersey, 1981) may not be valid. If the objective of such studies is ultimately to identify the substrate-dependency of acid secretion in the human adult stomach, then it would probably be better to use a mature animal whose dietary

habits more closely resemble that of man. Such an animal is the rat, which has the added advantage of low purchasing and maintenance costs and for these reasons, this animal will be used in these studies. However, it should be pointed out that the physiology of the rat stomach does not entirely mirror that of the human, and some of the differences known to exist have been mentioned (1.1.1).

Another problem with previous studies on the substrate-dependency of acid secretion has been the tendency by most workers to test substrates alone and at concentrations above those which are normally presented to the parietal cell *in vivo*. Therefore, this study will attempt to elucidate the substrate-dependency of acid secretion by examining the situation as far as possible *in vivo* before testing individual substrates at physiological concentrations with an isolated cell system *in vitro*. Ultimately it is intended to answer the questions, "Does the process of acid secretion by the rat stomach derive energy from the metabolism of any particular substrate or group of substrates and if this is the case what are these substrates?"

1.3 STIMULUS-SECRETION COUPLING IN THE PARIETAL CELL

1.3.1. Receptors.

The use of receptor antagonists has demonstrated the existence of histamine and cholinergic receptors on parietal cells (Soll, 1978; Soll, 1980). Dissociation constants for cimetidine inhibition of histamine action and for atropine inhibition of carbachol action indicate the parietal cell possesses typical H_2-histamine and

muscarinic receptors. The fact that neither of these inhibitors blocks the action of gastrin (Soll, 1982), suggests the existence of specific gastrin receptors on canine parietal cells. Although no specific antagonists to gastrin are available, work with radioiodinated gastrin strongly suggests that gastrin receptors exist on canine parietal cells and have a specific role in mediating acid secretion (Rutten & Soll, 1981). The above work, with isolated canine parietal cells, has led to a fairly comprehensive understanding of hormonal interaction with parietal cells, although there is a possibility that differences exist in other species. For example, no data appears to be available on stimulation of rat parietal cells by gastrin, and the response of parietal cells from different species to carbachol may differ (Soll, 1981a).

1.3.2. Stimulus Transduction.

The effect of a hormone binding to its receptor is eventually to change the concentration of a second messenger in the target cell. Two types of second messenger, namely cAMP and Ca^{2+}, appear to be important in stimulation of parietal cells. The effect of histamine binding to H_2-receptors and raising intracellular cAMP levels is well-established (Soll & Wollin, 1979) and this presumably occurs via activation of adenylate cyclase closely associated with the receptor protein in the plasma membrane. Indeed the activity of adenylate cyclase in parietal cells is correlated with acid secretion (Scholes et al., 1976) and acid secretion is stimulated by inhibitors of phosphodiesterase, the enzyme catalysing the breakdown of cAMP, (Fromm et al., 1975).

Changes in cAMP levels would appear to be related to parietal cell functioning since the stable analogue of cAMP, dibutyrylcAMP, stimulates O_2 consumption, aminopyrine accumulation and morphological transformation in parietal cells (Soll & Wollin, 1979; Soll, 1980, Michealangeli, 1976). However, the mechanism by which cAMP mediates the characteristic responses of the parietal cell to histaminergic stimulation remains to be established, although it was shown recently that histamine-induced stimulus-secretion coupling in parietal cells involves activation of cAMP-dependent protein kinases (Jackson & Sachs, 1982).

Neither carbachol nor gastrin has been shown to activate gastric adenylate cyclase or to affect cAMP levels in either unstimulated or histamine-stimulated parietal cells (Soll & Wollin, 1979), and therefore their second messengers are thought to be different from that of histamine. Thus, stimulation of parietal cells by acetylcholine may elevate cytosolic Ca^{2+}levels. Stimulation of canine parietal cells with carbachol was highly dependent upon the concentration of extracellular calcium and also caused $^{45}Ca^{2+}$ uptake by the cells (Soll, 1981b). Work with the flourescent Ca^{2+} indicator, Quin 2, also indicates that carbachcl raises intracellular levels of Ca^{2+} by stimulating Ca^{2+} influx across the cell membrane of rabbit parietal cells (Sachs, 1984). Gastrin also appears to influence Ca^{2+} levels in the parietal cell, but its action may involve mobilisation of intracellular pools of Ca^{2+}. Thus, gastrin raises intracellular Ca^{2+}in rabbit parietal cells, as indicated with Quin 2, but the effect, although dependent on the presence of extracellular Ca^{2+}, is not inhibited by lanthanum ions (Sachs, 1984).

The mechanism by which histamine, acetylcholine and gastrin act synergistically to stimulate acid secretion both *in vivo* and *in vitro* (Soll, 1981) remains to be established. However it seems likely that the point or points of synergism occur distal to the production of second messenger, since histamine does not alter intracellular Ca^{2+} (Sachs, 1984) and carbachol has no effect on the stimulation of cAMP by histamine (Soll,1981b).

The ultimate target of the second messengers is to switch on the process of acid secretion i.e. membrane rearrangement and the pumping of protons across secretory surfaces. Work with vesicles isolated from resting and stimulated rabbit fundic mucosa has shown that stimulated vesicles possess K^+ permeability whereas control vesicles require the presence of valinomycin for K^+ transport, (Wolosin & Forte, 1982). Thus, the ultimate target of one or both second messengers may be to alter the permeability of the secretory membrane to K^+ and activate the $K^+ + H^+$ ATPase (Fig 1.4).

What is not clear, at this stage, is the sequence of events linking increased second messenger concentration with the events associated with acid secretion. For instance, does Ca^{2+} activate a calcium-or calmodulin-dependent regulatory protein, such as a protein kinase? In addition, if histamine activates a cAMP-dependent protein kinase (Jackson & Sachs, 1982), then what is the substrate subsequently phosphorylated?

It is the intention, in the second part of this work, to attempt to define more clearly the actions of secretagogues in parietal cells, by investigating the potential

role of protein phosphorylation induced by the calcium-sensitive, phospholipid-dependent protein kinase (protein kinase C), an enzyme implicated in the stimulus-secretion coupling of many other cell types (see Chapters 7 & 8).

CHAPTER 2.

ARTERIOVENOUS DIFFERENCES ACROSS THE CONTROL AND ACID-SECRETING STOMACH.

2.1. INTRODUCTION.

Several attempts have been made to assess the metabolism of the parietal cell using *in vitro* techniques. These have produced conflicting results (see Chapter 1) as to the relative importance of carbohydrate and lipid fuels as substrates for generating the energy required by the acid-secreting parietal cell. A problem with such experiments is that potential substrates are often presented alone to the tissue or at non-physiological concentrations. Measurement of arteriovenous differences *in vivo* is a good procedure for the study of overall metabolism by a particular tissue. In this technique the concentrations of potential substrates are measured in the blood before and after it has perfused the tissue being studied. A decrease in the concentration of a particular substrate in the blood upon passage through the tissue indicates that this substrate has been taken up and probably metabolised, while an increase in the concentration of a substrate in venous compared with arterial blood, suggests that the substrate may be a product of tissue metabolism. The technique in isolation does not, however, provide an absolute measure of the rate of substrate metabolism, nor can the products of such metabolism in the venous blood be unequivocally indentified. Rate of metabolism can be estimated by simultaneously measuring the rate of blood flow through the tissue and there are several techniques available for this type of measurement in the stomach as reviewed by Guth (1982).

To establish the origin of metabolic products in venous blood, further studies using radiolabelled substrates must

be carried out (Windmueller & Spaeth, 1978) and also measurements of enzyme activities associated with likely metabolic pathways must be performed. The main advantage of the measurement of arteriovenous differences *in vivo* is that substrates are presented together and at physiological concentrations, provided the measuring procedure does not alter intermediary metabolism (see 2.3.3). Furthermore, the cells are present in their normal topographical relationship (c.f. isolated cells, 3.1) and are well oxygenated.

Arteriovenous difference measurements to assess metabolism in tissues can be performed in various ways. A potential problem, if venous and arterial samples are taken sequentially is that taking the first sample may influence the substrate concentrations in the second sample. To surmount this problem, Windmueller & Spaeth 1978) simultaneously sampled blood from the aorta and a vein draining segments of rat jejunum. On the other hand, Yamamoto et al. (1974), when studying amino acid metabolism in and between several organs in the rat, used different donors for arterial and venous samples. If it is not possible to sample simultaneously, the time between sampling arterial and venous blood should be reduced to a minimum. Despite this, it would probably be necessary to assess any changes taking place in metabolite levels during the withdrawal of the first sample. Trauma associated with rupturing a vein is likely to be less than that associated with an artery and so it is best to withdraw the venous sample first, if simultaneous sampling is not practicable. These techniques use needle-cannulation of vessels for blood sampling, but Hawkins et al. (1971a) demonstrated

that it is also possible, and valid, to sample venous blood in the brain of rats by puncturing the dura and removing blood from the sinuses below, through a needle. The above methods all use anaesthetised animals, but it is possible to use conscious volunteers as has been demonstrated by Aoki et al. (1971) who measured amino acid levels across forearm muscle in man.

The main problems concerning the measurement of arteriovenous differences across the rat stomach are the complexity and size of vessels draining the organ (Fig 2.1). The splenic vein is the main vein but this also receives tributaries from the pancreas and spleen. Therefore, before sampling from this vessel can occur, the occlusion of pancreatic and splenic branches, which effectively removes these organs from the circulation, is necessary. In addition, the splenic vein is small and relatively inaccessible. Although the portal vein is large and easy to sample from, using it for venous sampling involves, to an even greater extent, tying off other organs before only blood draining the stomach is flowing through it. Indeed such a procedure severely limits hepatic blood flow. The other possibility is to use a vein which is more accessible and does not require tributaries to be tied. Such a vessel is the coronary vein (Fig 2.1) which has the additional advantage of draining blood from the acid-secreting portion of the stomach only. It is, however, of small diameter than the portal or splenic vein. Sampling arterial blood is less complicated. Generally, the abdominal aorta is used because it is easy to cannulate and relatively accessible.

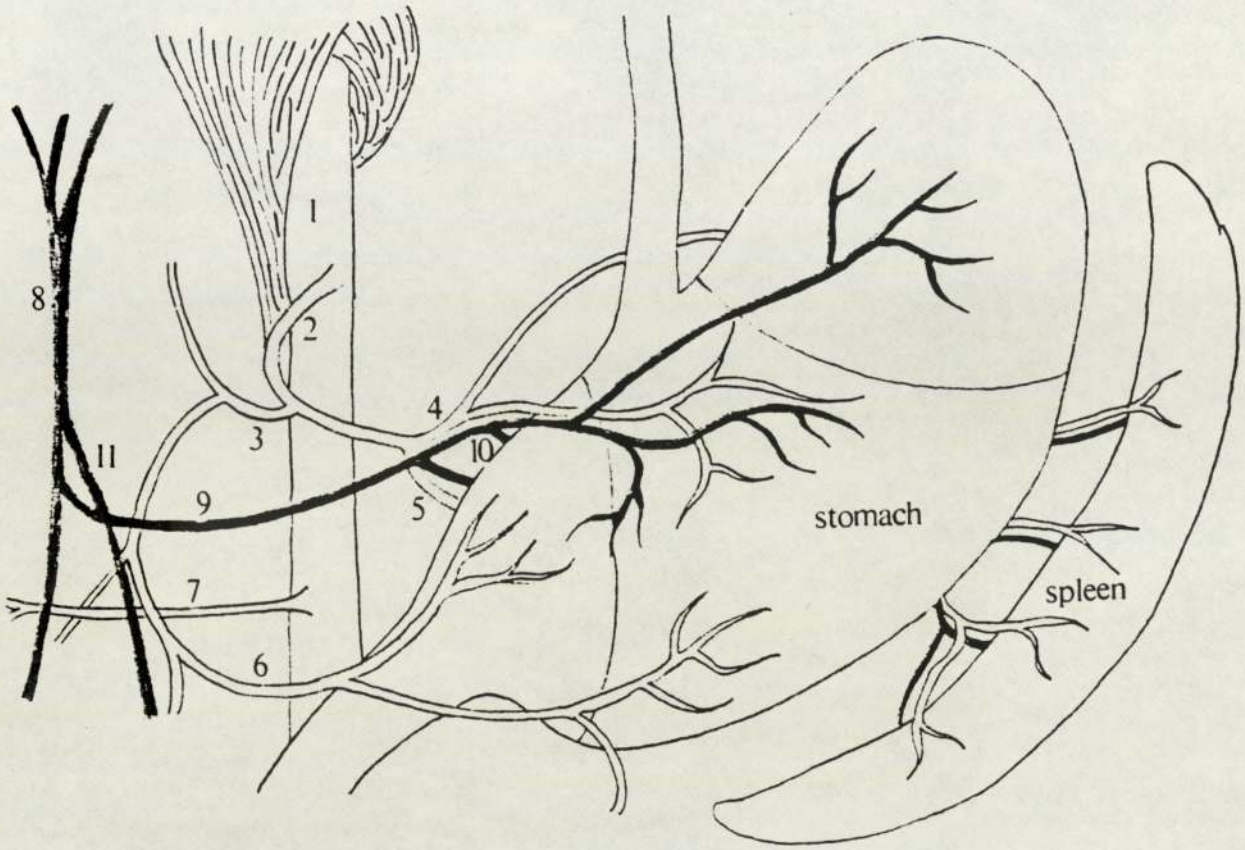


Fig 2.1 Vasculature comprising the blood supply to and drainage from the rat stomach. The intestines and liver have been displaced laterally.

1 Abdominal aorta
2 Coeliac artery
3 Hepatic artery
4 Left Gastric artery
5 Splenic artery
6 Right Gastric artery
7 Superior Mesenteric artery
8 Portal vein
9 Splenic vein
10 Coronary vein
11 Pyloric vein

Terminology of Greene(1955)

Because acid secretion involves the generation of a 10^6-fold proton gradient across the apical membrane of the parietal cell (Berglindh et al., 1980), the process requires large amounts of energy. Therefore it is pertinent to ask the questions: "Does substrate metabolism in the gastric mucosa increase to meet these high energy requirements?" and "Does any change take place in the overall pattern of metabolism?" To observe any changes that might take place when acid secretion is induced, it is necessary to increase the rate of acid secretion by the stomach. This can be achieved by injection of pentagastrin, a pentapeptide, N-tert-butyloxycarbonyl-alanyl-tryptophanyl-L-methionyl-L-aspartyl-L-phenylalanine, which is an analogue of the C-terminal tetrapeptide of gastrin, and possesses all the physiological properties of the natural secretagogue (Barrett, 1966).

The work described in this chapter will also attempt to answer two more specific questions arising from previous studies on gastrointestinal metabolism. "Does the high activity of branched-chain amino acid aminotransferase (EC 2.6.1.42) found by Ichihara et al.(1975) in the stomach compared with other gastrointestinal tissues (Table 2.1) reflect an importance of branched-chain amino acid metabolism in the stomach?" and "Is glutamine a major metabolic fuel in the stomach as it is in the small intestine (Windmueller & Spaeth, 1978)?"

2.2. STIMULATION AND MEASUREMENT OF ACID SECRETION *in vivo*.

2.2.1. Methods.

A simple method of inducing gastric acid secretion *in vivo* is the subcutaneous injection of pentagastrin.

Table 2.1 Activities of branched chain amino acid aminotransferase in various tissues of the rat digestive tract (Taken from Ichihara et al., 1975).

Tissue	Enzyme activity (μ moles keto acid formed/ min/g wet wt. tissue)
Eosophagus/Forestomach	0.46
Glandular stomach	13.33
Small Intestine - upper ⅓	0.92
middle ⅓	0.64
lower ⅓	0.83
Caecum	0.46
Colon	0.41

This is preferable to other secretagogues because its major site of action is the stomach, where it causes release of histamine locally, which acts directly on the parietal cell to induce acid secretion. It has also been postulated that the parietal cell possesses specific gastrin receptors (Rutten & Soll, 1981). To ensure that injection of pentagastrin induced secretion of acid by the rat stomach, above the resting rate, the following procedure was carried out to measure the amount of acid secreted in response to pentagastrin administration. This involved modifications to the method of Puurunen (1978).

Rats, which had been starved overnight, received an intraperitoneal injection of sodium pentobarbitone (Sagatal, 50-60mg/kg body weight). A mid-line incision was made to expose the stomach which was dissected free from connective tissue. The liver was then pushed back using saline (NaCl,9g/l)-soaked tissues to expose the stomach which was cannulated at the cardiac and pyloric ends as described below. Cardiac cannulation was carried out by making a small incision in the oesophagus, followed by the insertion and tying-in of a glass cannula (i.d. 1.6mm, o.d. 2.5mm) bent at 90^{o} and connected by a 25cm length of pvc tubing (i.d. 1.75mm) to a 5ml plastic syringe. Care was taken to ensure that the ligature around the oesophagus did not occlude the two branches of the vagus nerve which has an important role in the regulation of gastric acid secretion (see Chapter 1). The syringe was filled with saline (0.9% NaCl) at 37^{o}C and the residual stomach contents washed out, via the duodenal incision, taking care not to distend

the stomach wall. The pyloric cannula, made from a 2cm length of polyethylene tube (i.d. 2.4mm, o.d. 4.2mm) connected to a 30cm length of pvc tubing (i.d. 3.3mm), which was bent round and supported in the vertical position distal to the cannula, was then inserted and tied in position. The stomach was refilled with warmed (37°C) saline (NaCl, 9g/l) via the cardiac cannula and the fluid allowed to fill to a marked position, 10cm above the stomach, in the vertical section of the exit cannulation tubing. After a period of 10 minutes the saline was expelled by carefully injecting air via the cardiac cannula to displace the fluid. The expelled solution was titrated by the addition of aliquots of 0.02M NaOH using phenolphthalein (1% solution in ethanol) as an indicator (end point:approx pH 7-pink colour), after refilling the stomach with fresh saline. After two control samples had been taken, pentagastrin (Peptavlon, ICI; 250 µg/kg body weight) was injected subcutaneously. Further samples were taken every ten minutes until the secretory response had subsided.

2.2.2. Results and Discussion.

Fig 2.2 represents the acid-secretory response of the rat stomach to a subcutaneous injection of pentagastrin (250 µg/kg body weight). This dose was used as it has been shown (Barrett, 1966) that it produces 80-90% of the maximal acid-secretory response, when given to rats in a bolus intravenous injection. The response to pentagastrin appeared to be fairly rapid and a plateau was reached twenty minutes after injection. Maximal secretion continued for about 40 minutes and represented on average, a

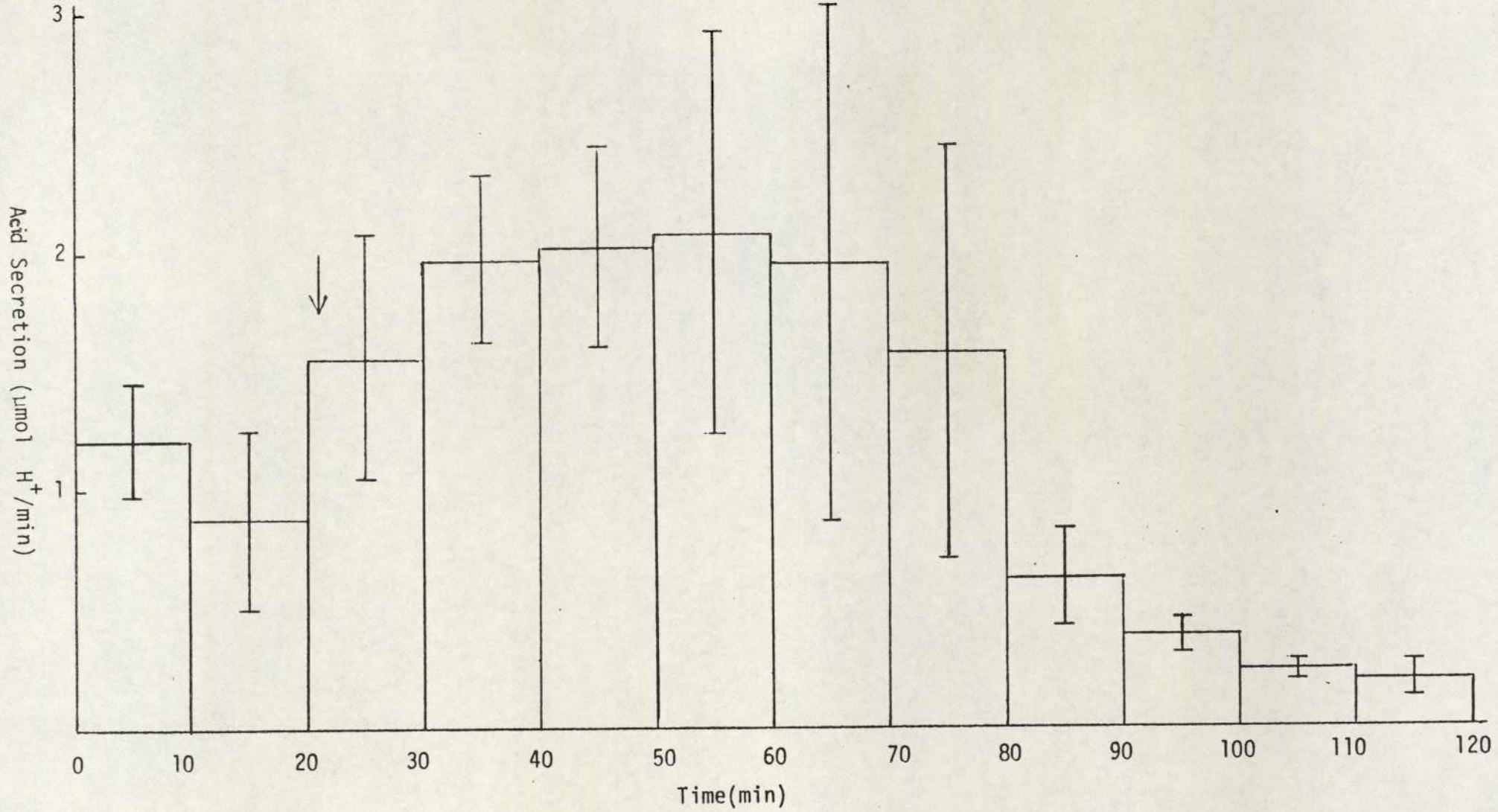


Fig 2.2 Gastric Acid Secretion After Injection of Pentagastrin. Pentagastrin (250μg/kg body weight)was injected subcutaneously into an anaesthetised rat. Arrow denotes time of injection. Values are means ± S.E.M. for four animals. Peak response (50-60 min period) is 2.36x 10-20 min period and 7.54x 110-120 min period.

7.5-fold elevation ($P<0.05$, paired t-test) above the basal level measured at the 110-120 minute period. Measurements made before injection of pentagastrin were probably elevated by reactions of the animals to surgery and manipulation of the stomach; consequently the reference basal secretory rate was taken to be that at 110-120 minutes. 20 minutes after injection there was an acid output that was at or near to the beginning of the plateau maximal rate, and was also subject to relatively small errors. Consequently, 20 minutes was chosen for the period between injection of pentagastrin and collection of blood in the arteriovenous difference studies. In addition, none of the aforementioned surgery and stomach manipulation took place when blood samples were withdrawn and so a more predictable response could be expected. 4-fold (Barrett, 1966) and 4.5-fold (Puurunen, 1978) stimulation of acid secretion in response to intravenous infusion of pentagastrin coupled with maximal secretory rates of 1.77μmol H^+/min (Barrett, 1966) and 0.8μmol H^+/min (Puurunen, 1978) have been obtained previously. In this work the maximal secretory rate was 2.0μmol H^+/min.

2.3. TECHNIQUES USED FOR THE MEASUREMENT OF ARTERIOVENOUS DIFFERENCES ACROSS THE RAT STOMACH in vivo.

2.3.1. Operative Procedure and Collection of Blood Samples.

Initially, venous samples were withdrawn by needle cannulation of the portal vein after tying off tributaries from the spleen, pancreas, duodenum and intestines and before removing a sample from the abdominal aorta. This involved a 60 second time interval between the mid-point of venous and arterial collections, and so in separate

experiments, two samples were withdrawn from the aorta at 60 second intervals to assess changes in arterial substrate levels during the time taken for venous sampling. Such control experiments involved performance of the entire procedure of vessel ligation in the same way as for the arteriovenous difference measurements. For reasons explained in 2.3.3. this procedure was discontinued and another method, involving simultaneous sampling from the coronary vein and abdominal aorta, was adopted. A few attempts were made to sample blood directly from the coronary vein by needle cannulation, but the low rate of success achieved with this procedure due to the size and fragility of the vessel, led to the use of the technique described below.

Rats were anaesthetised with sodium pentobarbitone (Sagatal, 60mg/kg body weight), and, where required, acid secretion was stimulated as in 2.2, 20 minutes before sampling. In control animals an equal volume of saline was injected. Body temperature was maintained at 37^{o}C throughout the operation by means of an automatically regulated heating blanket (Searle Bioscience, Sheerness, Kent, U.K).

The operation began with a mid-line abdominal incision and two lateral incisions just posterior to the diaphragm. The small and large intestines were displaced laterally and covered with plastic film to prevent evaporative cooling. After clearing the abdominal aorta, the stomach was turned over to expose the coronary vein which was also cleared from surrounding tissue. To prevent clotting during blood sampling, a bolus injection of sodium heparin (500IU in 0.2ml 0.9% NaCl, 37^{o}C) was administered

into the right common iliac vein. Heparin was injected just prior to blood sampling to minimise its effect on plasma free fatty acid levels brought about by release of lipoprotein lipase from capillary epithelia (Robinson & Jennings, 1965). Simultaneous sampling was effected by withdrawing blood from the two vessels by means of tubing mounted on a peristaltic pump (Gilson HP4MF, Anachem, Luton, U.K). 0.5 to 0.8ml of arterial and venous blood was collected in this way in one minute. Arterial samples were withdrawn via a 25-gauge bent cannulation needle connected to a 30cm length of polyethylene tubing (i.d 0.58mm). Venous blood was collected through a micro-funnel (o.d. 3.9mm), fashioned from the narrow end of a 200μl disposable pipette tip (GK2, Gordon Keeble, Cambridge, U.K) and joined to a 35cm length of polyethylene tubing (i.d. 1.2mm). Both samples were trapped in plastic tubes (LP3, Luckham, Burgess Hill, Sussex, U.K) surrounded by ice, (Fig 2.3).

Each sampling procedure began by cannulating the aorta, followed by carefully nicking the coronary vein with fine scissors. The pump was switched on and the venous blood collection funnel placed over the wound. The pump rate was such as to collect most of the blood issuing from the wound whilst avoiding 'over-suction.'

2.3.2. Treatment of Blood Samples.

For the assay of amino acids and non-esterified fatty acids, portions of whole blood were centrifuged in a Beckman Microfuge (10,000g, 1min) to separate the plasma.

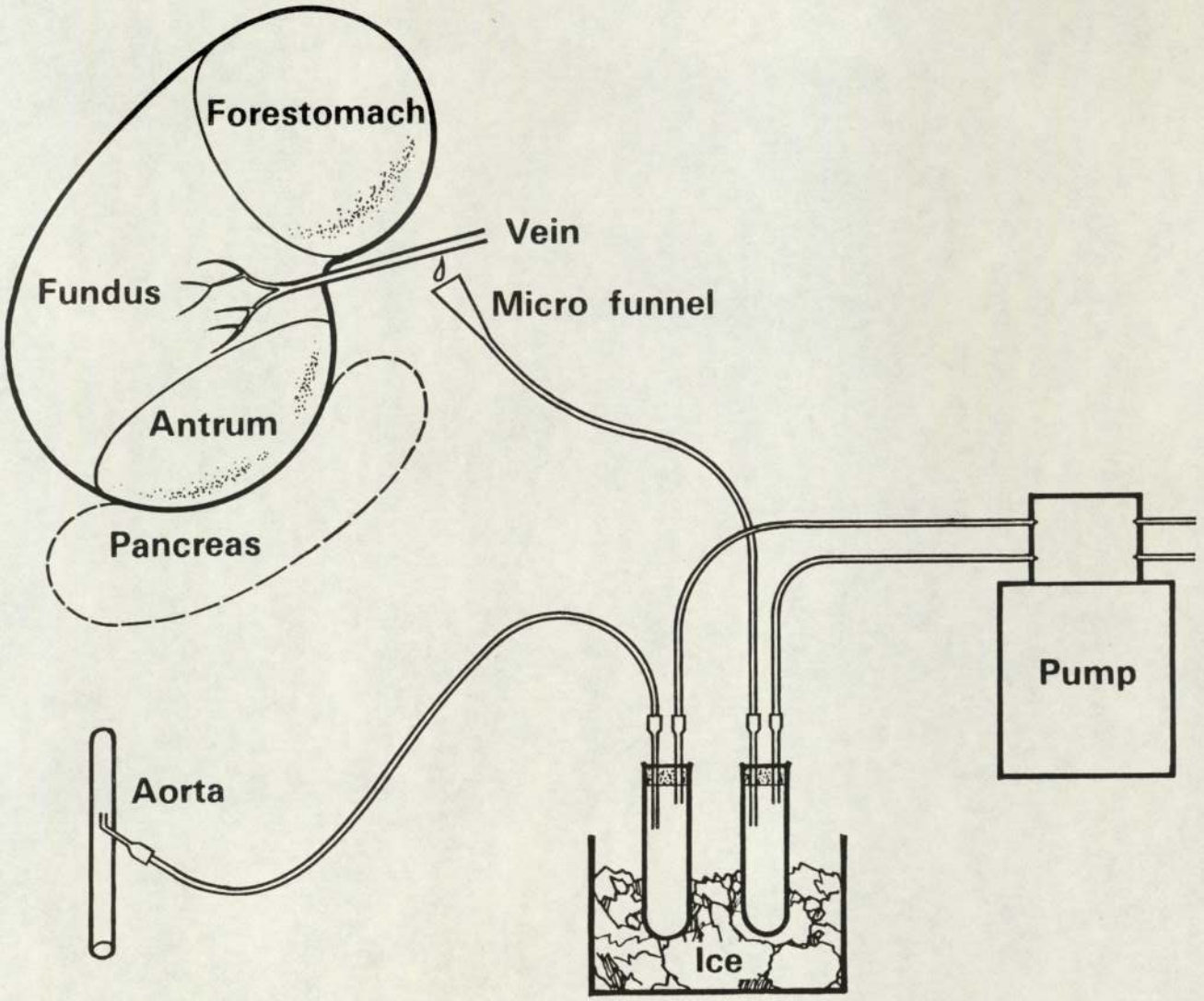


Fig 2.3 The simultaneous collection of blood from the abdominal aorta and coronary vein of the rat.

Amino acids were measured by automated ion-exchange chromatography (see A.4). Before analysis, the plasma was deproteinised by the addition of 5 volumes of 3% (w/v) sulphosalicylic acid. The supernatant after centrifugation at 4°C (1065g, 15min) was removed and analysed or stored at -20°C. The acid-lability of tryptophan, glutamine and asparagine prevented their determination by this method. Non-esterified fatty acids were assayed in plasma without deproteinisation (see A.3.10). Samples were stored at 4°C, but always analysed on the same day.

Other substrates and metabolites; glucose, lactate, glutamate, glutamine, pyruvate, ammonia, glycerol, acetoacetate and D-3-hydroxybutyrate, were analysed in neutralised perchloric acid extracts (see A.3). Approximately equal volumes of arterial or venous blood (0.5-0.7ml) were added to 4ml 1M-perchloric acid on ice, and immediately mixed on a vortex mixer. Samples were then centrifuged at 4°C (2790g, 15min) to sediment precipitated protein. To prevent deamination of glutamine and oxidation of acetoacetate by the acid, the time of exposure to perchloric acid was never more than 20 minutes and at all stages samples were kept on ice. An aliquot of the supernatant solution (3.75ml) was then neutralised to pH 7 using 4M-KOH and universal indicator. A pale green colouration indicated a pH of close to 7 and this was taken as the end point in all neutralisations. The resultant precipitate of $KClO_4$ was allowed to sediment and the clear supernatant solution withdrawn for analysis. Usually samples were assayed on the same day, and in the case of pyruvate this was essential as pyruvate is not

cold-stable. If storage was necessary, the extracts were stored at 4°C overnight, except for samples for glutamine and ammonia analysis where storage at -20°C was required.

2.3.3. Results and Discussion of Techniques.

Anaesthesia was induced throughout by an intraperitoneal injection of sodium pentobarbitone. This was used because of its ease of use compared with other anaesthetics e.g. ether, and also because of its cost and speed of action; sufficient anaesthesia was usually produced within ten minutes of the administration of 60mg/kg body weight. Unlike ether, which elevates blood lactate and glucose levels (Brewster et al., 1952), the effect of pentobarbitone on blood metabolite levels is minimal. Only slight elevations in blood lactate (Hawkins et al., 1971b) have been observed. The concentrations of metabolites in arterial blood found in this work (Tables 2.3 & 2.4) are similar to those found elsewhere in starved, anaesthetised rats e.g. Windmueller & Spaeth (1978), although in some animals, the concentrations of lactate and ammonia were elevated for reasons discussed in 2.4 and 2.5.

Blood samples from the coronary vein and aorta were usually taken simultaneously according to the method described in 2.3.1. Comparison between values for arteriovenous differences obtained by successively withdrawing coronary venous and arterial samples by a needle and those made using the method described in 2.3.1 showed a qualitative similarity (Table 2.2). Therefore, as the latter procedure was less subject to technical failures, it was used routinely, and gave results

Table 2.2 Comparison of two methods for the collection of venous blood:- effect on arteriovenous differences under control conditions for six metabolites. The arteriovenous differences for glucose, lactate, glutamate, isoleucine, leucine and valine under control conditions measured by using the technique described in 2.2.1, were compared (Student's t-test) with results obtained when venous blood was collected by needle cannulation of the coronary vein. In all cases there is no significant difference between the two methods, ($P>0.05$).

Metabolite	Venous-Arterial Concn. (mM)		t value.
	Needle cannulation of coronary vein	Pump collection of coronary vein outflow	
Glucose	-0.364 ± 0.242 (3)	-0.149 ± 0.022 (8)	1.556
Lactate	0.225 ± 0.113 (3)	0.160 ± 0.035 (10)	0.747
Glutamate	0.014 ± 0.003 (3)	0.007 ± 0.005 (5)	2.058
Isoleucine	-0.007 ± 0.001 (3)	-0.007 ± 0.003 (5)	0.047
Leucine	-0.010 ± 0.002 (3)	-0.011 ± 0.002 (5)	0.165
Valine	-0.012 ± 0.005 (3)	-0.012 ± 0.002 (5)	0.065

(Tables 2.3 & 2.4) which, with the exception of certain substrates, showed a high degree of repeatability between animals.

2.4 ARTERIOVENOUS DIFFERENCES FOR AMINO ACIDS AND AMMONIA.

Most amino acids were measured by Automated Amino acid analysis in plasma samples rather than whole blood, in an attempt to amplify any concentration changes which would be less when measured in whole blood, due to the potential for uptake or release of amino acids from erythrocytes, during passage of blood across the capillary bed, to buffer changes in plasma amino acid concentration (Felig et al., 1973). Were such exchanges between erythrocytes and plasma to occur, then the observed differences measured on plasma alone would be underestimates (Felig et al., 1973), with the exception of glutamine which was found by Felig et al., (1973) to exchange in the direction that would cause an overestimate of uptake of this amino acid by a particular tissue. Since glutamine was measured in whole blood, such exchanges of this amino acid would not affect arteriovenous difference measurements.

Table 2.3 shows the arteriovenous differences for amino acids and ammonia. A negative value indicated net removal of that metabolite by the stomach wall and a positive difference, a net output. Values for arterial concentrations represent the pooled data from acid-secreting and control animals. Injection of pentagastrin did not induce a significant change in the arterial concentration of any of the amino acids measured.

Table 2.3 Arteriovenous differences for amino acids across the rat stomach. Results are expressed as means ± S.E.M. with the number of animals in parentheses. Measurements were made with plasma except for glutamine and glutamate and ammonia where whole blood was used (see 2.3). P, where differences significantly different from zero (paired t-test) denoted as follows:

*, P<0.05; **, P<0.025; ***, P<0.01; ****, P<0.001
n.d. not determined.

Amino acid	Plasma/blood concn. (nmol/ml)	Venous-arterial concn. (nmol/ml)	
		Control	Pentagastrin-stimulated
Valine	117 ± 6 (15)	-11.7 ± 2.3 *** (8)	-7.4 ± 1.6 *** (7)
Leucine	95 ± 5 (15)	-10.5 ± 1.5 **** (8)	-7.4 ± 1.3 *** (7)
Isoleucine	68 ± 3 (15)	-7.1 ± 1.6 *** (8)	-6.5 ± 1.2 *** (7)
Cysteine	43 ± 4 (14)	-5.9 ± 0.8 **** (7)	-9.3 ± 2.3 *** (7)
Arginine	119 ± 4 (14)	-8.8 ± 2.8 *** (7)	-5.7 ± 1.3 *** (6)
Tyrosine	41 ± 2 (14)	-4.7 ± 1.0 *** (7)	-2.5 ± 0.6 *** (7)
Phenylalanine	43 ± 2 (15)	-4.4 ± 0.8 *** (8)	-2.9 ± 0.3 **** (7)
Proline	83 ± 2 (15)	-0.8 ± 2.4 (8)	3.1 ± 2.9 (7)
Methionine	25 ± 1 (15)	-1.9 ± 0.8 * (8)	-1.3 ± 0.8 (7)
Histidine	55 ± 2 (15)	-0.5 ± 1.7 (8)	-0.7 ± 0.5 (7)
Lysine	216 ± 12(15)	6.2 ± 3.7 (8)	7.2 ± 3.7 (7)
Glycine	216 ± 7 (15)	7.6 ± 4.2 (8)	10.4 ± 6.4 (7)
Aspartate	7 ± 1 (14)	2.7 ± 0.6 *** (7)	4.2 ± 0.7 *** (7)
Ornithine	31 ± 1 (15)	11.5 ± 3.7 ** (8)	6.7 ± 2.0 (7)
Alanine	214 ± 13(14)	15.9 ± 4.9 ** (7)	19.7 ± 5.3 *** (7)
Ammonia	113 ± 10(23)	n.d.	17.4 ± 11.1(23)
Glutamine	475 ± 17(20)	n.d.	-22.4 ± 9.5 * (20)
Glutamate	165 ± 7(20)	n.d.	8.1 ± 2.3 *** (20)

Stimulation of acid secretion did not significantly affect the arteriovenous differences, although no control values were established for glutamate, glutamine and ammonia for the reasons discussed in 2.4.2. Thus, there was no evidence for the metabolism of any one amino acid, or group of amino acids, being linked specifically with the acid-secretory process. It seems likely that a general increase in amino acid metabolism takes place with the onset of acid secretion since blood flow, as indicated by clearance of [^{14}C]-aniline (Main & Whittle, 1973), increases to the gastric mucosa upon stimulation, and amino acid metabolism would have to increase to a comparable extent for arteriovenous differences to remain unchanged.

2.4.1. Branched-Chain Amino Acids.

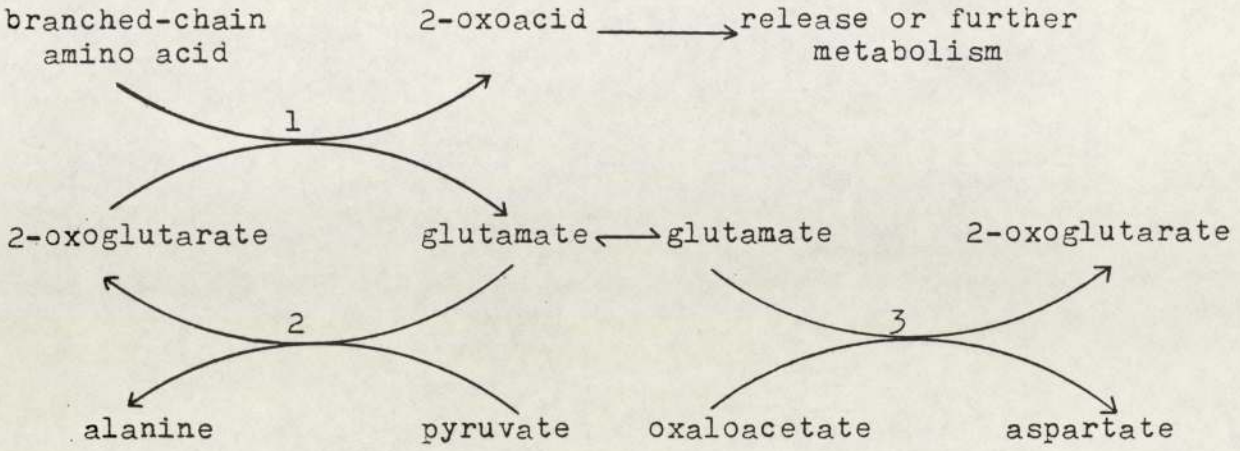
All three branched-chain amino acids were taken up by the stomach wall. Uptake of amino acids by any organ can be attributed to protein synthesis or metabolic degradation. If the former were true for the stomach, one would expect uptake of most or all amino acids, and also those taken up to the highest degree to appear in high proportions in the amino acid composition of major proteins synthesised by the stomach. The amino acid composition (A.7) of two such proteins, gastric mucin (Kim & Horowitz, 1971) and pepsinogen (Dayhoff, 1972) does not reflect the amino acid arterio-venous differences observed in this study. Furthermore, if protein synthesis were taking place to any great extent, one might expect to see an overall negative arteriovenous nitrogen balance. There was no evidence of this. Thus, the nitrogen balances (ng atoms Nitrogen/ml)

were -52.0 ± 23.9 (7) for acid-secreting rat stomachs and + 75.2 ± 96.2 (8) for control rat stomachs, the two results being insignificantly different from each other and from zero (t test). Furthermore, the output of alanine under both control and acid-secreting conditions was highly indicative of amino acid metabolism (see below). Therefore, protein synthesis is unlikely to be responsible for the bulk of the uptake of those amino acids removed from the blood to a substantial extent by the stomach.

The glandular mucosa of the stomach contains a high activity of branched-chain amino acid aminotransferase (Ichihara et al., 1975), suggesting that branched-chain amino acids taken up are transaminated to the corresponding 2-oxoacid and glutamate (Fig 2.4). This is supported in part by the output of glutamate, and also the outputs of aspartate and alanine, both of which are metabolically linked to glutamate by transamination (Fig 2.4). However, it is also possible that the outputs of these amino acids are related to the metabolism of glutamine (see below). 2-oxoacids produced by transamination might be further metabolised to provide energy and *in vitro* gastric mucosa has been shown to oxidise leucine (Ichihara et al., 1975). Alternatively 2-oxoacids could be released into the bloodstream as is the case in skeletal muscle (Livesy & Lund, 1980). A more detailed consideration of the pathways of 2-oxoacid metabolism is given in Chapter 6.

2.4.2. Glutamine, glutamate and ammonia.

Arteriovenous differences across the stomach for these metabolites varied quite considerably between animals and for this reason, the work was restricted to an investigation

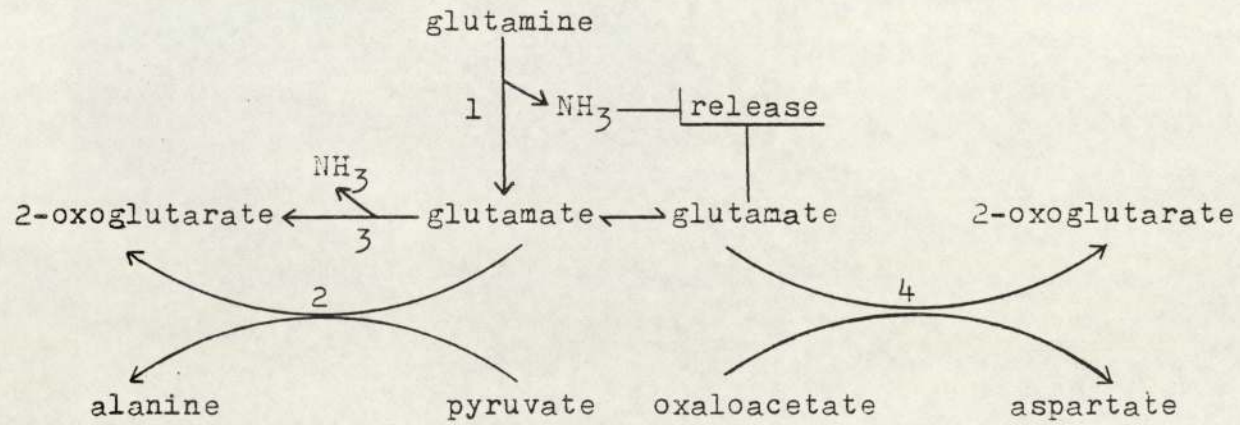


1 branched-chain amino acid aminotransferase (EC 2.6.1.42)

2 alanine aminotransferase (EC 2.6.1.2)

3 aspartate aminotransferase (EC 2.6.1.1)

Fig 2.4 Possible pathways for the metabolism of branched-chain amino acids in the gastric mucosa.



1 glutaminase (EC 3.5.1.2)

2 alanine aminotransferase (EC 2.6.1.2)

3 glutamate dehydrogenase (EC 1.4.1.3)

4 aspartate aminotransferase (EC 2.6.1.1)

Fig 2.5 Potential pathways for the metabolism of glutamine in the gastric mucosa.

of a large number of acid-secreting rats. In the small intestine glutamine uptake is dependent on the arterial glutamine concentration (Windmueller & Spaeth, 1974; Hanson & Parsons, 1977), but this could not be shown for the stomach (correlation coefficient, $r=0.436$, $p>0.05$). Glutamine uptake was also not dependent on the arterial ammonia concentration, (correlation coefficient, $r=0.573$, $p>0.05$). In 20 experiments, 15 showed an uptake and 5 an output of glutamine and there was, overall, a significant uptake (Table 2.3).

Metabolism of glutamine by gastric mucosal glutaminase (EC 3.5.1.2; Pinkus & Windmueller, 1977) should produce glutamate and ammonia (Fig 2.5). Indeed, the arteriovenous difference for glutamine was related to that for ammonia, so that when the stomach took up glutamine, ammonia was released and vice-versa (Fig 2.6), linear regression:

(venous-arterial) ammonia concn. = 0.003-0.709x
(venous-arterial) glutamine concn.;

correlation coefficient, $r= -0.851$; $p<0.01$. Thus, it seems that an uptake of glutamine (arteriovenous difference <-0.004 nmol/ml) results in an output of ammonia. Furthermore a regression line could be drawn relating the arteriovenous difference for glutamine and glutamate (Fig 2.7);

(venous-arterial) glutamate concn. = 0.004-0.174x
(venous-arterial) glutamine concn.;

correlation coefficient, $r= -0.746$, $p<0.01$). This result suggests that about half the total glutamate output (8.1 nmol/ml) was derived from glutamine. The remainder of the glutamate might have been further transaminated to

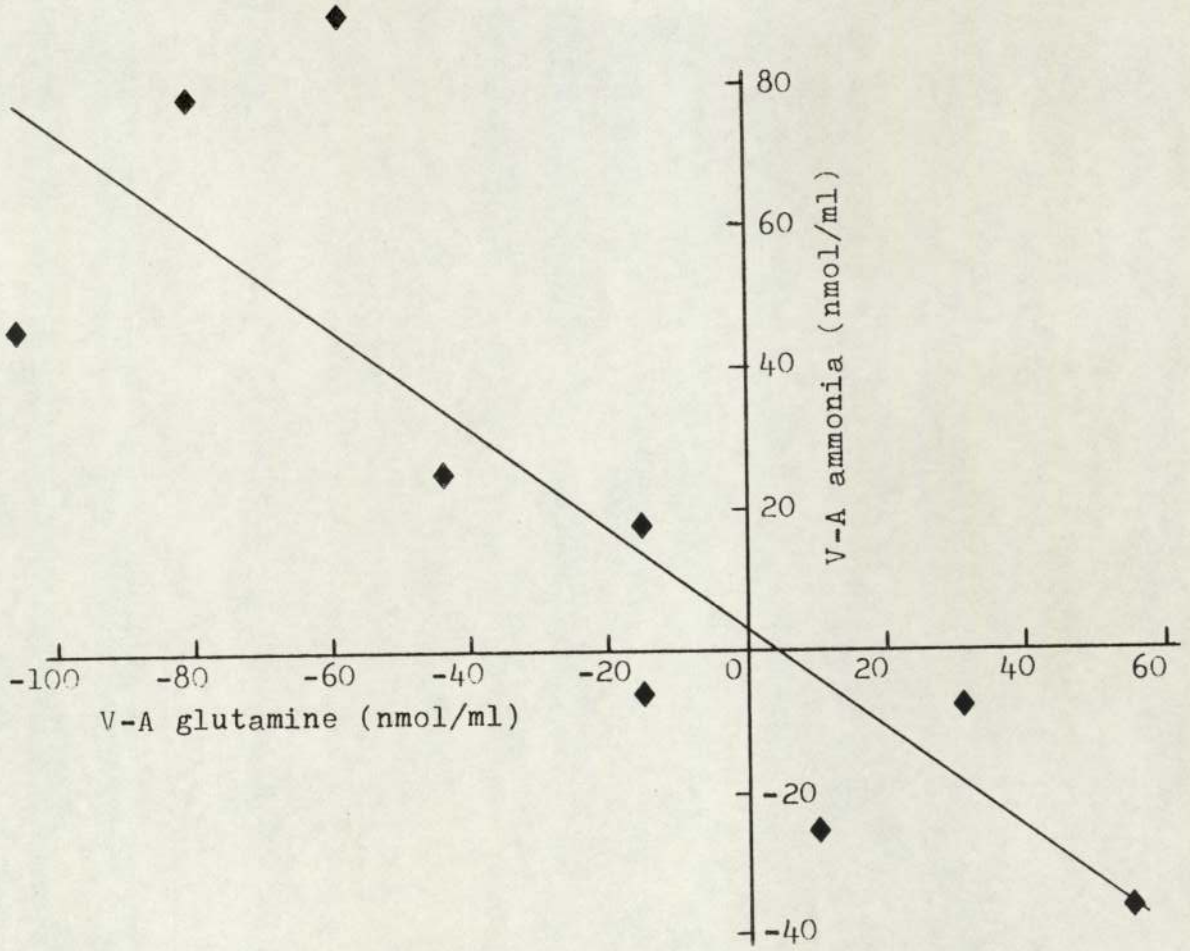


Fig 2.6 The relationship between the arteriovenous differences for glutamine and ammonia. Results are from nine rats injected with pentagastrin (250 μg/kg body wt). The line was drawn by linear regression analysis of the data, the regression equation being $y = 0.003-0.709x$; correlation coefficient, $r = -0.851$

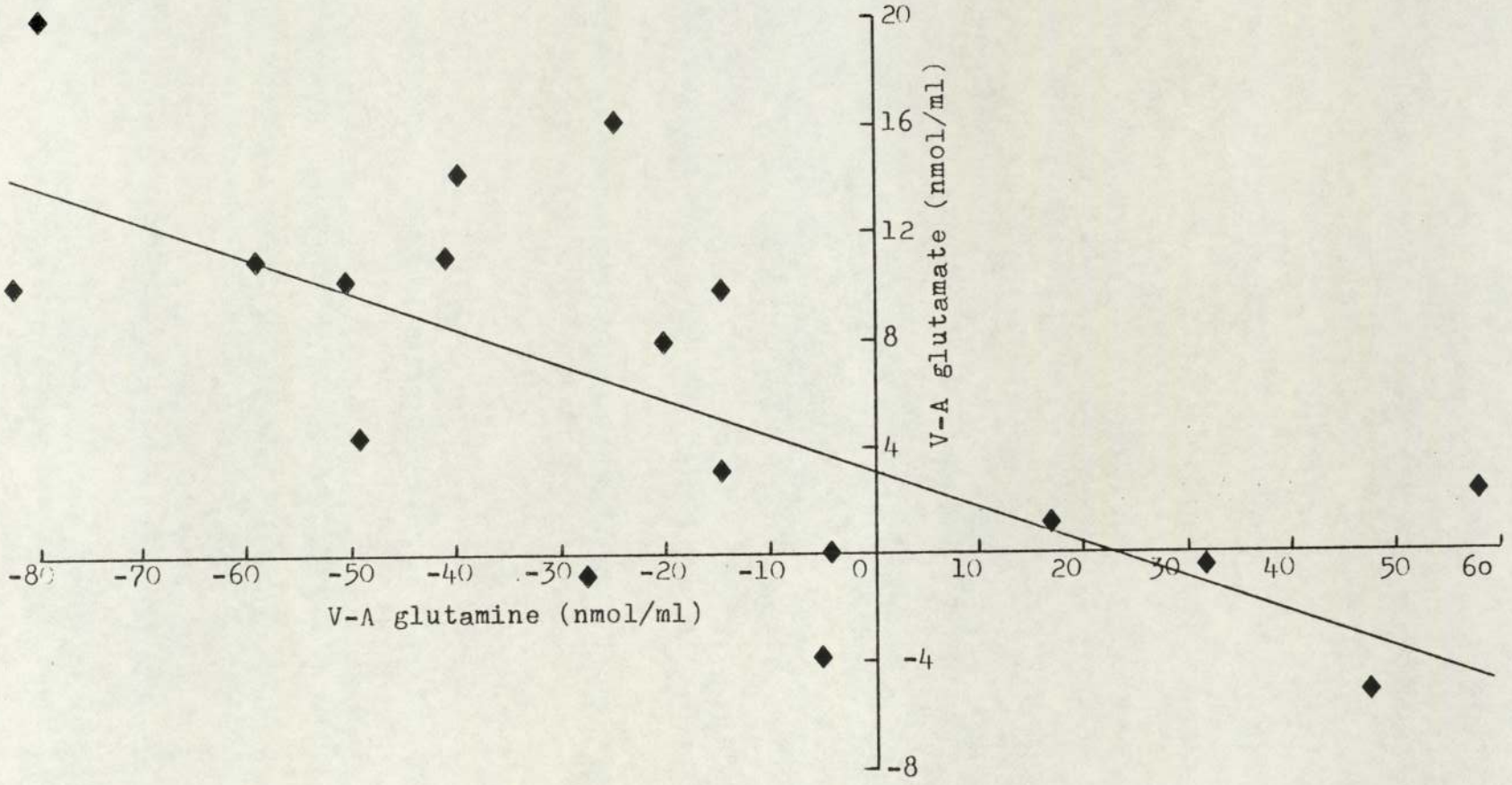


Fig 2.7 The relationship between the arteriovenous differences for glutamine and glutamate. The results are from 17 rats injected with pentagastrin(250μg/kg body wt). The line was drawn by linear regression analysis of the data, the regression equation being $y = 0.004-0.174x$; correlation coefficient, $r = -0.746$

give alanine and aspartate, both of which showed significant outputs (Fig 2.5).

A significant relationship also existed between the arteriovenous difference for ammonia and the arterial ammonia concentration (Fig 2.8) such that when the arterial ammonia concentration was above 0.15mM it was taken up by the stomach. Overall, there was no significant change in ammonia concentration across the stomach. Ammonia concentrations, elevated above what is considered to be physiological i.e. approximately 0.04mM (Windmueller & Spaeth, 1974) might have been due to stress associated with surgical manipulation and it is interesting to note that Windmueller & Spaeth (1980) also obtained elevated blood ammonia concentrations in animals necessarily subjected to considerable surgical manipulation.

As mentioned above, the variation in glutamine uptake could not be related to the concentration of ammonia in the arterial blood and it was possible that, when blood ammonia concentrations were high, an uptake of ammonia was effected via an increased activity of glutamate dehydrogenase, rather than glutaminase (Fig 2.5). Also, although the relationship between the arteriovenous difference for ammonia and the arterial ammonia concentration is statistically significant, a correlation coefficient of 0.504 means that only about 25% of the variation in the arteriovenous difference can be ascribed to changes in arterial ammonia concentration (Snedecor & Cochran, 1967).

The mean fractional extraction of glutamine (4.7%) was very much lower than that found in the small intestine

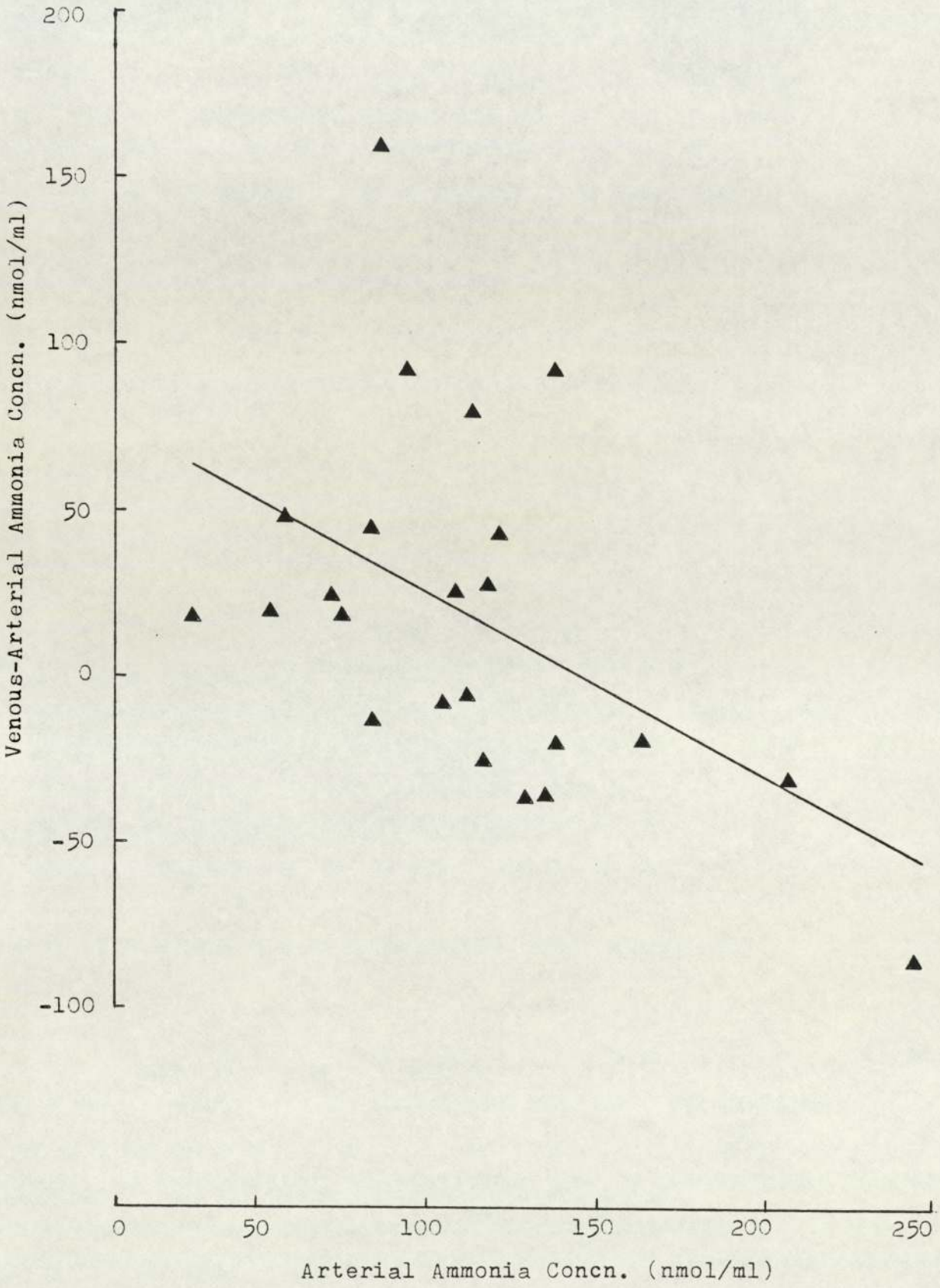


Fig 2.8 The relationship between the arteriovenous difference for ammonia and the arterial ammonia concentration. Results are from 23 rats injected with pentagastrin (250μg/kg body weight). The line was drawn by linear regression analysis of the data, the regression equation being $Y=0.08 - 0.560x$; correlation coefficient, $r= -0.504$ ($P < 0.05$).

in vivo (35%; Windmueller & Spaeth. 1975), yet the blood flow to the two tissues is similar (Malik et al., 1976). In addition, the mean arteriovenous difference for glutamine (Table 2.3) was much smaller than that for glucose (Table 2.4), whereas in the jejunum the two substrates are metabolised at similar rates (Windmueller & Spaeth, 1978). These results are consistent with the activity of glutaminase in the stomach being ~10% of that found in the small intestine (Pinkus & Windmueller, 1977). It seems unlikely that glutamine is as important a fuel in the stomach mucosa as it is in the mucosa of the small intestine.

2.4.3. Other Amino Acids.

The significant uptakes of cysteine and the aromatic amino acids under both control and acid-secreting conditions (Table 2.3) suggest their possible metabolic roles. Cysteine may be degraded to produce pyruvate and the aromatic amino acids degraded to produce acetoacetate, but whether the necessary enzymes are present in the gastric mucosa is not clear. Finally, the significant uptake of arginine was approximately balanced by the output of ornithine and it is possible that the gastric mucosa contains arginase (EC 3.5.3.1). However, the significance of such a conversion remains unexplained and may be of little importance to the supply of energy for gastric acid secretion.

2.5. ARTERIOVENOUS DIFFERENCES FOR CARBOHYDRATES AND LIPIDS.

The arteriovenous differences for the range of potential carbohydrate and lipid substrates are shown in Table 2.4. Only the arterial concentration of glucose appeared to alter significantly between control and pentagastrin-

stimulated rats. It seems unlikely that induction of acid secretion should effect hypoglycaemia, and it is possible that this alteration was produced as a result of random variations between animals. Also, there was no evidence to relate the arteriovenous difference for glucose with arterial blood glucose concentration; acid-secreting experiments - regression equation:

(Venous-arterial) glucose concn.= -0.328 + 0.044 (arterial glucose concn.).

correlation coefficient, r= 0.609, $p>0.05$, not significant, and regression coefficient small and not different from zero; control experiments regression equation:

(venous-arterial) glucose concn.= 0.113 - 0.041 (arterial glucose concn.).

correlation coefficient, r= -0.601, $p>0.05$, not significant, and again regression coefficient small and not different from zero. Therefore, even if the effect on arterial glucose concentration was genuine, this would not have had any major effect on the arteriovenous difference for glucose.

The net uptakes of glucose and D-3-hydroxybutyrate under both control and acid-secreting conditions were significant, as was the output of lactate when the arterial concentration of this metabolite was within the accepted resting physiological range i.e. within 2 S.D. of the value found by Hawkins et al., (1971b) i.e. less than approximately 1mM. It has been found that sodium pentobarbitone increases lactic acid levels in the blood (Hawkins et al., 1971b), and elevation might also occur through anoxia in another tissue. A significant relationship was found between the arterial lactate concentration

Table 2.4 Arteriovenous differences for metabolites excluding amino acids across the rat stomach. Values are means ± S.E.M. with the number of animals in brackets.

METABOLITE	ARTERIAL CONCENTRATION (μmol/ml)	V - A (μmol/ml)	
		CONTROL	ACID
Glucose	5.950 ± 0.241 (8)[1] 4.971 ± 0.389 (8)[2]	-0.149 ± 0.022 (8)**	-0.109 ± 0.026 (8)*
Lactate †	0.666 ± 0.037 (18)[3]	+0.160 ± 0.035(10)	+0.170 ± 0.028 (8)**
Pyruvate	0.088 ± 0.009 (6)[1]	+0.001 ± 0.001 (6)	n.d.
Glycerol	0.117 ± 0.006 (14)[3]	-0.004 ± 0.009 (7)	+0.024 ± 0.011 (7)
N.E.F.A	0.661 ± 0.033 (21)[3]	-0.001 ± 0.039(11)	+0.008 ± 0.023(10)
Acetoacetate	0.175 ± 0.027 (7)[2]	n.d.	+0.005 ± 0.011 (7)
D-3-Hydroxybutyrate	0.358 ± 0.057 (7)[2]	n.d.	-0.086 ± 0.020 (7)*

[1]Control, [2]Acid Secreting, [3]Pooled values, † denotes that values for lactate exclude 'non-physiological' results (i.e. Arterial Lactate > 1mM). See Fig. 2.9 for alternative expression of lactate results.
*P<0.01 ** P<0.001

and the arteriovenous difference for lactate, so that when arterial lactate was raised above 1.3mM, the stomach wall exhibited a net uptake (Fig 2.9). This phenomenon was present under both control and acid-secreting conditions and is indicative of good oxygenation of the stomach; anoxic tissues cannot utilise lactate. However, it seems likely that net lactate utilisation by the stomach, although possible, does not occur physiologically, in the same way as, for example, in the heart (Newsholme & Leech, 1983). Nevertheless, the possibility that some cells can use lactate at resting physiological concentrations, and that this is masked by the output of lactate from other gastric cells, cannot be excluded.

The arteriovenous differences, under acid-secreting conditions, for D-3-hydroxybutyrate, although showing only small variations in that they were always negative, were nevertheless, dependent on the arterial D-3-hydroxybutyrate concentration. This relationship was not present when the stomach was in the resting state (Fig 2.10). This might indicate that the uptake of D-3-hydroxybutyrate by the stomach is regulated by different mechanisms depending on whether secretion of acid has been stimulated or not. Thus, when the stomach has been stimulated to increase acid secretion, the uptake of D-3-hydroxybutyrate could be governed by the amount available in the arterial blood supply, but at rest, an internal regulatory mechanism may be present involving the intracellular NAD/NADH ratio which controls the acetoacetate/D-3-hydroxybutyrate ratio. No net uptake of acetoacetate was observed under either control or acid-secreting conditions and it is possible that

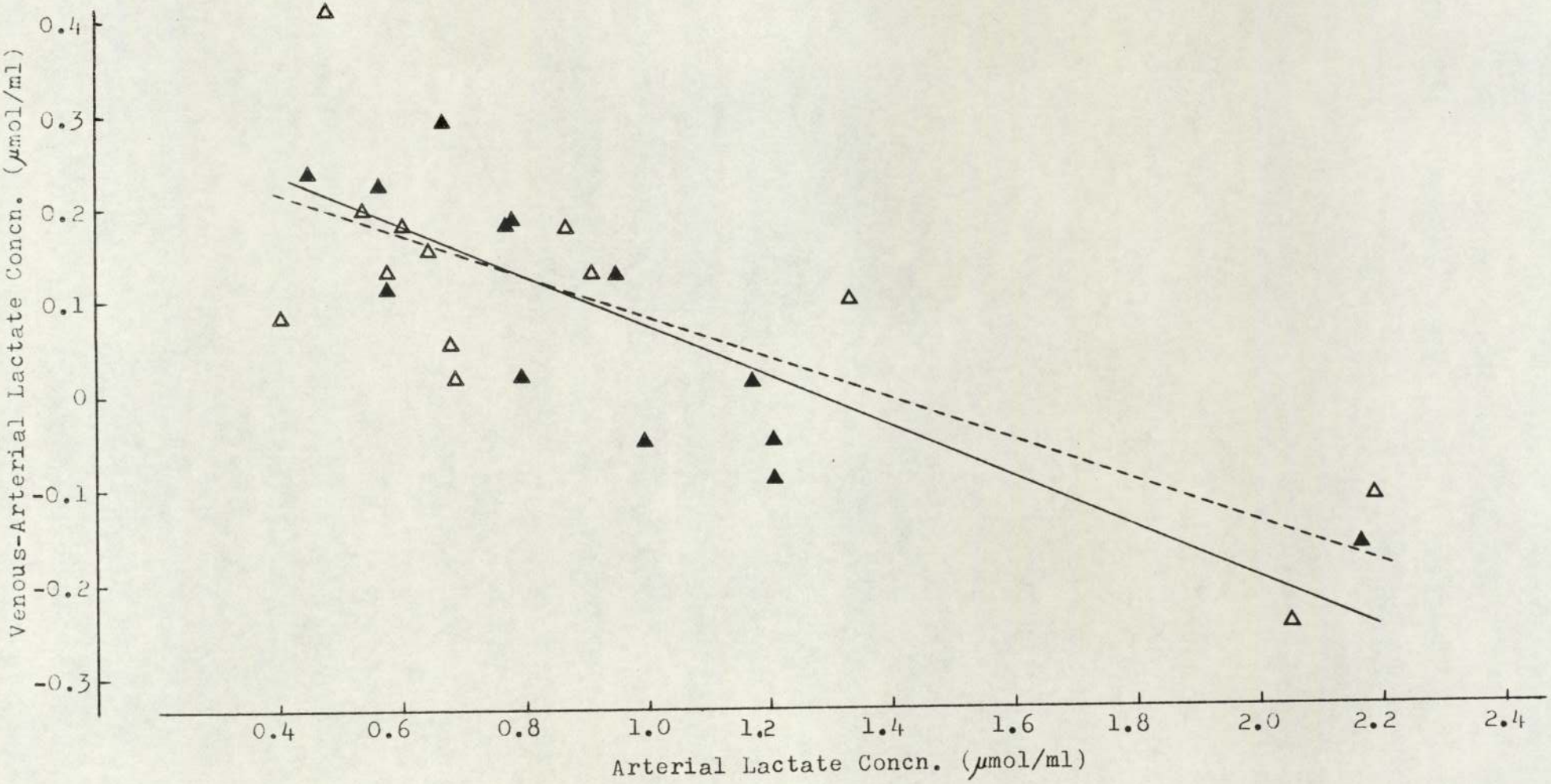


Fig 2.9 The relationship between the arteriovenous difference for lactate and the arterial lactate concentration. Under control (△----△) conditions the regression line has a correlation coefficient, r = -0.806 and under acid secretory conditions (▲——▲) r = -0.863. The two lines are not significantly different from each other.

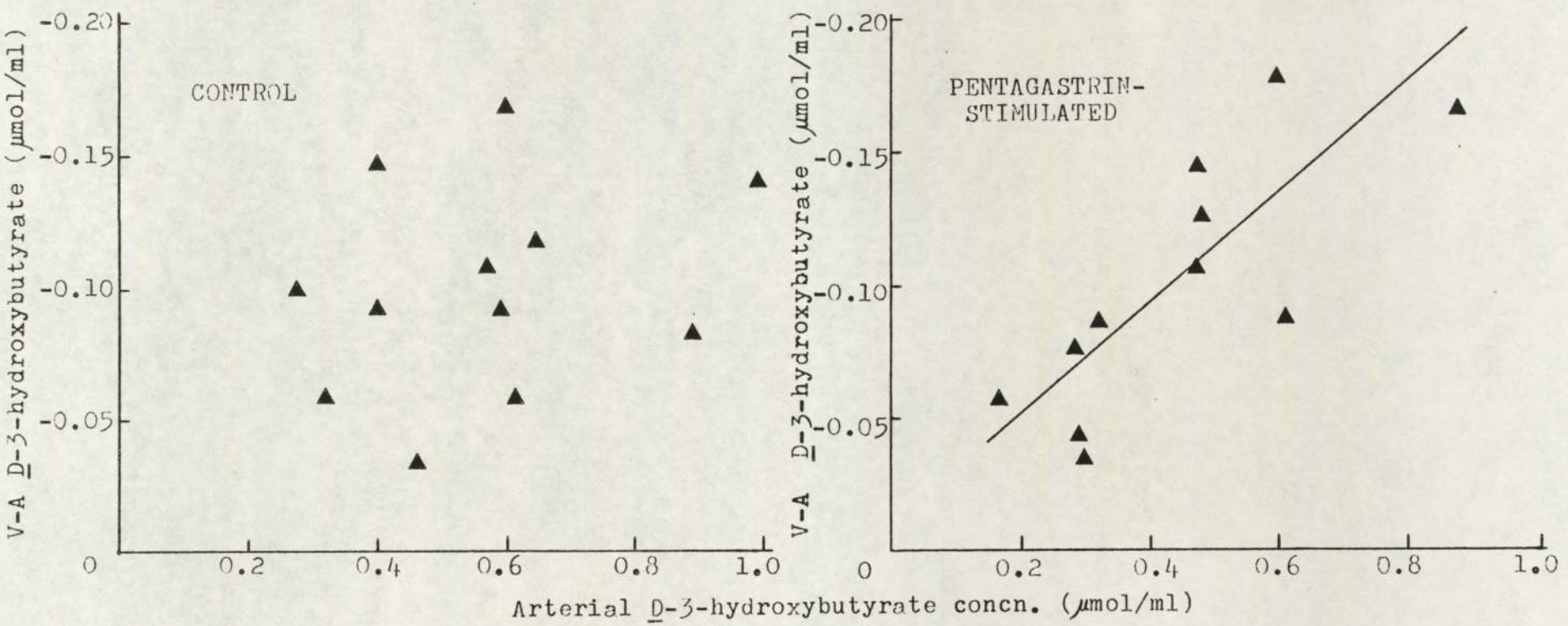


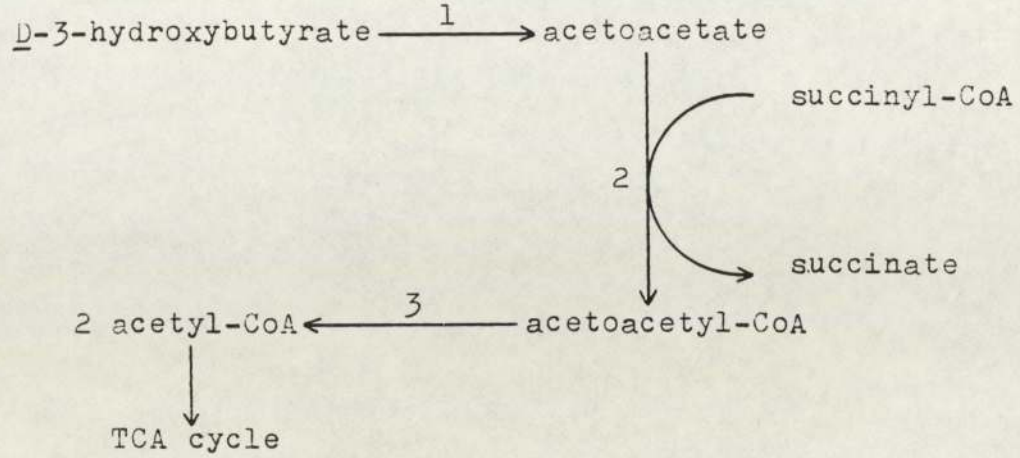
Fig 2.10 The relationship between the arteriovenous difference for D-3-hydroxybutyrate and the arterial D-3-hydroxybutyrate concentration. The line was drawn by linear regression analysis of the data, the regression equations being, for 12 control rats, $y = -0.07 - 0.05x$ and for 11 pentagastrin-stimulated rats, $y = -0.01 - 0.208x$. The correlationcoefficient was only significantly different from zero for rats injected with pentagastrin and therefore a line has only been drawn in this instance.

D-3-hydroxybutyrate is able to enter the tissue more rapidly than acetoacetate. In other tissues, for example brain (Hawkins et al. 1971a) and small intestine (Windmueller & Spaeth, 1978), acetoacetate and D-3-hydroxybutyrate are taken up equally. However, it is possible to explain the absence of acetoacetate uptake in the stomach if one considers the interconversion of acetoacetate and D-3-hydroxybutyrate catalysed by the mitochondrial enzyme D-3-hydroxybutyrate dehydrogenase (EC 1.1.1.30) in an NAD-linked redox reaction, thus:-

$$\text{Acetoacetate} + \text{NADH} + H^+ \rightleftharpoons \text{D-3-hydroxybutyrate} + NAD^+$$

In the parietal cell and possibly other gastric cells, mitochondria will be active in ATP production, especially during secretion of acid, and this might lead to a depletion of NADH and a resultant increase in the ratio NAD/NADH. This would result in the conversion of some D-3-hydroxybutyrate to acetoacetate, and so some of the D-3-hydroxybutyrate taken up could be returned to the bloodstream as acetoacetate. Therefore, although both ketone bodies might be taken up, no net acetoacetate removal would be observed. Evidence for this can be extracted from the arteriovenous data by observing the change in the ratio acetoacetate/D-3-hydroxybutyrate in acid-secreting rats:- the arterial ratio is 0.499 $\pm$ 0.064 (7) and the venous ratio 0.681 $\pm$ 0.065 (7), the increase being significant. $p<0.025$, (student's paired t-test).

Conversion of ketone bodies to acetyl-CoA occurs as shown in Fig 2.11. High activities, relative to most other rat tissues, of D-3-hydroxybutyrate dehydrogenase (EC 1.1.1.30) and 3-oxoacid-CoA transferase (EC 2.8.3.5)



1 D-3-hydroxybutyrate dehydrogenase (EC 1.1.1.30)

2 3-oxoacid-CoA transferase (EC 2.8.3.5)

3 acetyl-CoA acetyltransferase (EC 2.3.1.9)

Fig 2.11 Potential pathways for the metabolism of ketone bodies in the rat stomach.

have been found in the gastric mucosa (Hanson & Carrington, 1981; MacGill et al., 1984) and this, together with the arteriovenous data in these studies, suggests an important role for ketone body metabolism in supplying energy to the stomach.

Glycerol shows no significant arteriovenous difference, although the average values suggest there may have been a change from uptake to output when acid secretion was stimulated by pentagastrin. Therefore, there exists the possibility that onset of acid secretion results in increased gastric triglyceride lipase activity. The differences for free fatty acids were variable, and therefore it is not possible to say definitely that the stomach does not use these fuels. The variation may mask small uptakes which would be important metabolically because, on a molar basis, fatty acids have a higher energy-providing capacity than glucose, ketone bodies or amino acids. The significance of the findings in this section and in 2.4 on energy provision for acid secretion will be discussed more fully in Chapter 6, where the data will be related to previous work by others, and to results obtained here on the substrate dependency of acid secretion by isolated parietal cells.

2.6 THE PRESENCE OF POTENTIAL SUBSTRATES IN THE GASTRIC LUMEN.

There is evidence that epithelial cells in both the small and large intestine can utilise luminally-derived substrates. It was therefore of interest to determine the concentrations of potential substrates in the gastric lumen after a meal.

2.6.1. Method.

Rats were starved for 24h, and allowed to feed for 1h (ad libitum) after which food was removed. 30 minutes later, the animal was killed and the total contents of the stomach removed and weighed. The contents were then centrifuged at 10,000g for 4 minutes (Beckman Microfuge) and the volume of supernatant solution recorded. A neutralised perchloric acid extract of the supernatant was then prepared as in 2.3.2, although non-esterified fatty acids were extracted directly from the untreated supernatant. Assays were then performed for amino acids, carbohydrates and lipids. In the case of amino acids, assays were carried out on extracts before and after hydrolysis by 6M-HCl *in vacuo* for 18h.

2.6.2. Results and Discussion.

Data are presented in three ways, necessary to convey the amounts of potential substrates in the stomach after a meal as well as the availability of these substrates to the cells of the gastric mucosa. Clearly, if a particular substrate is present at a high concentration, this is of little importance physiologically if there is only a small amount in total. In addition, the amount eaten by the animal varies and so it was essential to relate the levels of substrate to the amount of food in the stomach.

Table 2.5 shows the results for the quantities of carbohydrate and lipid substrates present in the gastric lumen. There are high concentrations of glucose, lactate and glycerol. In Table 2.6 the concentrations of free amino acids are low, but after acid hydrolysis of the extract, using 6M-HCl, these concentrations are increased

Table 2.5 Concentration of potential carbohydrate and lipid substrates in the fluid present in rat stomach 1h after a meal. Results are means ± S.E.M. for four rats in eachcase, and are expressed in three ways: (i) concentration in fluid (mM), (ii) concentration with respect to total wet weight of gastric contents (μmol/g) and (iii) total amount of susbtrate present in fluid (μmol). The volume of fluid obtained by centrifugation of the stomach contents was 0.679 ± 0.068ml (13).

Substrate	Fluid concentration (mM)	Amount per g wet weight (μmol/g)	Total amount (μmol)
Glucose	19.47 ± 1.60	2.78 ± 0.52	15.07 ± 3.32
Lactate	5.44 ± 0.41	0.87 ± 0.08	4.39 ± 1.06
D-3-hydroxybutyrate	undetectable	undetectable	undetectable
Acetoacetate	0.16 ± 0.06	0.013 ± 0.005	0.12 ± 0.05
Glycerol	5.71 ± 0.69	0.43 ± 0.03	3.60 ± 0.25
N.E.F.A.	0.44 ± 0.06	0.049 ± 0.005	0.27 ± 0.02
Ammonia	0.92 ± 0.14	0.08 ± 0.01	0.61 ± 0.12

Table 2.6 Concentration of amino acids in the fluid present in rat stomach after a meal. Results are means ± S.E.M. expressed in three ways, before and after hydrolysis of the fluid by 6M-HCl for 18h *in vacuo*: (i) concentration in the fluid (mM), (ii) concentration with respect to total wet weight of gastric contents (μmol/g) and (iii) total amount of amino acid present in the fluid (μmol). The volume of fluid obtained by centrifugation of the stomach contents was 0.679 ± 0.068ml (13).

Amino acid	Fluid concentration (mM)		Amount per g wet weight (μmol/g)		Total Amount (μmol)	
	Before	After	Before	After	Before	After
Alanine	2.22 ± 0.21	22.34 ± 1.39	0.29 ± 0.04	2.19 ± 0.33	1.35 ± 0.11	13.67 ± 1.22
Arginine	0.88 ± 0.08	9.84 ± 0.50	0.12 ± 0.02	1.29 ± 0.16	0.54 ± 0.06	6.01 ± 0.45
Aspartate	1.33 ± 0.09	15.99 ± 0.40	0.18 ± 0.03	2.14 ± 0.36	0.82 ± 0.11	9.97 ± 1.38
Cysteine	0.20 ± 0.02	---	0.03 ± 0.003	---	0.12 ± 0.001	---
Glutamate	1.00 ± 0.08	33.52 ± 1.17	0.13 ± 0.02	4.43 ± 0.63	0.61 ± 0.05	20.78 ± 2.49
Glutamine	0.18 ± 0.04	---	0.03 ± 0.01	---	0.14 ± 0.07	---
Glycine	0.87 ± 0.11	32.32 ± 2.52	0.11 ± 0.01	4.23 ± 0.59	0.52 ± 0.04	19.92 ± 2.50
Histidine	0.45 ± 0.02	2.81 ± 0.18	0.06 ± 0.01	0.37 ± 0.05	0.28 ± 0.03	1.73 ± 0.19

Table 2.6 continued

Amino acid	Fluid concentration (mM)		Amount per g wet weight (µmol/g)		Total Amount (µmol)	
	Before	After	Before	After	Before	After
Isoleucine	0.57 ± 0.03	5.33 ± 0.24	0.08 ± 0.01	0.70 ± 0.09	0.35 ± 0.04	3.28 ± 0.34
Leucine	1.16 ± 0.09	10.08 ± 0.48	0.15 ± 0.02	1.33 ± 0.18	0.71 ± 0.06	6.25 ± 0.75
Lysine	0.69 ± 0.08	7.05 ± 0.41	0.09 ± 0.01	0.92 ± 0.10	0.42 ± 0.03	4.32 ± 0.39
Methionine	0.57 ± 0.07	3.18 ± 0.17	0.07 ± 0.01	0.42 ± 0.06	0.35 ± 0.04	1.98 ± 0.28
Phenylalanine	0.42 ± 0.02	4.60 ± 0.23	0.06 ± 0.01	0.53 ± 0.12	0.26 ± 0.02	2.43 ± 0.46
Proline	0.78 ± 0.10	21.18 ± 1.06	0.10 ± 0.02	2.89 ± 0.56	0.47 ± 0.05	13.32 ± 2.07

at least 10-fold in most cases indicating a high content of protein and peptide material in the stomach. Clearly, the short time of exposure of the gastric contents to pepsin secretion will not lead to a wholesale breakdown of protein and therefore high levels of amino acids in the lumen are unlikely. Probably most of these substrates, with the exception of ammonia, are derived from the diet (Heygates breeding diet (modified 41b diet), Pilsbury, Birmingham, U.K). One experiment was performed to measure the amounts of substrates present in a crude water soluble extract of the diet. The extract was made by grinding a known weight of food with water and analysing the fluid portion of the mixture after centrifugation. High concentrations of glucose (6.7mM), lactate (2.8mM) and glycerol (2.8mM) were found which strongly supports the suggestion that substrates found in the gastric contents are diet-derived. However, Remesy et al., (1977) showed the lactic acid concentration is higher in the non-glandular region of the stomach of rats when compared to the acid-secreting region (non-secretory 78mM; secretory 25mM). This could indicate that fermentative bacteria, which are more likely to proliferate in the less acidic environment, are the source of some of the luminal lactate.

Parietal cells are located in gastric glands, away from direct access to the luminal nutrients and also their arrangement is such as to present only the apex of the cell to the contents of the gland lumen (Fig 1.1), Furthermore, during acid secretion, substrates would have to reach the cells against an opposing flow of acid. Consequently it is considered unlikely that

substantial uptake of substrates by parietal cells through this route takes place. Other gastric cells, such as epithelial mucous cells may be able to utilise some of the potential metabolic fuels present in the gastric lumen in the same way as short-chain fatty acids are utilised in the colon (Roediger, 1982) and glutamine is in the small intestine (Hanson & Parsons, 1977; Windmueller & Spaeth, 1980).

2.7 SUMMARY.

1. A technique has been described whereby satisfactory measurements of arteriovenous differences across the oxyntic portion of the rat stomach under control and acid-secreting conditions can be performed.

2. Notable results were the uptakes of glucose, D-3-hydroxybutyrate and branched-chain amino acids.

3. Glutamine was also taken up, but its fractional extraction (4.7%) was very much less than that in the small intestine.

4. Arteriovenous differences for ammonia, lactate and D-3-hydroxybutyrate were related to their concentrations in the arterial blood.

5. No significant differences were found between measurements made on control and acid-secreting animals. It is suggested that substrate metabolism and blood flow may increase in parallel when acid secretion is stimulated.

6. The concentrations of potential substrates were measured in the gastric lumen after feeding. Glucose,

lactate and glycerol were present in high concentrations.

7. The relationship of the above results to data obtained by others on stomach metabolism, and to results on the substrate dependency of acid secretion by isolated parietal cells will be discussed in Chapter 6.

CHAPTER 3.

PREPARATION OF ISOLATED PARIETAL CELLS AND THEIR CHARACTERISATION

3.1 INTRODUCTION.

3.1.1. Advantages and Disadvantages of Using Isolated Parietal Cells.

The main question which requires consideration before employing an isolated cell system is, "Why is it desirable to use isolated parietal cells?" Possibly the main advantage of using isolated parietal cells is that cell suspensions may be incubated under *in vitro* conditions where oxygenation and nutrient supply can be easily controlled and maintained. Consequently, the design of quite complex experiments using a single preparation of cells for each "run" is facilitated. Such experiments might only be performed *in vivo*, or with the intact stomach *in vitro*, by using a very large number of experimental animals e.g. experiments on the substrate-dependency of acid secretion (Chapter 5). A preparation of isolated cells greatly simplifies the system being studied since the number of cell types under investigation is usually reduced in comparison with the intact organ, and if enrichment of a particular cell type can be achieved then more detailed information about the physiology of that cell may be obtained which would not be possible with studies *in vivo*. The limitations of work performed *in vivo* are evident from the discussion of the results presented in Chapter 2 concerning substrate metabolism in parietal cells; definite conclusions being prevented by the complexity of the system. Therefore, in this work, where both the substrate-dependency of acid secretion (Chapter 5), and intracellular events involved in the mechanism of acid secretion (Chapters 7 & 8) are

under investigation, the use of a preparation of isolated parietal cells is indicated. It should not be necessary to prepare purified fractions of parietal cells to study the substrate-dependency of acid secretion, since the parietal cell response can be distinguished in an impure preparation by using the aminopyrine accumulation test (3.1.2.5) to assess acid secretion. However, studies on the intracellular mechanism of acid secretion will require enrichment of the parietal cell content of an impure preparation.

There are, of course, some disadvantages in using isolated cells, not least of which, in the case of isolated parietal cells, is the loss of cell polarity. Isolated parietal cells are exposed on all surfaces to agents which might only interact with the basolateral membrane *in vivo*. This problem cannot easily be surmounted and so all results presented here have to be considered against a background of the loss of polarity. It is also difficult to assess artifacts caused by the isolation procedure. Treatment of cells with proteolytic enzymes can have deleterious effects on physiologically important sites on the cell surface, and so it is essential to determine the viability of cells produced, by using several valid criteria. Although it may be advantageous, and indeed necessary, to isolate the cell under study in order to remove the influence of other cell types, such interactions constitute the parietal cell microenvironment *in vivo*. The physiological relationship of histamine- and somatostatin- releasing cells to parietal cells is lost with the isolated parietal cell system, and this could affect the nature of the response of parietal cells

to acid secretagogues. The loss of cell-cell connections could have general effects on cell function as these connections, if they exist in parietal cells as they do in pancreatic acinar cells (Iwatsuki & Peterson, 1977) may allow transport of molecules of a variety of sizes and provide low resistance pathways for ionic conductance.

3.1.2. Criteria of Viability.

The usefulness of any preparation of isolated cells depends on their viability, and ways of assessing this are therefore presented before a consideration of the various methods available for the isolation of parietal cells.

3.1.2.1. Dye exclusion.

The retention of structural cell integrity after the isolation procedure can be assessed in several ways. Probably the commonest, and certainly the simplest test available is that of the ability of cells to exclude a dye, which reflects essentially the integrity of the plasma membrane. Most workers use this test and usually trypan blue (mol.wt. 961 da) is the dye of choice. However, a number of factors may result in falsely high values of dye exclusion, and therefore cell viability (Elliot, 1979). For example, cells must be given sufficient time to take up the dye and a representative sample of the cell population must be counted. Falsely high values for viability may also be obtained in the presence of high concentrations of bovine serum albumin, which is known to bind trypan blue (Seglen, 1976).

3.1.2.2. Leakage of cellular constituents.

Intact cells should also be able to retain their complement of enzymes, metabolites, ions etc., and leakage of such molecules might indicate a breakdown in structural integrity. Leakage of K^+ and adenine nucleotides was used by Baur et al. (1975) to assess the viability of liver cells but problems arise in the interpretation of results. Thus, only a few cells might be damaged, but damaged severely, and a great deal of leakage from those cells might result. But the same degree of leakage might be observed when a high proportion of cells were damaged, and their function impaired, but only so as to cause slight leakage.

3.1.2.3. Metabolic integrity.

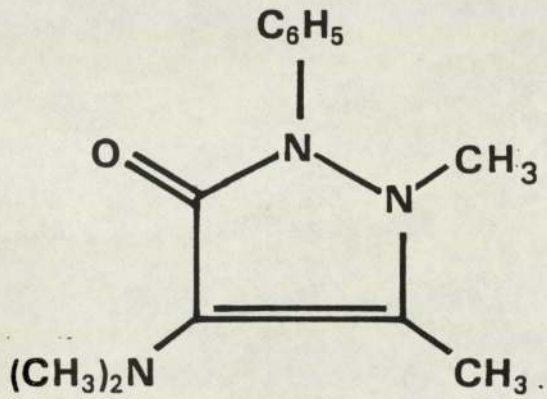
Viability can also be assessed by testing the metabolic integrity of a cell preparation, and this has been used for gastric cells as well as for many other cell types (Soll, 1978; Saladino, 1982). Since acid secretion by parietal cells is highly dependent on energy, reflected by the high density of mitochondria in parietal cells, stimulation of a preparation of cells, by acid secretagogues should increase the rate of respiration. Usually respiration can be measured using different parameters such as O_2 consumption and CO_2 production, and usually linearity of such processes with time is taken as evidence of normal cell functioning. Soll (1978) showed increased consumption of O_2 by a population of isolated canine parietal cells in response to several secretagogues. Using isolated guinea pig gastric glands, respiration was measured by Saladino et al., (1982) as $^{14}CO_2$

production from either glucose, glutamine or isoleucine and shown to increase 15-30% on addition of histamine. If intact parietal cells exhibit increased acid secretion and energy consumption when stimulated with physiological secretagogues, this is indicative not only of a functional intracellular machinery, but also of at least partially intact membrane receptors for secretagogues. However, unless the secretagogue concerned is a specific stimulant of parietal cells then the test may give misleading results when performed with unenriched parietal cell preparations, since increased O_2 consumption might also result from, for example, pepsinogen secretion from chief cells.

3.1.2.4. Aminopyrine accumulation.

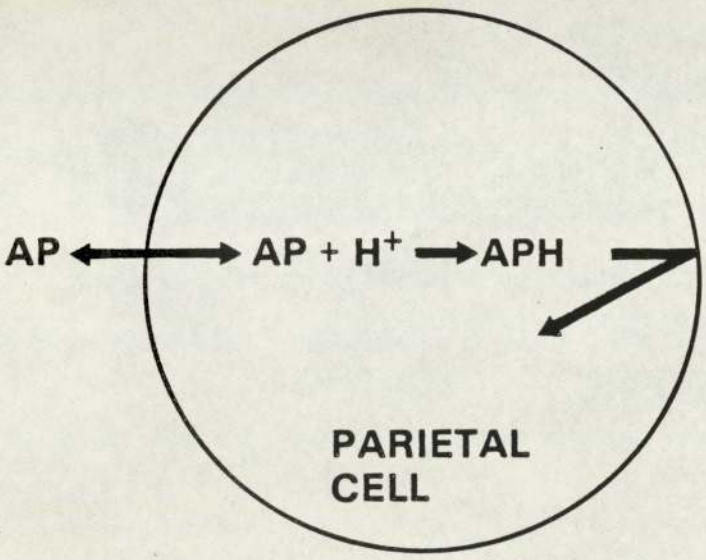
When Berglindh et al.(1976) demonstrated that their preparation of rabbit gastric glands accumulated [^{14}C]-aminopyrine, a method of assessing the responsiveness of parietal cell preparations to acid secretagogues became available and thereafter great advances were made in the understanding of parietal cell physiology. The basis of aminopyrine accumulation lies in the pH partition hypothesis. Weak bases such as aminopyrine are able to pass freely across cell membranes at normal physiological pH (i.e. $\sim$ pH 7.4). But if the pH falls below the value of the pKa of the base, which is 5.0 in the case of aminopyrine, the base becomes ionised and cannot diffuse across lipophilic cell membranes (Fig 3.1). In the acid-secreting parietal cell, when aminopyrine diffuses into the highly acidic environment of the secretory canaliculi and tubulovesicles it becomes trapped.

Aminopyrine



pKa = 5

WEAK BASE



PROTONATED FORM OF AMINOPYRINE IS TRAPPED INSIDE THE CELL

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

Fig 3.1 Intracellular accumulation of aminopyrine (AP) as an index of acid secretion by parietal cells.

Berglindh et al., (1980b) showed that acid does indeed accumulate in these structures by using fluorescence and Nomarski microscopy to detect the entrapment site of another weak base, acridine orange. Using radiolabelled aminopyrine, the amount sequestered intracellularly can be measured, by separating the cells from the medium. Therefore, it should be possible to demonstrate an increase in the accumulation of aminopyrine when the parietal cells are stimulated to secrete acid. The absolute degree of accumulation does not, however, provide a measure of the acid secreted, but rather an index of the acid sequestered within the parietal cell, and so aminopyrine accumulation data may not entirely agree with other indices of parietal cell response, such as O_2 consumption. Nevertheless, aminopyrine accumulation serves as a simple and reliable index of the response of parietal cells to stimulation, and enables the construction of secretagogue-dose versus secretory-response curves (Soll, 1980).

Often data are expressed as the amount of aminopyrine accumulated by a given number of cells e.g. Ecknauer et al. (1981). Alternatively, the aminopyrine accumulation ratio, which depends on the acidity of the intracellular compartment where the aminopyrine accumulates, is quoted. This is essentially the concentration of aminopyrine trapped intracellularly divided by the concentration of aminopyrine present in the medium. This is a preferable means of expressing aminopyrine accumulation as the ratio is related to the "averaged" intracellular pH by the formula in Fig 3.1, although assumptions must be made, before the pH can be estimated, and also comparison between different cell preparations are made easier. The aminopyrine accumulated/

10^6 cells is dependent on the extracellular aminopyrine concentration as well as the responsiveness of the cells to stimulation.

3.1.2.5. Ultrastructural changes.

Another method which can be used to assess the response of parietal cells to secretagogues, is the investigation of ultrastructural changes. Such changes (Chapter 1) take place as the cell secretes acid and can be observed using electron microscopy. Several investigators (Berglindh et al., 1976; Michelangeli, 1976; Soll, 1980) have demonstrated ultrastructural changes in parietal cells induced by secretagogues. However, although this technique can assess what proportion of the parietal cell population have responded to secretagogues, it is not easy to perform routinely to assess day-to-day viability of each cell preparation, and has tended to be used only once, when first characterising a population of cells isolated by a new method.

3.1.3. Methods for Preparing Isolated Parietal Cells.

Oxyntic mucosal cells have been isolated from many species, but only since about the mid-1970's have there been major advances in the understanding of parietal cell physiology. Until then, many isolated parietal cell preparations were amphibian (Blum et al., 1971; Forte et al., 1972; Michelangeli, 1976) and although some methods of preparing mammalian oxyntic cells were described (McDogual & Decosse, 1970; Romrell et al., 1975) it was difficult to assess the viability of these preparations in terms of their response to secretagogues.

Tables 3.1.A and 3.1.B provide a summary of the methods which have been used to isolate parietal cells and gastric glands from various species. Table 3.1 A shows some of the methods employed before the advent of the rabbit gastric gland preparation (Berglindh & Obrink, 1976) on which the aminopyrine accumulation test was used to provide a reliable index of the acid-secretory response. Preparations prior to this date showed no unequivocal response to secretagogues and therefore either cell viabilities must be questioned or membrane receptors were damaged during isolation. Since 1976 (Table 3.1.B), many highly viable mammalian preparations have been described and a survey of some of these methods is presented in 3.3 in an attempt to find the most convenient and useful for the experiments mentioned in 3.1.1. Ideally, a preparation using rats would be most suitable, as the studies in vivo (Chapter 2) were made using this animal, which is also less expensive to purchase and maintain than either rabbits or dogs. Therefore, some of the preparative methods investigated below were adapted from the original method involving a different species. Purification of parietal cells is dealt with in Chapter 4.

3.2. METHODS USED FOR TESTING CELL VIABILITY AND FOR DETERMINING THE PROPORTION OF PARIETAL CELLS.

The main test for viability of isolated parietal cells or gastric glands used in this work was aminopyrine accumulation in response to secretagogues. This test was carried out routinely on all batches of cells isolated, as was the trypan blue exclusion test.

Table 3.1.A. Methods used to isolate viable parietal cells (until 1976)

Authors	Animal	Method	Tests of Viability*
Croft & Ingelfinger (1969)	Dog	Sequential digestion of minced mucosa with pronase and collagenase.	1,2,3
McDogual & Decosse (1970)	Rabbit	Collogenase digestion of minced mucosa	1
Blum et al., (1971)	Frog	Pronase digestion of whole mucosa	1,2,3
Lewin et al., (1974)	Rat	Pronase digestion of an everted stomach sac with transient exposure to EDTA.	1,2
Romrell et al. (1975)	Mouse	Pronase digestion of whole mucosa	1,2
Michelangeli (1976)	Frog	Pronase digestion of whole mucosa	3,4,5

* 1. Dye exclusion
2. O_2 consumption
3. Maintenance of levels of cell constituents
4. Increased O_2 consumption in response to secretagogues
5. Ultrastructural transformation.

Table 3.1.B. Methods used to isolate viable parietal cells or gastric glands (since 1976)

Authors	Animal	Cells/Glands	Method	Test of viability*
Thomson et al. (1976)	Rat	cells	Pronase digestion of whole mucosa	1,6,7
Trotman & Greenwell (1979)	Rat	cells	Pronase digestion of an everted stomach sac with transient exposure to EDTA.	2
Gespach et al. (1980)	Rat	glands	Ca^{2+}depletion (EDTA) & mechanical dispersion	1,7
Berglindh et al. (1976)	Rabbit	glands	Intraarterial perfusion of mucosa followed by collagenase digestion	1,4,5,6
Batzri & Gardner (1978)	Guinea Pig	cells	Collagenase digestion of mucosa	1,3,6
Soll (1978)	Dog	cells	Collagenase digestion of mucosa with transient exposure to EDTA	1,4,5,6
Fellenius et al. (1982)	Human	glands	Collagenase digestion of mucosal biopsies	1,6

* 1. Trypan blue exclusion
2. Linear incorporation of leucine into protein with time
3. Maintenance of the level of cell constituents
4. Increased O_2 consumption in response to secretagogues
5. Ultrastructural transformation.
6. Increased aminopyrine accumulation in response to secretagogues
7. Increased intracellular cAMP levels in response to secretagogues

3.2.1. Aminopyrine Accumulation.

Isolated cells (usually 2-5 x 10^6 cells/ml.) were incubated, normally in a total volume of 1.5ml at 37°C in 20ml glass scintillation vials which had previously been siliconised using dimethyldichlorosilane. The cell suspensions were pregassed with O_2/CO_2 (95/5) and shaken (120cycles/min) in a water bath, usually for a period of 30 minutes. Each vial contained [^{14}C] aminopyrine (0.1μCi/ml; 0.9μM), [^{3}H] polyethylene glycol (0.4μCi/ml) and test secretagogues and substrates as required. The radiolabelled compounds and other test agents were all added to the incubation vessels in small volumes (often 20 or 30μl) to avoid any major dilution of the cell suspension, which was subsequently introduced. The final 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. Cells were suspended in Eagle's minimum essential medium (pH 7.35) containing HEPES (20mM) and bovine serum albumin (30mg/ml.) except in experiments where the substrate-dependency of acid secretion was being investigated. In the latter case the incubation medium was Krebs-Ringer bicarbonate buffer (pH7.4) containing dialysed fatty acid-free bovine serum albumin(30mg/ml) the preparation of which is described in A.2.2. Addition of 1.5ml of cell suspension to scintillation vials containing the radiolabelled compounds and test agents was taken as the starting time of the reaction. After the desired period, duplicate (or occasionally triplicate) aliquots were removed and centrifuged in 1.5ml tubes (L.I.P. (Equipment & Services) Ltd., Shipley, W. Yorks, England) at 10,000g for 30 sec.

(Beckman Microfuge) to separate the cells from the medium. Samples of the supernatant were removed for determination of radioactivity. The cell pellets were washed once using Eagle's minimum essential medium and then dissolved in 1ml Protosol at 40°C overnight. Protosol was also added to the supernatant samples followed by overnight incubation. 10ml of Econofluor was then added to all cell and supernatant samples and radioactivity was determined as described in A.5.

Calculation of the aminopyrine ratio was carried out as described below. Thus, the total ^{14}C dpm in the tissue samples represents the intracellular aminopyrine and also that trapped in the extracellular fluid (ECF) associated with the cell pellet. To correct for this, the volume of the ECF was estimated using [^{3}H]PEG. This does not, due to its high molecular weight (~4000da), penetrate the cells, and therefore, when trapped in the cell pellet provides a reliable index of the amount of ECF present, and enables a correction of extracellular aminopyrine to be made.

The intracellular fluid (ICF) was related to the dry weight of each preparation (see below). Dry weights were measured by centrifuging known volumes of the cell suspension in duplicate (10,000g, Beckman Microfuge), removing the supernatant, followed by cutting off the tip of the tube containing the cell pellets and drying in an oven at 90°C overnight. The dried cell pellets were transferred to tared foil boats and weighed on a micro-balance (accurate to 0.5 μg). The measured weight was corrected for the contribution of salts and albumin in the ECF associated with the cell pellet as, for example

medium B (3.4.1) contains 46.5mg dissolved salts and albumin/ml. Berglindh et al. (1976) found that the ratio, intracellular water (mg):dry weight (mg) was approximately 2.0 for rabbit gastric glands. On two occasions this ratio was determined here, by measuring both the wet and dry weights of cell pellets to calculate the volume of ICF present (wet weight-dry weight = total fluid; total fluid-ECF = ICF), and found to be 1.915 and 2.275. Therefore, since these ratios were very similar to those of Berglindh & Obrink (1976) and because the convention 2µl ICF/mg dry wt. has been used by others (Sack & Spenney, 1982; Hersey et al., 1981), it was used routinely here.

A sample calculation of an aminopyrine ratio is given below.

$$\text{Aminopyrine ratio} = \frac{A - \frac{BC}{D}}{CE}$$

A Total pellet $[^{14}C]$ AP (dpm)

B Total pellet $[^{3}H]$ PEG (dpm)

C Supernatant $[^{14}C]$ AP (dpm/µl)

D Supernatant $[^{3}H]$ PEG (dpm/µl)

E Volume of intracellular fluid (µl)

In a typical example for stimulated cells, the values for the formula might be 20,000 dpm (A), 2,500 dpm (B), 200 dpm/µl (C), 1000 dpm/µl (D) and 2µl (E) giving an aminopyrine ratio of 48.75.

3.2.2. Exclusion of Trypan Blue.

0.5 - 0.7 ml of cells suspended in medium B were centrifuged at 10,000g for 1 second. The cell pellet was gently resuspended in a known volume of medium

C(4.2.1) to which an equal volume of trypan blue solution (4mg/ml 0.9% NaCl) was added. The mixture was then viewed under the microscope. Only cells which showed no sign of having taken up the blue dye were counted as intact and viable.

3.2.3. Glucose Oxidation.

Although this technique was not used routinely, it was employed on two occasions to assess overall cell viability in terms of the retention of metabolic activity.

The test was carried out using the method of Cuendet et al., (1976) in which the production of $^{14}CO_2$ by the cells from $[U\text{-}^{14}C]$ glucose was determined.

After pregassing, the suspensions were incubated in Erlenmeyer flasks in a medium containing 5.5mM-glucose, bovine serum albumin (25mg/ml) and $[U\text{-}^{14}C]$ glucose (0.5μ Ci/ml). The flasks were sealed with subaseal stoppers to isolate the system. After incubation at 37^{o}C in a shaking water bath (100 cycles/min) for 60min, 100μl of 1M-sodium hydroxide was injected from a Hamilton syringe onto a fluted filter paper positioned in the centre well of the flask. Immediately after, 300μl of 30% perchloric acid was similarly injected into the medium surrounding the well, in order to stop the reaction. The flask was then allowed to stand at room temperature for 3h to ensure maximal absorption of the CO_2 onto the filter paper. Finally the filter papers were removed and transferred to scintillation vials. Radioactivity was determined using 10ml Aquasol as the scintillation fluid. Results were expressed as the amount of glucose converted to CO_2 per minute per mg. dry weight of tissue.

This was calculated by transforming the amount of $^{14}CO_2$ trapped on the filter paper (dpm) to the amount of U-^{14}C glucose converted and then calculating the total glucose converted, as shown below.

$$\frac{^{14}CO_2 \text{ trapped (dpm)}}{\text{specific activity of } [U\text{-}^{14}C] \text{ glucose (dpm/}\mu\text{mol)}} = \mu\text{mol glucose converted to } ^{14}CO_2$$

3.2.4. Cell Counting and Identification of Parietal Cells.

Cells suspensions were prepared for microscopy as described in 3.2.2. On a few occasions nitrotetrazolium blue dye was used to identify the parietal cells as described by Pearse (1960). This involved incubating the cells in a medium containing 13mM-2-(4-indophenyl)-3-(4-nitrophenyl) -5- phenyltetrazolium chloride, 60mM-sodium succinate and 80mM-sodium chloride, for 1h at 37°C. The dye is reduced by the mitochondrial enzyme succinic dehydrogenase (EC 1.3.99.1) and causes the mitochondria to stain blue. Since parietal cells have a very high mitochondrial content [about 40% of their cell volume, which is about 8 times more than in chief cells (Hellander, 1969)], they can be distinguished as dark blue cells. A problem encountered with the use of nitrotetrazolium blue was that many parietal cells did not stain (see Fig 3.3.e); unstained parietal cells could be identified by size and morphology. The reason for this may be that the technique is dependent on the penetration of succinate and the dye into the cell, a process which may not normally occur, in intact cells. It was therefore assumed that parietal cells stained with nitrotetrazolium blue might have been damaged in some way,

and a better method of identifying parietal cells was by size and morphology (see below).

Cells were counted in a haemocytometer containing a well of known volume (0.1 μl) which was subdivided by a grid to facilitate counting. At least 200 cells were counted on each occasion to obtain a good estimate of cell concentration, parietal cells being identified chiefly from their size and appearance (see 3.4.3.1).

3.3. ISOLATION OF PARIETAL CELLS BY TECHNIQUES SUBSEQUENTLY REJECTED.

The ideal method for preparing parietal cells would involve the isolation of large quantities of cells from rats in a way which caused minimum disruption to the cells' integrity, and was relatively quick and easy to perform routinely. This section relates those methods which were rejected for a variety of reasons, and which are therfore described without great detail. The method finally adopted is described in 3.4.

3.3.1. Digestion of Minced Mucosa Using Collagenase.

The first method attempted involved digestion of finely minced gastric mucosa with collagenase (Type A , Sigma). This technique was used by Batzri & Gardner (1978) to isolate gastric mucosal cells from guinea pig and by Soll (1978) with dogs and has been used successfully in other tissues e.g. adrenal cortex (Richardson & Schulster, 1972) and pancreatic islets (Lacy & Kostianovsky, 1976). In this work, both rats and guinea pigs were used and slightly different techniques were employed with the two species. In rats the procedure was based on that

used by Soll (1978) with dogs, and was as follows.

Stomachs were removed from animals anaesthetised by an intraperitoneal injection of sodium pentobarbitone (Sagatal 60mg/kg body wt.). The fundic portion of the stomach was dissected free and, after rinsing, the oxyntic mucosa was scraped off using a microscope slide. The mucosa was minced using fine scissors in a small volume of Krebs-Ringer bicarbonate medium. The minced mucosa was then incubated for a total of 30 minutes at 37°C in a shaking water bath, with continuous gassing (O_2/CO_2; 95/5) in two incubation media. For the first ten minutes, the medium was Krebs-Ringer bicarbonate (pH 7.4) containing 2mM-EDTA, 10mM-glucose and 1mg bovine serum albumin/ml. After washing the minced mucosa once with Krebs-Ringer bicarbonate buffer, the EDTA - containing buffer was replaced with one containing collagenase. This consisted of Krebs-Ringer bicarbonate buffer containing 10mM-glucose, 1.25 mM-$CaCl_2$, 1mg bovine serum albumin/ml and 1mg collagenase/ml. After 20 minutes the supernatant was collected, filtered through nylon mesh (150μm pore size) and centrifuged (200g,5min). The cell pellet was washed once in Krebs-Ringer bicarbonate containing 10mM-glucose and 1mg bovine serum albumin/ml. Two further twenty minute digestions of the minced mucosa were carried out and the total cells were pooled. This procedure was performed on only one occasion and produced a very low yield of cells, the majority of which did, however, exclude trypan blue, (Table 3.2). The low yield did not permit the testing of response to secretagogues and because of this, the procedure was not pursued.

Although it was not ideal to use cells isolated from another species since the *in vivo* studies had been performed using rats, the technique described by Batzri & Gardner (1978), using guinea pigs, has been shown to produce a preparation of gastric mucosal cells containing parietal cells which are responsive to histamine, and carbachol. Consequently an attempt was made to reproduce the technique, which is now described. Animals were anaesthetised with sodium pentobarbitone, as above, and the stomachs were removed. The fundic portion was dissected free and the mucosa scraped off using a glass microscope slide, and transferred into Hanks'buffer (A.2.4). The mucosa was then finely minced with scissors and the resulting suspension centrifuged (50g, 7min). The pellet was then resuspended and incubated for a period of 15 min. in Hanks' buffer containing 10mM-EDTA, 1mg bovine serum albumin/ml and 1mg collagenase/ml, at 37^{o}C in a shaking water bath (120 cycles/min) with continuous gassing (O_2/CO_2: 95/5). The supernatant was then discarded and the tissue incubated for three periods of 20 minutes in Hanks' buffer containing 1mg bovine serum albumin/ml and 1mg collagenase/ml. After each digestion period, the supernatant was filtered through nylon mesh (82μm pore size) and centrifuged (100g, 10min). The cell pellets were washed twice in ice-cold Hanks' buffer and finally pooled and stored in Hanks' buffer. The yield of cells obtained (Table 3.2) was higher than that from the rat and the parietal cells retained high viability as assessed by the exclusion of trypan blue. However, although this method followed, almost exactly, the procedure of Batzri & Gardner (1978), the cells did not respond to

secretagogues, and the method was rejected. No reason for the difference compared with Batzri & Gardner (1978) was apparent.

3.3.2. Isolation of Gastric Glands from Rat Using EDTA in the Cold.

Gespach et al. (1980;1982) have prepared isolated rat gastric glands which were viable in terms of trypan blue exclusion and in the response of adenylate cyclase to stimulation by various hormones. The preparation of these glands is now described.

The stomachs of 3 or 4 rats, anaesthetised as above, were removed and the fundic portions transformed into everted sacs as described in detail in 3.4.1. The fundic sacs were injected and fully inflated with an ice-cold solution consisting of 2.5mM-EDTA in 0.25mM-NaCl,pH 7.5. The sacs were then kept in 40ml of the same medium for a period of 20 minutes on ice. The gastric glands were separated from the mucosa by vigourously shaking the suspension for 15 successive 10 sec. periods by hand. The solution was then filtered through nylon mesh (360μm pore size), centrifuged (200g, 3 min) at 4^{o}C, and washed twice with Krebs-Ringer bicarbonate buffer. Three further periods of 20 minutes in ice-cold EDTA/saline were carried out and the pooled glands finally suspended in Krebs-Ringer bicarbonate medium containing 1.25mM-$CaCl_2$, 10mM-glucose and 1mg bovine serum albumin/ml, gassed with O_2/CO_2(95/5).

The yield of glands was quite high (Table 3.2) although less than that obtained by Gespach et al. (1980). The viability of the glands, as determined by trypan blue

exclusion, was also good, but no increase in aminopyrine accumulation in response to secretagogues could be achieved. Gespach et al. (1980;1982) have never reported aminopyrine accumulation data with these glands.

At this stage in the developmental work it was thought that the aminopyrine accumulation test might not be functioning, due to it being performed incorrectly. Therefore, one other viability test was carried out on the gastric glands, namely the response of glucose oxidation to stimulation by secretagogues. In the only experiment performed, there was a suggestion that respiration increased upon addition of 0.1mM carbachol. The amount of glucose converted (pmol/min/mg dry wt. glands) was 296.4 under resting conditions and 348.2 when stimulated. However, such an elevation was small and may have been due to cholinergic stimulation of another cell type, and so no further work was performed with this method.

3.3.3. Isolation of Parietal Cells using Pronase and DNase.

Thomson et al. (1981) described a method for producing viable fractions of rat parietal cells using digestion of the gastric mucosa in a medium containing pronase. This procedure for cell isolation will now be described.

As above, the stomach of an anaesthetised rat was removed and the oxyntic region dissected free. After rinsing in distilled H_2O, the whole tissue was placed in a flask containing 20ml of RPMI 1640 medium (A.2.5) at 37^{o}C with 1mg Pronase (BDH)/ml. After 30 minutes the fundus was rinsed with Hanks' buffer and the

mucosa was scraped off using a microscope slide. The scrapings were then placed in 20ml Hanks' buffer containing 0.25mg DNase (type I)/ml, dispersed using a pasteur pipette, and incubated at room temperature for 30 minutes. After incubation the suspension was again dispersed, filtered through nylon mesh (200μm pore size) and centrifuged (200g, 10min). The cells were washed once in Hanks' buffer, recentrifuged (50g, 15min) and finally resuspended in RPMI 1640 medium.

As shown in Table 3.2, the response of the cells to dbcAMP was substantial despite the apparently low exclusion of trypan blue. However, the reason for not pursuing this method of isolation was the low yield of cells produced. This would have entailed the use of many animals to produce enough material to carry out the type of experiments envisaged. The typical yields quoted by Thomson et al. (1981) - 7-8mg dry weight per stomach - are higher than found here, but the reason for the discrepancy is not known.

3.3.4. Perfusion of the Gastric Vasculature with Collagenase.

Although perfusion of an organ with enzyme-containing buffer is a recognised method for the isolation of cells from other tissues e.g. liver (Berry, 1974) and heart (Farmer et al., 1977) it did not appear to have been tried with the stomach, although Berglindh & Obrink (1976) used high pressure perfusion with saline buffer as a first stage in isolating gastric glands from rabbits. Perfusion of the gastric vasculature has previously been performed in studies on acid secretion *in vivo*

(Saffouri et al., 1980; van Huis & Kramer, 1981). Adaptations to the latter two methods of perfusion were used to design a system for the digestion of gastric mucosal cells by perfusion of the organ *in situ*. The reader is referred to Fig 2.1 where the main vessels involved in the procedure are identified.

The operation began with clearing the abdominal aorta of an anaesthetised rat which had been starved overnight. The spleen and pancreas were then tied off to prevent medium perfusing these organs through vessels arising from the coeliac artery. Next, the hepatic artery was ligated, and loose ligatures were placed around the aorta proximal to the point of origin of the coeliac axis, and distal to the juncture of the superior mesenteric artery. The superior mesenteric artery and right renal artery were then ligated and the rat received an intravenous injection of heparin (250 I.U.) into the right common iliac vein. A temporary clip was placed on the aorta just proximal to the superior mesenteric artery and the aorta was then cannulated with nylon tubing (o.d. 0.94mm) containing perfusion fluid up to the tip. The cannula was tied in place, the clip around the aorta removed, and the ligature proximal to the coeliac axis tied. Finally the splenic vein was cut to allow the blood and eventually perfusion fluid to drain away. Two perfusion media were used and were supplied from reservoirs maintained at 37^{o}C by water jackets. The flow rate was 5ml/min. which produces a perfusion pressure of 20-35mm Hg (Salvati & Whittle, 1981). The system also contained a bubble-trap to remove any air trapped in the medium before entry to

the vasculature. Perfusion began with Krebs-Ringer bicarbonate medium (pH 7.4) containing 0.5mM-EGTA and continued for 5 minutes. Chelation of calcium causes loosening of cell-cell junctions and enables lower collagenase concentrations to be employed for shorter digestion periods (Elliot, 1979). The perfusion medium was changed after 5 minutes to one consisting of Krebs-Ringer bicarbonate (pH 7.4) with 1.25mM-$CaCl_2$ and 1mg collagenase (Type AIV, Sigma)/ml, which was continued for between 10 and 30 minutes. The stomach was then removed, opened, and the glandular mucosa scraped free with a microscope slide. The scrapings were agitated and minced with fine scissors in a few ml. of warmed (37°C) Krebs-Ringer bicarbonate containing 1.25mM-$CaCl_2$. The suspension was then filtered through nylon mesh (360μm pore size) and centrifuged (200g, 10min). The resulting pellet of cells was washed twice with Krebs-Ringer bicarbonate containing 1.25mM-$CaCl_2$ and finally resuspended in Krebs-Ringer bicarbonate medium containing 1.25mM-$CaCl_2$, 10mM-glucose and 1mg bovine serum albumin/ml.

The yield of tissue obtained by vascular perfusion was very poor (Table 3.2), although the cells were viable in terms of trypan blue exclusion, and response to dbcAMP. However, the complexity of the procedure and the low yield obtained precluded its use for the routine isolation of parietal cells.

3.4. ROUTINE PREPARATION OF ISOLATED PARIETAL CELLS AND THEIR CHARACTERISATION.

3.4.1. Method.

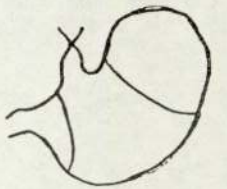
This method is closely related to that of Trotman &

Table 3.2. The yields, viabilities and aminopyrine responses to stimulation of isolated cells and glands prepared by methods subsequently rejected.

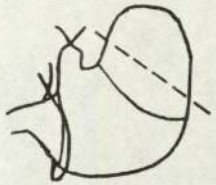
Section	Method	Yield (mg dry wt/ stomach)	Viability (% parietal cells excluding trypan blue)	Secretagogue used	Stimulation $\frac{\text{stimulated AP accumulation}}{\text{control AP accumulation}}$
3.3.1	Collagenase digestion of minced rat mucosa	0.4	85	-----	-----
	Collagenase digestion minced guinea pig mucosa	10.7	94	dbcAMP H + IMX	0.998 0.961
3.3.2	EDTA/vigorous shaking	5.9	88	Carbachol H + IMX dbcAMP	1.061 1.118 0.997
3.3.3	Pronase digestion of mucosa followed by DNase dispersion	1.2	70	dbcAMP	7.134
3.3.4	Vascular perfusion with EDTA and collagenase	2.1	86	dbcAMP	7.560

Greenwell (1979) which in turn is a modification to that of Lewin et al. (1974).

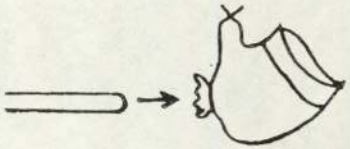
Rats (usually 2 or 3 per experiment) were anaesthetised by an intraperitoneal injection of sodium pentobarbitone (Sagatal; 60mg/kg body wt.). A mid-line incision was made, and the stomach was cleared of surrounding connective tissue. Before removing the stomach, the oesophagus was occluded with a ligature. Everted stomach sacs were then prepared as shown in Fig 3.2, according to the method first described by Dikstein & Sulman (1965). The stomach was rinsed in ice-cold saline (NaCl, 9g/l) and a ligature tied along the border of the fundus and antrum. This line was not distinct and was judged to transverse the glandular portion of the stomach one-third of the distance between the pylorus and the border with the non-glandular region. The line dividing the non-glandular and glandular portions is obvious since the non-glandular region has a white appearance, whereas the glandular region is grey-pink in colour. The pyloric region was cut away below the ligature and then an incision was made in the non-glandular part opposite the ligature. The fundus was then everted by pushing the tied end through the aperture in the non-glandular portion using a blunt-ended glass rod. The interior of the fundic wall (now exteriorised) was rinsed in ice-cold saline (NaCl, 9g/l) and the sac was sealed by tying a ligature across the open end on the non-glandular/ glandular border. Finally, the excess non-glandular tissue was removed and the sacs fully inflated with Pronase solution introduced via a 26-gauge hypodermic needle. The enzyme was present at a concentration of



STOMACH REMOVED WITH OESOPHAGUS TIED



LIGATURE TIED ALONG BORDER OF FUNDUS AND ANTRUM AND NON-GLANDULAR REGION SECTIONED PARALLEL TO THE GLANDULAR/NON-GLANDULAR BORDER



EVERSION OF THE FUNDUS USING A BLUNT GLASS ROD

EVERTED SAC SEALED BY A LIGATURE TIED ALONG THE GLANDULAR/NON-GLANDULAR BORDER

Fig 3.2 Formation of an everted sac of the fundus from the rat stomach

1000U/ml in medium A, which consisted of Eagle's minimum essential medium (A.2.2) containing 20mM-HEPES, 2mM-EDTA, 0.1mg Soybean trypsin inhibitor/ml and 30mg dextran/ml. The sacs were then placed in 40ml medium A and incubated for 30 min at 37°C in a shaking water bath (60 cycles/min) with continuous gassing (O_2/CO_2;95/5). After digestion, the incubation medium was discarded and the sacs were blotted on filter paper to remove excess fluid and transferred to 20ml medium B which consisted of Eagle's minimum essential medium containing 20mM-HEPES and 30mg bovine serum albumin/ml. The sacs were then stirred gently, using a magnetic follower, for 30 minutes at room temperature to remove the cells. Isolated cells were harvested by filtering the stirring medium through nylon mesh (195μm pore size, Sericol Group Ltd., London, U.K) before centrifuging (100g, 10min). The resulting cell pellet was carefully resuspended in approximately 5ml medium B and stored at 37°C in a shaking water bath with continuous gassing (O_2/CO_2;95/5). The sacs were incubated for a further two hours with changes from medium A to medium B every 30 minutes. Finally, the three fractions of cells were pooled and centrifuged (100g, 10min) before resuspension in fresh medium B, where the cells were stored at 37°C in a shaking water bath with gassing (O_2/CO_2;95/5).

The above method was used routinely and gave rise to a population of cells whose characteristics are described in 3.4.3. It differs from that of Trotman & Greenwell (1979) in the use of Eagle's minimum essential medium (1.8mM-Ca^{2+}) rather than Eagle's minimum essential medium for suspension cultures (Ca^{2+}free) as the basis

of medium A and in the addition of Soybean trypsin inhibitor to this medium. It also differs with respect to the incubation periods of the everted sacs in medium A. Trotman & Greenwell (1979) appear to incubate the sacs in medium A for 90 minutes with a change of medium A every 30 minutes, whereas here, cells were removed in medium B after each 30 minute incubation in medium A.

3.4.2. Effects of Different Modifications to the Cell Isolation Procedure.

Certain modifications to the isolation procedure were carried out before the method described in 3.4.1. was adopted. In an attempt to maximise both the yield of cells and their responsiveness to secretagogues, the effects of altering pronase concentration, lot number and type were examined, as well as incubation times and the usefulness of adding trypsin inhibitor protein to the digestion mixture.

3.4.2.1. Pronase and the use of trypsin inhibitor protein.

The effects of altering the isolation procedure in terms of pronase and trypsin inhibitor are included in Table 3.3. Using pronase obtained from Boeringer rather than BDH appeared to produce cells with a reduced response to secretagogues (cf lines 1 & 3, Table 3.3) and the use of a batch of BDH pronase different from that used routinely also seemed to be deleterious to the cells (c.f. lines 1 & 4, Table 3.3). If the normal concentration of pronase (1000U/ml) was reduced to 200U/ml, or if the enzyme was removed completely, a decreased yield of cells

Table 3.3 The effects of modifications to the cell isolation procedure on the yield and viability of the cell preparation.

Modification	Yield (mg dry wt/ stomach)	Viability (%cells excluding trypan blue)	Response to secretagogues* (stimulated APR/control APR)
1. Control†	17.73±4.67(42)	96.1±3.24(42)	18.57±12.99
2. Alteration of Pronase concn.			
††(U/ml) 0	3.4	---	1.00
200	10.0	---	4.60
3. Use of Boeringer Pronase (1000U/ml)	28.6	95.7	4.23
4. Use of BDH Pronase (Batch no.5978373c)	18.5	95.8	1.25
5. Addition of SBTI to 4.	25.7	94.4	7.10
6. Exclusion of SBTI from medium A	25.3	93.8	26.85
7. Incubation in medium A for a single period of 90 minutes	18.7	91.9	1.46
8. Storage of cell fractions at 4°C.	----	96.8	4.66

*histamine(0.5mM), IMX(0.1mM), carbachol(0.1mM);APR = aminopyrine ratio

†cells isolated by method described in 3.4.1. Data presented as mean±S.D. with number of animals in brackets.

†† 1unit Pronase (PUK) is that which produces after hydrolysis of casein at 40°C in phosphate buffer pH7.4 for 10 minutes followed by precipitation of excess protein with trichloroacetic acid, an absorbance of 1.0 at 660nm with Folin/Ciocalteau's reagent.

resulted and these cells also exhibited a poor response to stimulation by secretagogues (line 2, Table 3.3).

Because pronase, which is a broad spectrum proteolytic enzyme isolated from Streptomyces griseus, may contain trypsin-like activity, damage to cell receptors could result, particularly if digestion periods are long. Hoffman & Kuksis (1979) demonstrated that the use of soybean trypsin inhibitor protected villus and crypt cells, isolated from the rat small intestine with hyaluronidase, against excessive damage caused by proteolytic enzymes present in the crude hyaluronidase or released from damaged cells during the isolation period. Table 3.3 shows that the omission of trypsin inhibitor from medium A may have increased both the yield and the response of the cells to secretagogues compared with the data shown for the average 'routine' isolation procedure (c.f lines 1 & 6, Table 3.3). However, trypsin inhibitor did clearly improve the effectiveness of the batch of pronase, which without trypsin inhibitor present, produced a poorly responsive population of cells (c.f. lines 4 & 5, Table 3.3). Consequently, soybean trypsin inhibitor was added to all cell isolations as a precaution against tryptic damage of cell receptors.

3.4.2.2. Incubation times in medium A and temperature of storage of individual fractions.

The method described by Trotman & Greenwell (1979) involves a 90 minute incubation of the everted sacs in medium A, with the replacement of fresh medium every 30 minutes, whereas here, a cell removal period in medium B was interspersed between each 30 minute

digestion period in medium A. The cells removed after 1h and 2h must therefore be stored, and ultimately three, possibly different, populations of cells are pooled. Therefore, the digestion period in medium A was modified so that a single incubation of 90 minutes in medium A was followed by three successive isolations in medium B. The main advantage of this modification is that it would produce an homogeneous population of cells and also reduce the preparation time by one hour. However, the result of this modification was to abolish the response of the cells to secretagogues (line 7, Table 3.3) which was probably due to the prolonged exposure of the cells to a low Ca^{2+} medium and also due to tryptic damage.

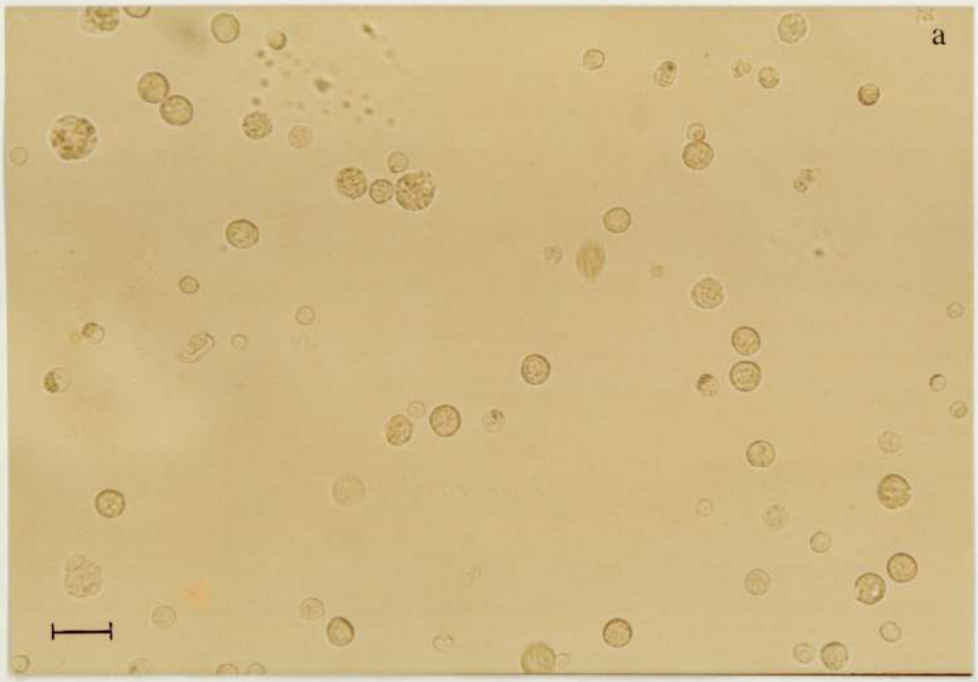
Because of the need to store the first two fractions of isolated cells, the temperature of storage of these fractions was investigated. Cold storage ($4^{o}C$) greatly reduced the subsequent response of the cells to secretagogues compared with cells stored at $37^{o}C$ (line 8, Table 3.3).

3.4.3. Characterisation of the Isolated Parietal Cells.

In this section the characteristics of the parietal cells, isolated by the method presented in 3.4.1, are described, and the results of the effect of incubation time, secretagogues and incubation medium composition on the accumulation of aminopyrine by these cells are presented.

3.4.3.1. Appearance, yields and exclusion of trypan blue.

Figs 3.2.a - 3.2.e. show the typical appearance of the isolated gastric mucosal cells under the microscope (see



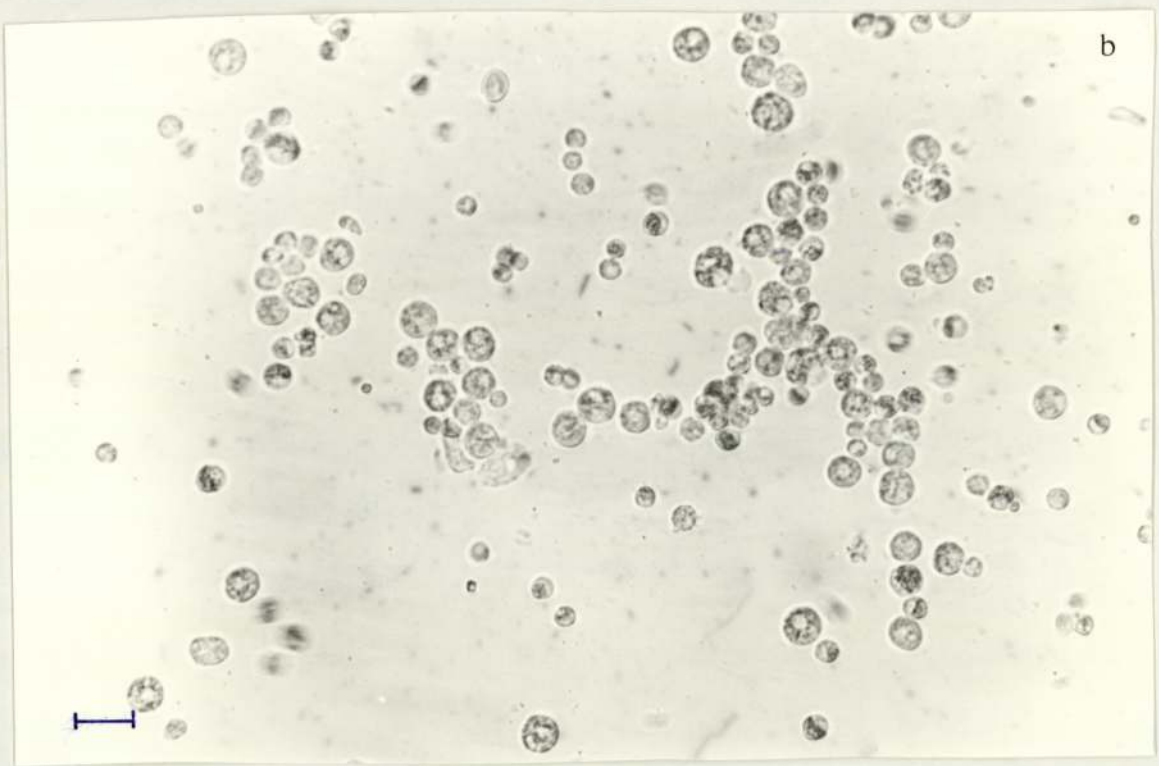
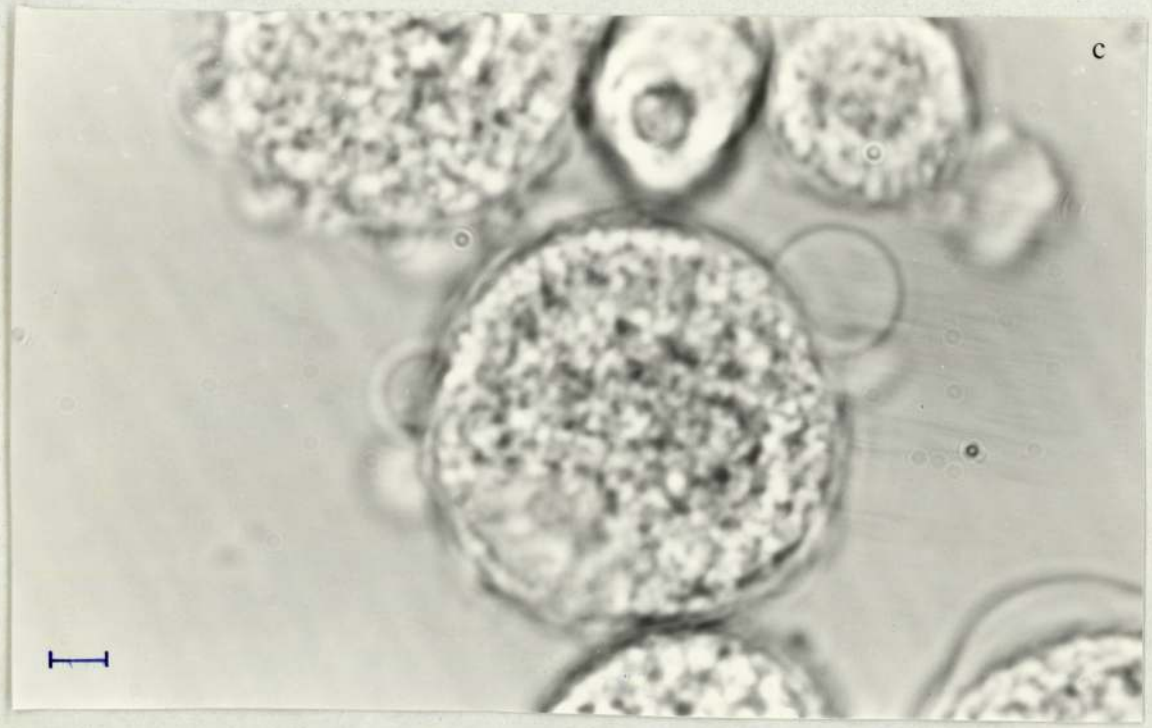


Fig 3.3 The appearance of isolated parietal cells under the light microscope. Details of the photography are given in A.8. a&b, an unstained preparation of isolated gastric mucosal cells. Bars represent 20μm



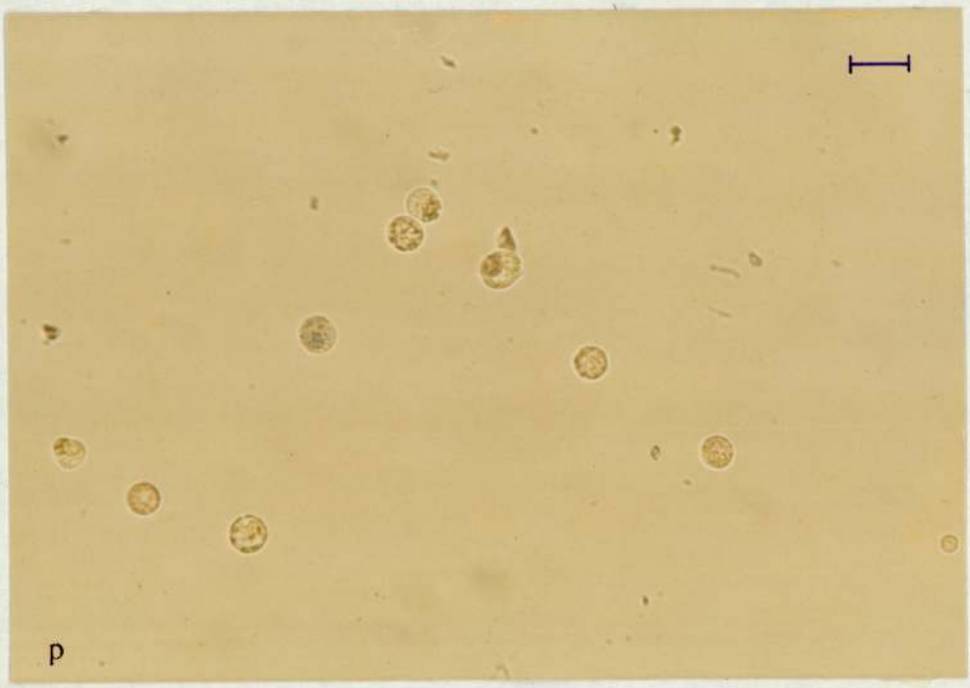


Fig 3.3 (contd.) c, a magnified view of a typical parietal cell. Bar represents 2 µm. d, a preparation of gastric mucosal cells stained with Trypan blue. Bar represents 20 µm.

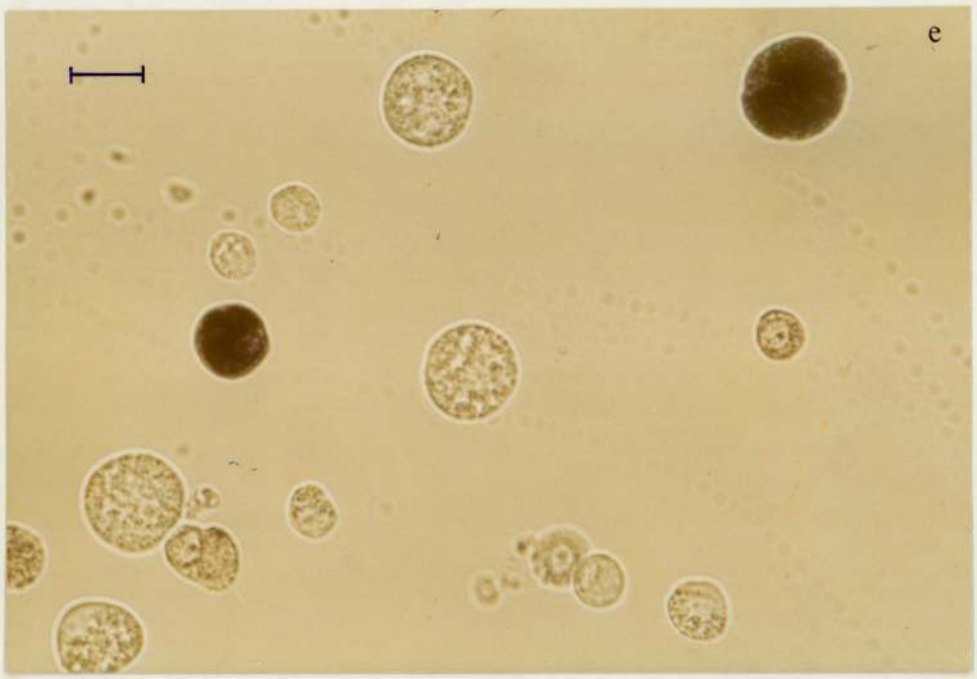


Fig 3.3 (contd.) e, a preparation of gastric mucosal cells stained with Nitrotetrazolium blue. Bar represents 10 μm.

A.8 for details of the camera and film used). Figs 3.3.a. and 3.3.b. show the appearance of an unstained preparation of cells. Parietal cells can be distinguished from other cell types on the basis of size and morphology. They are the largest cells and have a diameter which is usually greater that 12μm. Their heavily granulated appearance, due to the high proportion of mitochondria is another distinguishing feature, as is the possession of a large concentric nucleus. Fig 3.3.c. is a magnified view of a parietal cell. The other cell types seen in Figs 3.3.a. and 3.3.b. presumably consist of chief cells, mucous neck cells, endocrine cells and mucous epithelial cells in varying proportions. In Fig 3.3.d. a damaged parietal cell has not excluded trypan blue. In the photograph only the nucleus appears to have taken up the dye, but staining of whole cells was usually observed, and a cell which showed any sign of having taken up the stain was considered to be non-viable. As mentioned above (3.2.4) nitrotetrazolium blue was not considered to be a reliable stain for identifying parietal cells. Clear evidence for this is shown in Fig 3.3.e, where at least four parietal cells have not stained to any degree and only two of the parietal cells in the field of view are stained.

The mean yield of cells was 17.73 $\pm$ 0.72mg. dry weight (42), substantially better than any of the other methods attempted (Table 3.2). The average total number of cells isolated per stomach was 5.8 $\pm$ 0.3 x 10^7 (42), and the mean parietal cell content was 20.7 $\pm$ 0.9% (42).

When all cells were taken together, 96.1 $\pm$ 0.5% (42)

excluded trypan blue. Only very rarely was viability, assessed in this way, found to be less than 90% and if cells were found to be less that 85% viable they were discarded. When the viability of parietal cells alone was calculated it was found usually to be slightly lower than that for the whole cell population, reflected in the average of 94.6 $\pm$ 0.6% (42) excluding trypan blue.

3.4.3.2. Response to secretagogues.

The response of the parietal cells, present in the heterocellular population, to stimulation by acid secretagogues was determined by aminopyrine accumulation, which is an index of acid secretion (3.1 & 3.2.1).

Cells were poorly responsive to histamine at a concentration of 0.5mM (Fig 3.4). However, if the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IMX) was present (0.1mM) a significant stimulation took place. IMX alone did not increase the aminopyrine accumulation above control levels. The time course of the response indicated that maximum secretion was achieved rapidly, within 20 minutes and continued maximally for at least a further 40 minutes.

Increased acid secretion was also found when 0.1mM-carbamylcholine (carbachol), a stable analogue of acetylcholine, was added to the cell preparation (Fig 3.5). The possibility that carbachol was causing release of histamine from another cell type, and was not acting directly on the parietal cell, was not supported since potentiation of acid secretion occurred between carbachol and what is a near-maximally effective concentration of histamine + IMX (Schepp et al., 1983a).

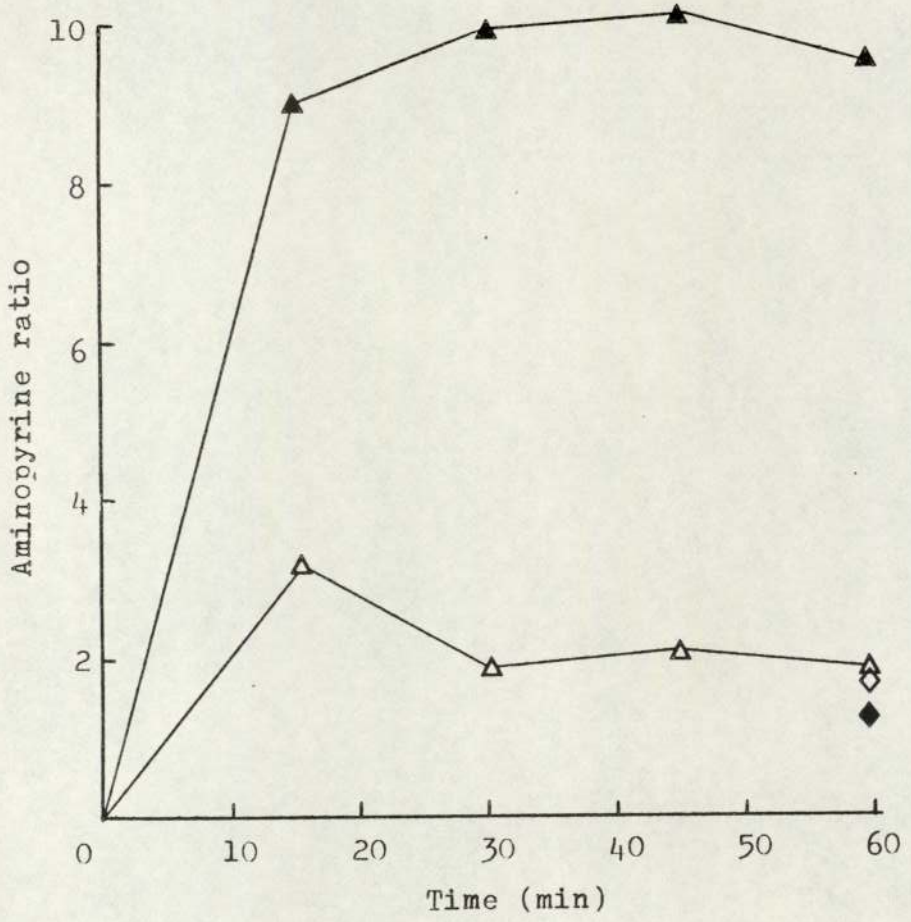


Fig 3.4 The aminopyrine accumulation by parietal cells in response to histamine (0.5mM) alone, (△——△), compared with histamine (0.5mM) plus IMX (0.1mM), (▲——▲). The aminopyrine accumulations by cells under control conditions, (◇), and in response to IMX alone, (◆), were measured at 60 min.

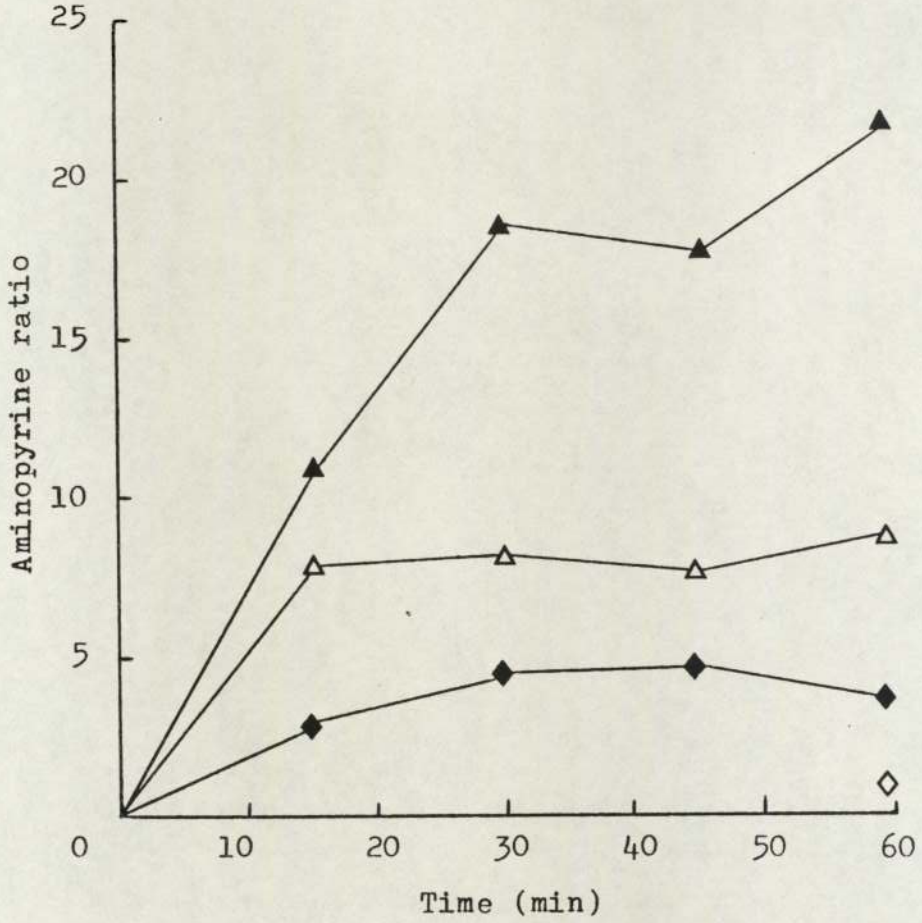


Fig 3.5 The aminopyrine accumulation by parietal cells in response tohistamine (0.5mM), IMX (0.1mM) and carbachol (0.1mM), (▲——▲) compared to that induced by histamine plus IMX, (△——△), and carbachol, (◆——◆). Aminopyrine accumulation under control conditions, (◇), was measured at 60 min.

Potentiation is defined as a response obtained with two secretagogues present together which is greater than the sum of the responses obtained with the two secretagogues alone, and potentiation between histamine + IMX and carbachol was clearly obtained with this preparation of parietal cells (Fig 3.5).

The above experiments were carried out with cells suspended in medium B (3.4.1) which contains a range of salts, amino acids, vitamins as well as glucose and probably non-esterified fatty acids associated with the bovine serum albumin. Because one of the aims of this work was to study the effectiveness of different substrates, supplied at known concentrations, in supporting the acid-secretory response, it was necessary to incubate cells in a medium containing no exogenous substrates. In these experiments, Krebs-Ringer bicarbonate was used as the basic medium supplemented with 1.25mM-$CaCl_2$ and 30mg. dialysed fatty-acid-free bovine serum albumin/ml (A.2.2). When cells were incubated in this medium, the response to secretagogues was increased when the medium was supplemented with three potential substrates for energy provision, namely glucose (10mM), D-3-hydroxybutyrate (1mM) and glutamine (1mM) (Fig 3.6). This combination of carbohydrate, ketone body and amino acid substrates was chosen as it reflected the results achieved with the studies *in vivo* (Chapter 2). The response in the absence of exogenous substrates was less, but still higher than unstimulated cells, probably due to the utilisation of endogenous fuel stores by the parietal cells (see also Chapter 5). The response, in the presence of added substrates, was not sustained at the maximal level and

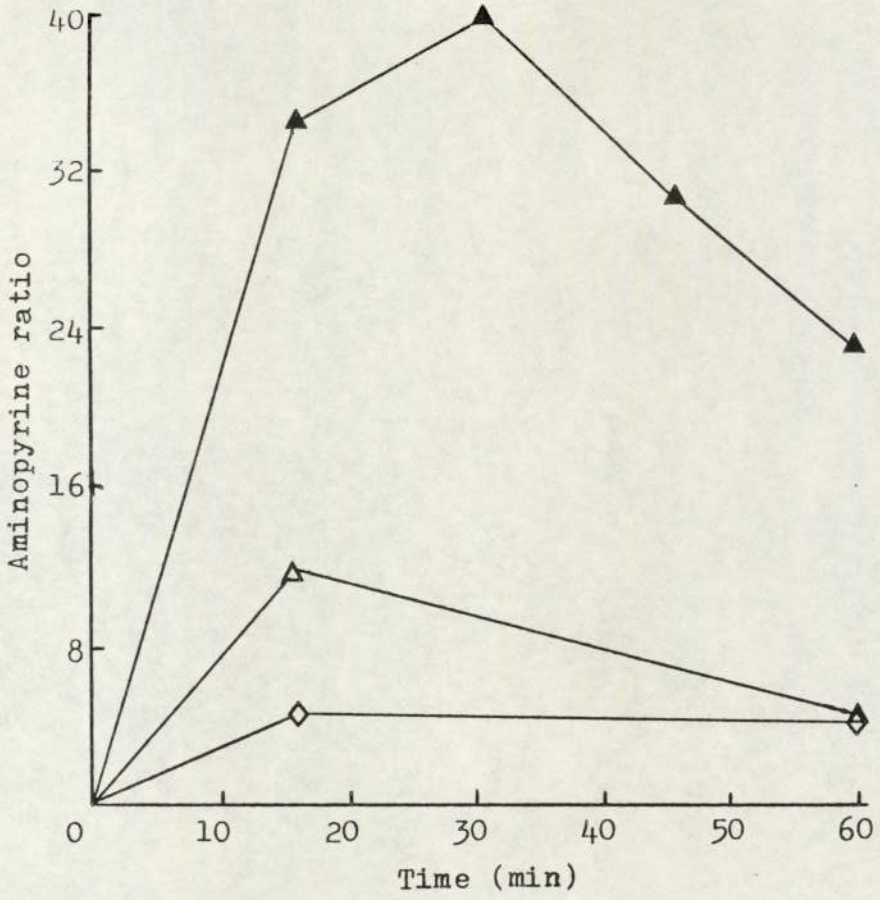


Fig 3.6 The aminopyrine accumulation by parietal cells incubated in Krebs-Ringer bicarbonate medium. Aminopyrine ratios were determined for cells incubated with secretagogues(histamine,IMX, carbachol)and substrates(glucose,D-3-hydroxybutyrate, glutamine),(▲—▲),with secretagogues only,(△—△) and with substrates only(◇—◇). Concentrations of secretagogues and substrates are given in the text.

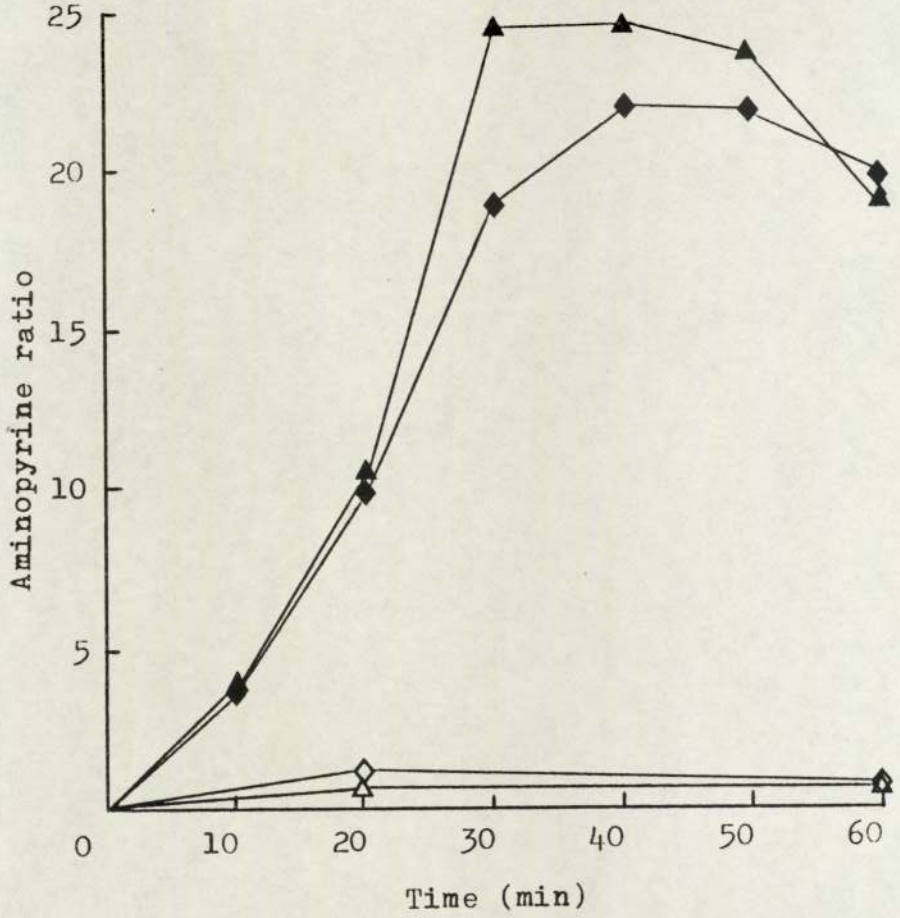


Fig 3.7 The effect of sodium oleate (0.2mM) on the aminopyrine accumulation by parietal cells incubated in Krebs-Ringer bicarbonate medium. Unstimulated,(△ , ◇) or stimulated,(▲ , ◆) cells were incubated with substrates (see Fig 3.6) in the presence,(▲ , △) or absence,(◆ , ◇) of sodium oleate.

fell off between 30 minutes and 60 minutes (Fig 3.6).

Inclusion of 0.2mM-sodium oleate in the Krebs-Ringer bicarbonate medium had no effect (two-way analysis of variance) on the time course of aminopyrine accumulation (Fig 3.7), although in this experiment the reduction in the response after 30 minutes of incubation, whether or not oleate was present, was less dramatic than that in Fig 3.6.

Finally, a direct comparison was made between the time course of aminopyrine accumulation when the same batch of cells were incubated either in Krebs-Ringer bicarbonate buffer with added substrates and dialysed fatty-acid-free albumin or in tissue culture medium (medium B) with undialysed bovine serum albumin not depleted of non-esterified fatty acids (Fig 3.8). There was a suggestion that the two curves were different (two way analysis of variance : $F = 7.998$; $p<0.05$) and that a greater fall-off in aminopyrine accumulation, after 30 minutes incubation, occurred with the Krebs-Ringer bicarbonate + substrates medium than with the tissue culture-based medium, although there seemed to be a greater response with cells incubated in the Krebs-Ringer bicarbonate buffer at 20 and 30 minutes. Despite the apparent limitations in the use of Krebs-Ringer bicarbonate based media for long term cell incubation it was still apparent that a substantial acid-secretory response could be achieved if the time of incubation was not continued for more than 30 minutes.

3.5. DISCUSSION.

3.5.1. Methods for Preparing Cells.

In section 3.3.1 an attempt was made to isolate

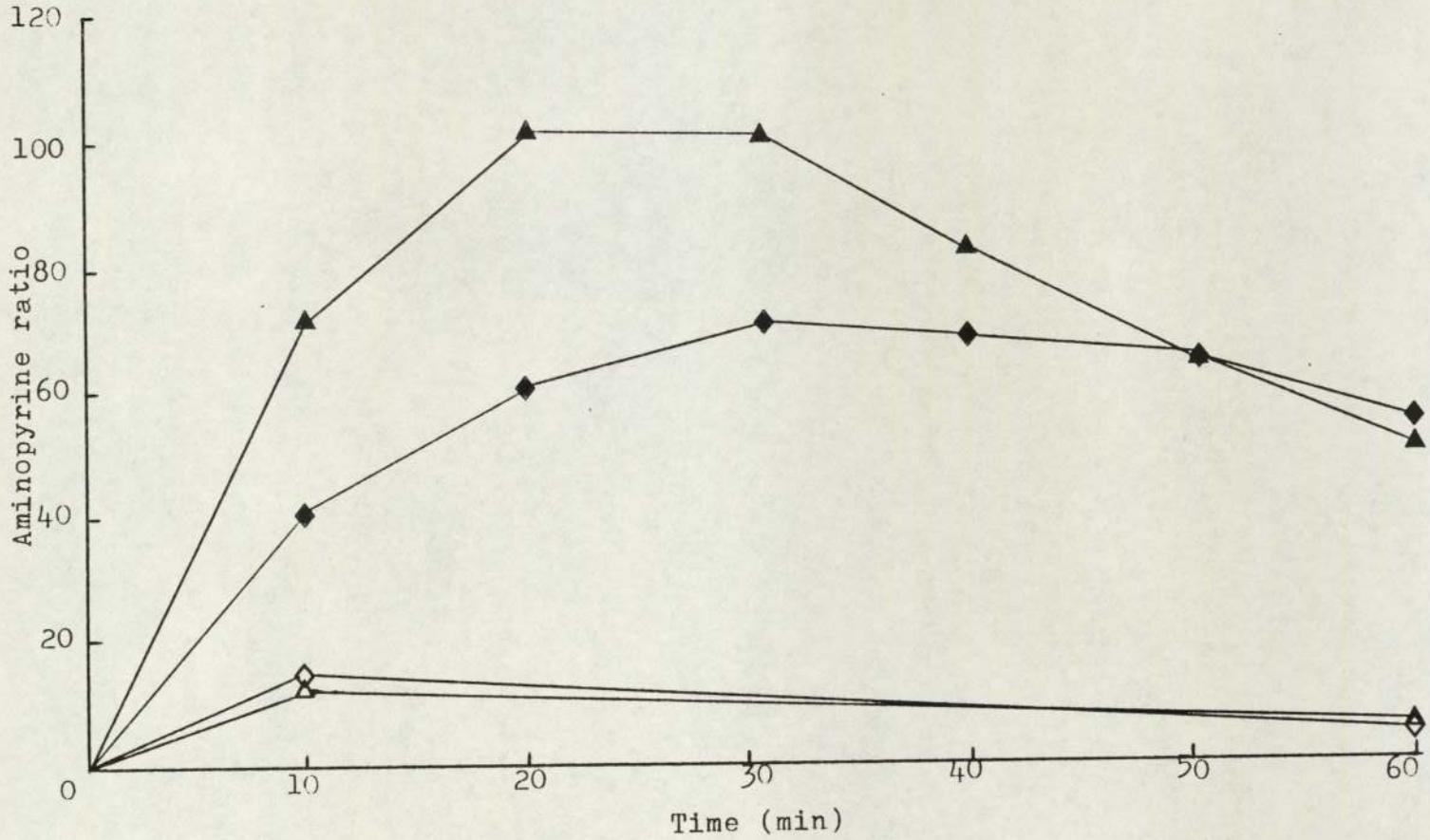


Fig 3.8 The aminopyrine accumulation by parietal cells incubated in Krebs-Ringer bicarbonate medium plus glucose(10mM),D-3-hydroxybutyrate(1mM) and glutamine(1mM),(▲,△) or medium B,(◆,◇). Aminopyrine ratios were determined for unstimulated cells (△,◇) and for cells stimulated with histamine(0.5mM),IMX(0.1mM) and carbachol(0.1mM),(▲,◆).

parietal cells from both rats and guinea pigs using a method involving digestion of the minced oxyntic mucosa with collagenase. Despite using similar procedures, in terms of collagenase concentration and time of digestion, the yield of cells from the guinea pig stomach was a great deal more than that from the rat. This suggests that the intercellular connective structures in the rat stomach are far less susceptible to both calcium chelation and collagenase digestion than in the guinea pig. Longer incubation times and/or higher collagenase concentrations might have lead to further release of individual cells from the rat tissue, but such cells are more likely to have been damaged.

The importance of not prolonging the depletion of Ca^{2+} from the isolation medium was demonstrated by the lack of stimulation of acid secretion by gastric glands isolated by the method described in 3.3.2, namely incubation of everted sacs in a medium containing 2.5mM-EDTA for a total of 80 minutes. Similarly, prolonged incubation (90 minutes) of everted sacs in medium A containing 2mM-EDTA (3.4.2.2) gave rise to a cell population which showed no response to stimulation by acid secretagogues. Thus, it would appear that prolonged depletion of Ca^{2+} is deleterious to cell integrity in terms of causing irreversible damage to some part of the secretory machinery.

The lack of response shown by gastric glands isolated by the method described in 3.3.2, might also have been caused by carrying out the isolation in an ice-cold medium. Mechanical dispersion of tissues can be particularly deleterious to cells if performed at low temperatures (Soll, 1981), since membrane fluidities are

restricted, which could impair sealing of defects created by tearing apart cell junctions, and the resultant shifts in ion composition may alter cell responsiveness. Storage of isolated cells at low temperatures also appears to reduce the responsiveness to secretagogues, (3.4.2.2).

3.5.2. Aminopyrine Accumulation.

Using the formula in Fig 3.1 and assigning a value of 1.2 as the pH in the intracellular canaliculi and tubulovesicular system of an acid secreting parietal cell *in vivo* (Sack & Spenney, 1982), a theoretical maximum aminopyrine ratio can be calculated to be approximately 6300. There are four basic reasons why theoretical ratios might not be achieved. Firstly, the secretagogues used may not cause maximal acid secretion. Secondly, the volume of the intracellular space where the acid is secreted could be overestimated. Thirdly, the conditions employed to carry out the aminopyrine test might not be optimal, and finally diffusion of acid out of the canaliculi into the medium might occur.

Schepp et al.(1983a), found 1mM-histamine in the presence of 0.1mM-IMX was maximally effective in increasing aminopyrine accumulation, using essentially the same method as here to isolate the parietal cells. 0.1mM-carbachol was shown by Ecknauer et al. (1981) to maximally stimulate aminopyrine accumulation in another rat parietal cell preparation, and therefore the combination of secretagogues used here (histamine, 0.5mM; IMX, 0.1mM; carbachol, 0.1mM) ought to be close to maximally effective in stimulating aminopyrine accumulation.

In this work calculations of aminopyrine ratios did not

include a correction for the volume of the intracellular space where acid did not accumulate. Since the unenriched parietal cell fractions used for much of this work contain an average of approximately 20% parietal cells and the acid-secretory compartments may represent only about 50% of the total parietal cell volume (Zalewsky & Moody, 1977), a substantial fraction of the intracellular space, calculated from measurement of the tissue dry weight (3.2.1), is not associated with acid secretion. However, since these studies are concerned more with a comparison of the acid secreted under different conditions than with obtaining an accurate measurement of the amount of acid secreted by the parietal cell, no corrections were made for the intracellular fluid associated with acid secretion.

Sack & Spenney (1982) have described some of the experimental conditions which it is important to optimise in order to achieve maximum aminopyrine accumulation. The two most important factors are the purity of the aminopyrine and the mixing of the cell suspensions during incubation with aminopyrine. In preliminary experiments here, both of these factors were investigated. Using the chromatographic analysis of $[^{14}C]$ aminopyrine as described by Sack & Spenney (1982) (see A.9) a radioactive impurity, amounting to 13.5% of the total radioactivity, was found in the initial batch of $[^{14}C]$ aminopyrine. Assuming that the radioactive contaminant does not accumulate in the acid intracellular space, the $[^{14}C]$ radioactivity measured in the extracellular medium is an overestimate of the amount of aminopyrine present, the degree of overestimation being dependent on the amount of contaminant present.

Consequently the aminopyrine ratio, calculated as in 3.2.1, will be underestimated. Thus, the apparent ratio of 48.75 shown in the sample calculation in 3.2.1, was an underestimate of the true ratio which is 56.55

Sack & Spenney (1982) stressed the importance of optimising the mixing of the cell suspension during incubation with aminopyrine, and a major factor determining the efficiency of mixing was the choice of incubation vessel. A preliminary experiment here showed that there was little difference between the maximum aminopyrine ratio achieved if incubation was carried out in a 20ml scintillation vial, or a 25ml plastic conical flask. The 20ml scintillation vial, with a base diameter of 2.5cm, produced a maximum stimulated aminopyrine ratio of 6.86 and the 25ml conical flask, with a base diameter of 3.5cm, produced a maximum stimulated aminopyrine ratio of 7.17. Since, there was no obvious advantage in using 25ml conical flasks to carry out incubations, scintillation vials, which were less expensive and more convenient to handle, were used routinely.

Generally, the maximum aminopyrine ratios were achieved with 20-30 minutes of incubation after which the stimulation either reached a plateau, if the cells were incubated in tissue culture medium, or fell off if the cells were incubated in Krebs-Ringer bicarbonate buffer with added glucose, D-3-hydroxybutyrate and glutamine. The reasons for such a reduction could be exhaustion of the substrate supply, the build-up of an inhibitory product of metabolism of one or more of the substrates or the absence of some component from the medium required to sustain acid secretion. It seems that the substrates provided in the Krebs-Ringer bicarbonate medium are at concentrations which would not be exhausted during the time of the experiment

(60 minutes) even by highly metabolically active cells. Indeed, the concentration of glucose in medium B is only 5.6mM and the only other potential metabolic fuels present are low concentrations of amino acids and fatty acids associated with bovine serum albumin. Because of the fall-off in response seen after 30 minutes incubation in Krebs-Ringer bicarbonate buffer, the studies on the dependency of acid secretion (Chapter 5) will use a 30 minute period of incubation to achieve the peak acid-secretory response. Other studies, where the concentration of exogenous substrate need not be controlled, will use medium B for cell incubations which appears to provide an environment allowing a nore sustained response to secretagogues.

3.5.3. Comparison of Results with other Preparations.

3.5.3.1. Yield of cells.

The total number of cells isolated per stomach here (6×10^7) is comparable with the yields obtained by Lewin et al. (1974) (5×10^7/stomach) and Schepp et al. (1983a) (8×10^7/stomach) who used a similar method of cell isolation. Lower yields were achieved by Ecknauer et al. (1981) (Table 3.4) with a different rat cell preparation. Other isolation procedures using different animals, such as those used by Soll (1978) and Berglindh & Obrink (1976) produced higher yields (Table 3.4) but these are offset by the expense of purchasing and maintaining dogs and rabbits. Also the procedure with rabbits (Berglindh & Obrink, 1976) produces gastric glands, which require further digestion (Chew & Hersey, 1982) to produce

Table 3.4. Comparison of the yields, viabilities and aminopyrine responses obtained by others using various preparations with those obtained here. The response to secretagogues represent the highest values quoted and may not represent maximal stimulation.

Preparation (animal;reference)	Yield (cells/stomach $x10^7$)	Yield (mg dry wt/ stomach)	Viability (%cells excluding trypan blue)	Basal APR	Response to secretagogues. (stimulated APR/ control APR) Hist.+IMX	Response to secretagogues. (stimulated APR/ control APR) Carbachol
Dog; Soll(1980)	70	---	94	1.83	24.4	23.4
Rabbit;Berglindh et al.(1976)	---	150	90	45.0	2.1	1.9
Rabbit;Fellenius et al.(1983)	---	---	--	5.8	50.0‡	---
Guinea pig;Batzri&Dyer(1978)	3.3	---	95	---	18.0‡	6.0
Human;Fellenius et al.(1983)	---	---	--	11.9	5.5‡	1.7
Rat;Sonnenberg et al.(1979)	5	---	90	---	2.0‡	3.3
Rat;Gespach et al.(1980)	---	10	80	---	---	---
Rat;Ecknauer et al.(1981) Dial et al.(1981)	2.5	---	85	2.4*	3.9	3.6
Rat;Schepp et al.(1983) Maslinski & Ruoff(1984)	8	---	90	1.2*	5.0	1.8
Present study	5.8±0.3(42)	17.73±0.72(42)	96.1±0.5(42)	2.6±0.1(24)	22.7±3.0(15)	5.6±1.0(9)

*Calculated from data expressed as $[^{14}C]$-aminopyrine accumulated/10^6 parietal cells
‡ Histamine alone

individual cells which would then have to be enriched in parietal cell content to allow studies on the mechanism of acid secretion (Chapters 7 & 8).

3.5.3.2. Response to secretagogues.

Sonnenberg et al. (1979) achieved a 2-fold increase in aminopyrine accumulation with histamine + IMX and a 3.3-fold increase with carbachol and the maximum aminopyrine ratio, 14, was only achieved when dbcAMP was also present. Schepp et al. (1983a) reported that aminopyrine accumulation was increased 5-fold by histamine + IMX. The above results were obtained with a cell population, prepared by a method similar to that used here. In this work the responses to both histamine + IMX and carbachol were greater than the above and also those of Ecknauer et al. (1981) (Table 3.4).

In this work the response to histamine alone was negligible (Fig 3.4) and this effect has presumably been found by others using rat parietal cells isolated with pronase, all of whom also can only demonstrate histamine stimulation in the presence of IMX (Dial et al., 1981; Schepp et al., 1983). Only Batzri & Dyer (1981) appear to have shown that a gastric mucosal preparation can be stimulated to substantially increase aminopyrine accumulation by histamine administered alone.

As discussed in 3.5.2, maximal stimulation was not sustained when cells were incubated in Krebs-Ringer bicarbonate medium. This was not found by either Sonnenberg et al. (1979) or Schepp et al. (1983) using essentially the same population of cells incubated in a similar medium, but reductions have been found by Soll (1980) who found that the acid-secretory response to both histamine + IMX

and carbachol subsided after 20-30 minutes and Berglindh et al., (1976) found only a transient response to carbachol after about 20 minutes. However the use of a tissue culture-based medium for cell incubation resulted, here, in a more sustained acid-secretory response.

Overall, using the three parameters of yield, viability (trypan blue exclusion) and response to secretagogues, the cell population, prepared and characterised here, compares favourably with other methods which are presently in use. The only limitations discovered were the negligible response to histamine alone, and the inability to sustain maximum levels of stimulation for periods of more than about 30 minutes, unless incubation was performed in a tissue culture based-medium. However, as discussed above, other preparations are also subject to these and further limitations, and therefore the use of the preparation of isolated cells described here would appear to offer the potential of providing genuine data on acid secretion which could be integrated with the work of others in the same field of study.

CHAPTER 4.

ENRICHMENT OF ISOLATED PARIETAL CELLS.

4.1 INTRODUCTION.

Having produced a population of viable isolated gastric cells from the rat stomach containing approximately 20% parietal cells (Chapter 3), a method of enriching the parietal cell content was required to enable a detailed investigation of the intracellular mechanisms involved in coupling secretagogue stimulation to acid secretion in the parietal cell. Generally, cell separation procedures harness a unique physical characteristic of the cell under investigation which distinguishes it from the other contaminating cell types.

4.1.1 Physical Properties of the Parietal Cell and General Methods Available for Separation.

In general, different cell types may be distinguished by both size and density. The gastric parietal cell is larger than any other cell in the stomach and also has a particularly low density due to its high complement of membranes (Soll, 1981). Thus, separation of parietal cells on the basis of size and density is feasible. Separation, using differing cell size, is normally achieved by velocity sedimentation, and this is based on Stokes law,

$$SV = (2/9)\ r^2\ (\rho p - \rho m)g/\eta$$

where SV is the sedimentation velocity, ρp and ρm are the densities of the cells and medium respectively, r is the radius of the cell, η is the viscosity of the medium and g is the gravitational field. Cell size is thus a major determinant of sedimentation velocity and the parietal cell, with its larger size, should sediment more rapidly

than any other cell type. Three basic techniques have been used to accomplish separation of parietal cells on the basis of sedimentation velocity. First, unit gravity sedimentation was used by Romrell et al. (1975) to enrich mouse parietal cells, Table 4.1, but this technique, despite producing high enrichment, also resulted in low recoveries of parietal cells and required a considerable time (90 minutes) in the cold room. Cold storage can result in changes in cellular ion composition and consequently cell responsiveness (see 3.4.2.2 and Soll, 1981). Second, velocity separation was performed by carrying out brief repeated centrifugations (Soumarmon et al., 1978) to enrich rat parietal cells to 75% purity (Table 4.1), but the yield of purified cells was poor. Third, separation of gastric cells on the basis of counterflow centrifugation has been used to enrich dog (Soll, 1978) and rabbit (Chew & Hersey, 1982) parietal cells. This involves the use of an elutriator rotor in which the separation chamber is continually perfused in a direction directly opposite to the action of the centrifugal force. Cells equilibrate within the chamber as a function of their sedimentation velocity, with the smaller, slower-sedimenting cells near the centre of the rotor, and the larger, faster cells at the periphery. As the flow rate is increased, successively larger cells are eluted. Elutriation has provided good parietal cell enrichment (Table 4.1) together with high yields, but equally good results have been achieved, without the expense of purchasing an elutriator rotor, using other techniques.

Density or isopycnic separations have also been widely used to enrich parietal cells. The principal of

Table 4.1 Methods used to enrich the parietal cell content of isolated gastric mucosal cell preparations.

Authors	Animal	Method	Final purity of parietal cells(%)
Romrell et al.(1975)	Mouse	Unit gravity velocity sedimentation at 4°C.	75-95
Soll (1978)	Dog	Counterflow centrifugation using elutriator rotor	85
Glick et al.(1974)	Rabbit	Isopycnic centrifugation on Ficoll gradient	60
Chew & Hersey (1982)	Rabbit	Counterflow centrifugation using elutriator rotor	70
Sewing et al.(1983)	Guinea Pig	Counterflow centrifugation using elutriator rotor	70-80
Lewin et al.(1974)	Rat	Isopycnic centrifugation on sucrose gradient	80
Soumarmon et al.(1978)	Rat	Iterative velocity centrifugation	75
Sonnenberg et al.(1979)	Rat	Isopycnic centrifugation in Percoll	70-90
Ecknauer et al.(1981)	Rat	Isopycnic centrifugation on discontinuous Percoll gradient	50-90
Thomson et al.(1981)	Rat	Isopycnic centrifugation on sucrose/Ficoll gradient	90

this type of separation involves the forcing of the cells through a medium with a range of densities by centrifugation until their sedimentation velocity reaches zero as they encounter a medium of a density equivalent to their own. Each cell type with a different density should reach its equilibrium position on the gradient provided that adequate centrifugal force and run-time are employed.

Density gradients have been constructed using a variety of media. A linear gradient of sucrose was used by Lewin et al. (1974) to isolate rat parietal cells, (Table 4.1). Other media such as Ficoll (Glick, 1974) and combinations of sucrose and Ficoll (Thomson et al., 1981) have also been employed with varying degrees of success (Table 4.1), and albumin and Nycodenz gradients have been shown to separate other cell types. A basic disadvantage of the above density gradient media is the problem of osmolality. In forming a gradient with a medium such as sucrose; a gradient of osmolality is necessarily present, and the densities of cells in hyperosmotic regions will be increased, causing difficulties in predicting the equilibrium position of a particular cell type, as well as possible deleterious effects on cell function. The development of Percoll (Pertoft & Laurent, 1977) overcame these osmolality problems, since it has a very low osmotic pressure and consequently density gradients are virtually iso-osmotic throughout.

4.1.3. Properties of Percoll and its Use in the Separation of Cells by Density Gradient Centrifugation.

Percoll consists of colloidal silica particles of 15-30 nm diameter which have been coated with polyvinyl-

pyrollidone (PVP). The concentration of free PVP is very low (1-2%) which retains the low osmolality of Percoll, (<20mOs/kg H_2O), and also a low viscosity allowing a more rapid isopycnic equilibration to take place. It also can provide a fairly wide range of densities i.e. 1.0-1.3g/ml which covers the densities of most cell types. Percoll has been shown to be non-toxic to cells and does not penetrate biological membranes (Pertoft et al., 1977). Separations of many cell types have been performed using Percoll density gradients including blood cells (Segal et al., 1980), liver cells (Seglen, 1979) and intestinal epithelial cells (Curman et al., 1979), and the technique has also been developed for the separation of parietal cells. Sonnenberg et al. (1979) used a solution of Percoll containing rat gastric mucosal cells and achieved separation of the parietal cells by employing the Percoll at a final density which allowed non-parietal cells to sediment under centrifugation, but retained the lighter parietal cells in suspension. Cell fractions with 70-90% parietal cells were thus obtained (Table 4.1). A different method was used by Ecknauer et al., (1981) to produce a similar enrichment of rat parietal cells (Table 4.1), where cells were loaded onto a discontinuous gradient of 10%, 20% and 30% Percoll. Centrifugation caused parietal cells to band at the 10%/20% interface. Both of the above procedures produced good yields of viable parietal cells which retained their responsiveness to secretagogues.

Because separation of cells with Percoll is an apparently uncomplicated and rapid method, involving minimal stress, it was chosen as the technique to enrich the parietal

cell content of the cell population prepared in Chapter 3.

4.2. METHODS AND RESULTS.

It was first necessary to examine the conditions required to bring about maximum purification coupled with good viability and yield of parietal cells. As in Chapter 3, a detailed methodology of the procedure finally adopted will be described first (4.2.1), followed by an account of the effect of various modifications subsequently rejected (4.2.2), and finally, the purified cell suspension will be characterised in terms of degree of purity, yield, viability and response to stimulation by secretagogues (4.2.3).

4.2.1. Routine Method Used to Prepare a Cell Suspension Rich in Parietal Cells.

Cells were enriched using a method based on that of Sonnenberg et al. (1979) involving isopycnic centrifugation in Percoll. Normally, the cells isolated from three stomachs, by the method described in 3.2.2, were used for enrichment. A suspension of crude cells in medium B (3.4.1) was centrifuged at 15°C (100g, 10min) and the cell pellet resuspended in 7ml medium C (medium B-see 3.4.1-without bovine serum albumin). To this, 3ml of stock Percoll solution was added to provide a 30% Percoll/cell suspension. Stock Percoll solution was prepared by adding 9 volumes of Percoll to 1 volume of 10x strength Eagle's minimum essential medium (composition 10X that in A.2.2) and adjusting the pH, after gassing with O_2/CO_2 (95/5), to pH 7.4 with 3M - HCl. The suspension of cells in 30% Percoll was then centrifuged (2000g, 1min) and the supernatant

collected. The supernatant cell suspension was diluted with 3 volumes of physiological saline (NaCl;9g/l) and centrifuged (100g, 5min) to sediment all cells present. The cell pellet was then resuspended in approximately 5ml medium C and recentrifuged (50g, 5min). This final centrifugation step, involving velocity sedimentation was repeated once to further enrich the parietal cell content, by resuspending the resulting cell pellet in approximately 5ml medium C and recentrifuging. The final cell pellet was resuspended in medium B and stored at 37°C in a shaking water bath (120 cycles/min) with continuous gassing (O_2/CO_2;95/5).

4.2.2. Modifications to the Purification Procedure.

In order to improve the results achieved using the method described in 4.2.1, a number of modifications were tested to examine their effects on purity, yield and viability.

4.2.2.1. The use of a preformed discontinuous gradient of Percoll.

A discontinuous Percoll gradient (Thomson et al., 1981) was formed by sequentially adding 3ml each of 30%, 20% and 10% Percoll solutions, via a Pasteur pipette to a plastic centrifuge tube. The solutions were prepared by adding the appropriate volume of stock Percoll solution to the appropriate volume of medium C. 2ml of medium C containing 4×10^7 impure gastric mucosal cells were carefully layered onto the top of the gradient (10% end) and the gradient was then centrifuged (50g, 10min). After centrifugation, samples were removed from the gradient by

by careful suction, and each density zone and interface was examined microscopically.

None of the layers contained a significant enrichment of parietal cell content, and indeed no accumulation of any cells occurred except at the 20%/30% interface. Both this band and the cell pellet contained the proportions of parietal cells present in the initial crude extract.

4.2.2.2. The use of a preformed linear gradient of Percoll.

A linear Percoll gradient was prepared by introducing, into a 10ml plastic centrifuge tube, a solution of linearly increasing Percoll concentration, beginning at 20% (w/v). The solution was introduced to the bottom of the tube so that as liquid having a higher Percoll concentration entered, it displaced the less-dense, lower concentration fluid upwards. The end result was a linear gradient of Percoll with limits of 20% at the top and 40% at the bottom of the tube.

The concentration gradient of Percoll was introduced via plastic tubing mounted on a peristaltic pump (Gilson HP4MF, Anachem, Luton, U.K) from a reservoir containing 20% Percoll initially. The rate of removal of fluid from this reservoir was twice the rate of the addition of 40% Percoll to the same reservoir, thus producing an outflow of linearly-increasing Percoll concentration. After formation, the gradient was overlayed with 2ml of cell suspension (5×10^7 cells) and centrifuged (800g, 15min). As before, layers were sequentially removed and analysed for parietal cell content. Only in the lower two-thirds of the gradient did any enrichment take place, where approximately 50% of the cells were parietal cells.

But this region contained very few cells in total, as most had been sedimented at the bottom of the tube. A possible explanation for the poor degree of enrichment found in this section and in 4.2.2.1, is the aggregation of cells at the cell suspension-Percoll interface. Cell aggregates would have a large mass and density which would not equilibrate with the separation medium and centrifugation would cause their sedimentation.

4.2.2.3. Modifications to the conditions employed for cell separation in a solution of Percoll.

Using 35% Percoll, which has a density of 1.051gcm^{-3} instead of 30% Percoll (1.043gcm^{-3}) was expected to increase the separation of the parietal cells, which in dog, have a density of 1.045 - 1.055gcm^{-3} (Soll, 1981). However, this was not achieved (line 2, Table 4.2) and the final purity of 61.5% was less than that usually achieved with 30% Percoll, although the parietal cell recovery, as expected, was higher.

Because Percoll had been shown to increase cAMP levels and stimulate acid secretion in isolated parietal cells (Sonnenberg et al., 1979), it was desirable to reduce the time exposure of the cells to Percoll. Consequently, an experiment was carried out where a preparation of crude cells were purified using three different density centrifugation protocols. In one, the conditions of Sonnenberg et al. (1979) i.e. 200g, 15min, were used. In the second, the same centrifugal force was applied for only 5 minutes and thirdly the conditions were altered to 2000g for 1 minute. Using 200g for only 5 minutes clearly did not produce the desired enrichment (line 3, Table 4.2).

Table 4.2.a. Modifications to the standard purification procedure. Results are presented in Table 4.2.b.

	Percoll concentration(%v/v)	Density Centrifugation	Velocity centrifugation
1	30	2000g, 1min.	50g, 5min. x 2
2	35	200g, 15min.	50g, 5min.
3	30	200g, 5min.	-------
4	30	200g, 15min.	50g, 5min. x 2
5	30	200g, 15min.	100g, 10min.
6	30	200g, 15min.	50g, 5min.

Table 4.2.b. Modifications to the standard purification procedure - results.

Modification (Table 4.2a)	Parietal Cell Content(%)			Purification factor*		Parietal cell recovery**
	Initial	After Density Centrifugation	After velocity Centrifugation	Density Centrifugation	Velocity Centrifugation	
1	19.9±5.8 (15)	65‡	77.3±8.9(15)	3.27	1.19	25.8±7.2(15)
2	15.8	49.3	61.5	3.12	1.25	70.1
3	17.9	41.6	---	2.32	---	---
4	17.9	62.2	87.5	3.47	1.39	10.4
5	17.8	56.0	69.5	3.15	1.24	43.4
6	17.5	59.1	82.2	3.38	1.39	43.7

* $\frac{\%\text{parietal cells after step}}{\%\text{parietal cells before step}}$

** $\frac{\text{no. of parietal cells in pure fraction}}{\text{no. of parietal cells in initial fraction}}$ %

‡ average figure from 2 determinations

The enrichments produced using the other conditions were better, and there appeared to be little difference between cell purification factors after centrifugation at 200g for 15 minutes (lines 4,5,& 6, Table 4.2) and at 2000g for 1 minute (line 1, Table 4.2). The high centrifugal force did not appear to affect either cell viability (2000g fraction - 90.2% of parietal cells excluded trypan blue; 200g fraction - 87.5% of parietal cells excluded trypan blue) or the recovery of parietal cells. Consequently the method using high centrifugal force and short time was adopted for routine purifications.

The parietal cell content of the supernatant fraction obtained after centrifugation in 30% Percoll was increased by further differential velocity centrifugation at low speed (Table 4.2, compare columns headed "after density centrifugation" and "after velocity centrifugation") probably because the larger parietal cells sedimented more rapidly than smaller cell types.

The enrichment effected by centrifugation at 50g for 5 minutes was higher than that effected by centrifugation at 100g for 10 minutes (cf. lines 4 & 5, Table 4.2). The use of a second velocity sedimentation under the same conditions (50g, 5min) appeared to produce a further increase in cell purity, but probably at the expense of cell yield (cf. lines 4 & 6, Table 4.2). The conditions best suited to produce the fraction of cells possessing the highest content of viable parietal cells were therefore those detailed in Table 4.2 (line 1).

4.2.3. Characterisation of the Purified Parietal Cell Fraction.

Yields and viabilities (trypan blue) were measured,

as described in Chapter 3. The average purity of the final fraction was 77.3 ± 2.3% (15) parietal cells. From the same 15 experiments the average yield of parietal cells was 0.66 ± 0.07mg (15) dry weight/stomach or approximately 4×10^6 parietal cells. This probably represents about 8% of the total parietal cells assuming 5×10^7 parietal cells per stomach (Hogben et al., 1974). The viability of the fraction, in terms of trypan blue exclusion was, on average, 90.3 ± 1.4% (15), and was rarely below 85%. Viability was further assessed by examining the response of the cells to secretagogue-stimulation in terms of aminopyrine accumulation (3.2.1). The number of cells was approximately 10^6 cells/ml. incubation medium and a combination of histamine (0.5mM), IMX (0.1mM) and carbachol (0.1mM) was used to maximally stimulate the cells. On two occasions the aminopyrine ratios for stimulated cells were not different from the unstimulated controls (the low level of the controls did not suggest that previous exposure to Percoll had produced a sustained stimulation of acid secretion). However, if purified cells were preincubated for 1h. at 37°C in medium B and gassed with O_2/CO_2 (95/5) continuously, the subsequent increase in aminopyrine accumulation induced by the secretagogue combination, mentioned above, (Fig. 4.1) indicated that the parietal cells had not been irreversibly damaged during purification.

4.3. DISCUSSION.

4.3.1. Parietal Cell Enrichment.

The average final purity of parietal cells (~77%) obtained here was in the same range as obtained by others

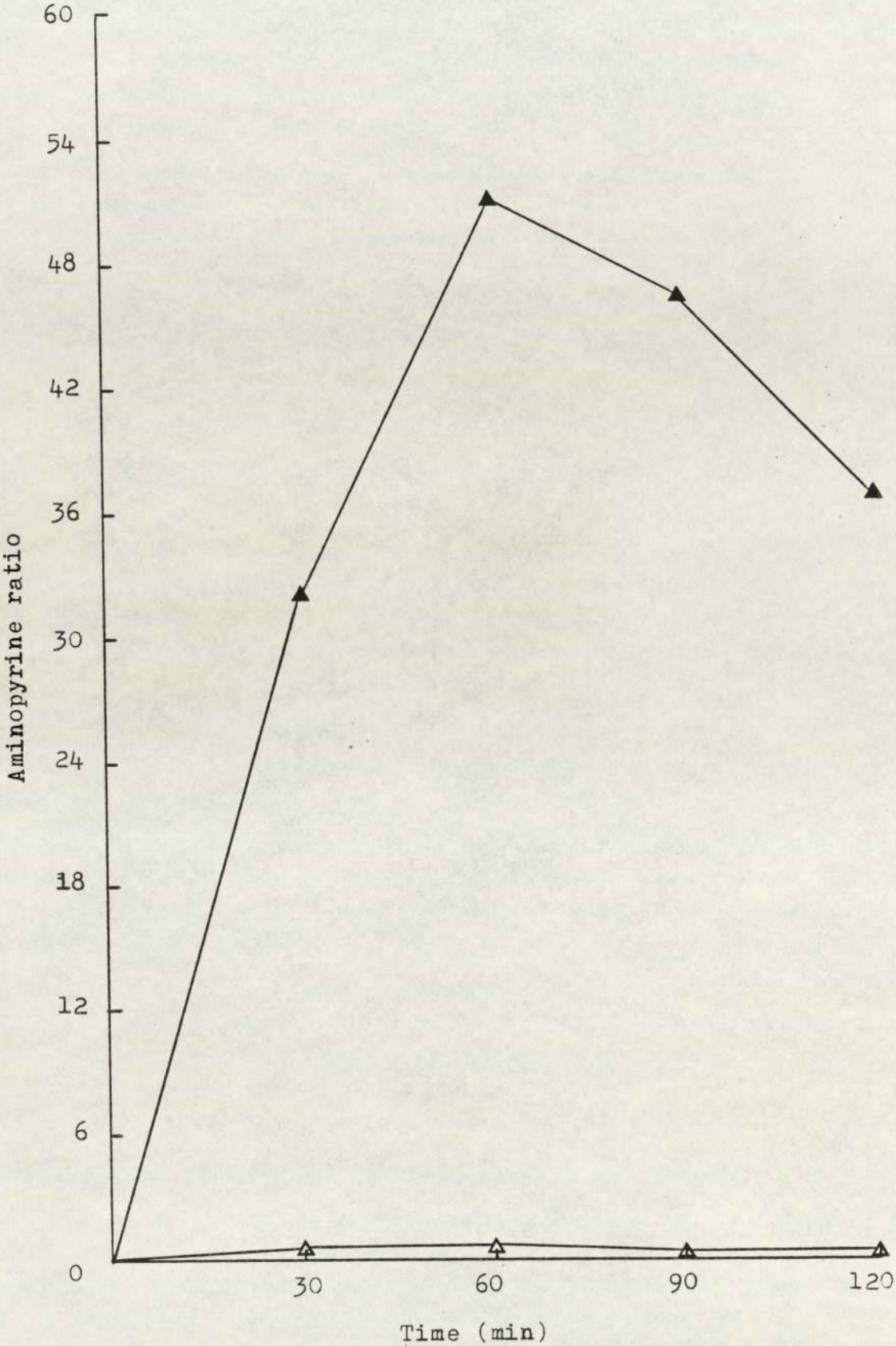


Fig 4.1 Aminopyrine accumulation by purified parietal cells under control conditions (△—△) and in response to histamine (0.5mM), IMX (0.1mM) and carbachol (0.1mM), (▲—▲). Cells were preincubated for 1 hour in medium B before stimulation.

using essentially the same purification procedure (Table 4.3; Sonnenberg et al., 1979: 70 - 90%; Schepp et al., 1983a: 65 - 85%). No other groups, using different purification methods, have consistently achieved higher purities (Table 4.3). Higher purities might have been achieved by introducing further separation steps, but it is likely that this would have resulted in a decreased yield. Thus, although a cell fraction containing 100% parietal cells is conceivable, the resultant recovery of cells from the initial population would probably be very low. Using less-than-totally pure fractions to study the physiology of the parietal cell should be valid provided copurification of another cell type does not occur. Copurification would only take place if another cell type possessed similar physical properties to that of the parietal cell. Soll et al. (1981) found canine histamine-containing cells had a similar density to that of parietal cells and therefore separation based solely on density might not enrich parietal cells with respect to the histamine-containing cells. However, because parietal cells are larger than histamine cells, an additional separation step based on differing velocities, as used in this work, should resolve the two cell types. Thus, it seems unlikely that the cells contaminating the enriched parietal cell fraction contained any cell type in a greater proportion than in the crude cell population and indeed superficial examination of contaminating cells microscopically did not indicate any copurification had occurred.

4.3.2. Yield and Trypan Blue Exclusion.

Approximately one quarter of the parietal cells in the

Table 4.3. A comparison of the purities, yields and viabilities of parietal cell fractions enriched by other methods with those obtained here.

Authors	Animal	Parietal cell content(%)		Parietal cell yield:- no.cells in purified fraction / no.cells in crude fraction	Viability (%parietal cells excluding trypan blue)
		Initial	Final		
Romrell et al.(1975)	Mouse	10-15	75-95	5-8	95
Soll (1978)	Dog	---	85	10-25	--
Glick et al.(1974)	Rabbit	---	60	---	60
Chew & Hersey (1982)	Rabbit	31	70	---	--
Sewing et al.(1983)	Guinea Pig	---	70-80	---	95
Lewin et al.(1974)	Rat	25	80	---	90
Soumarmon et al.(1978)	Rat	25-27	75	---	93
Sonnenberg et al.(1979)	Rat	25	70-90	---	--
Ecknauer et al.(1981)	Rat	20-25	50-90	30-50	85
Thomson et al.(1981)	Rat	36	90	47	75
Schepp et al.(1983a)	Rat	25	65-85	19	95
Present study	Rat	19.9±1.5(15)	77.3±2.3(15)	25.8±1.9(15)	90.3±1.4(15)

crude preparation were recovered after the purification procedure. Such a recovery is in the order of that achieved by Soll (1978); 10 - 25%, Ecknauer et al.(1981); 30 - 50% and Schepp et al. (1983a);19%.

The viability of the purified cell fraction, as assessed by trypan blue exclusion (90.3 $\pm$ 1.4%) was significantly lower than that obtained with the unenriched cell population (Student's t-test, $P<0.001$) suggesting an impairment of membrane integrity caused by certain aspects of the purification procedure. Percoll was chosen as the density gradient medium, amongst other reasons, because it has been shown not to be cytotoxic (Pertoft et al., 1977). However, the possibility of membrane damage by individual Percoll particles cannot be excluded without the use of electron microscopy. The time of exposure of the cells to the Percoll medium was reduced to a minimum, but this entailed using a high centrifugal force (2000g) to achieve separation, and this was another possible cause of membrane damage. However, despite these conditions the overall viability (~90%) was not so low as to invalidate the use of the enriched fractions.

4.3.3. Response to Secretagogues.

As described in 4.2.3, acid secretion by purified cells was only stimulated by secretagogues when a 1h recovery period was interspersed between purification and stimulation. Such a recovery may have been required to allow the resynthesis of membrane proteins which might have been damaged during the purification procedure. Sonnenberg et al. (1979) found that Percoll (6% w/v) increased acid secretion by purified parietal cells by raising intracellular cAMP

levels. This might occur because polyvinylpyrrolidone, present in the Percoll, has a structure somewhat similar to that of histamine (Fig. 4.2). Little evidence was obtained here for a stimulatory effect of Percoll on acid secretion, but it is nevertheless possible that Percoll, in some way, desensitised or 'down-regulated' the histamine receptor. Indeed, both Sonnenberg et al. (1979) and Schepp et al. (1983b) found that centrifugation in Percoll caused a decrease in the susceptibility of the parietal cell adenylate cyclase to stimulation by histamine.

The time course of the aminopyrine response of purified parietal cells to secretagogues (Fig 4.1) may differ from that of a typical unenriched fraction (Fig 3.8) in that the response appears to be sustained for longer with purified parietal cells, but no systematic investigation of this possibility was performed.

In conclusion, the population of enriched parietal cells, produced here, has been shown to be highly viable both in terms of trypan blue exclusion and response to secretagogues. Therefore, further studies on the mechanism of acid secretion (Chapters 7 & 8) should produce results which are valid and able to be integrated with data obtained by other groups.

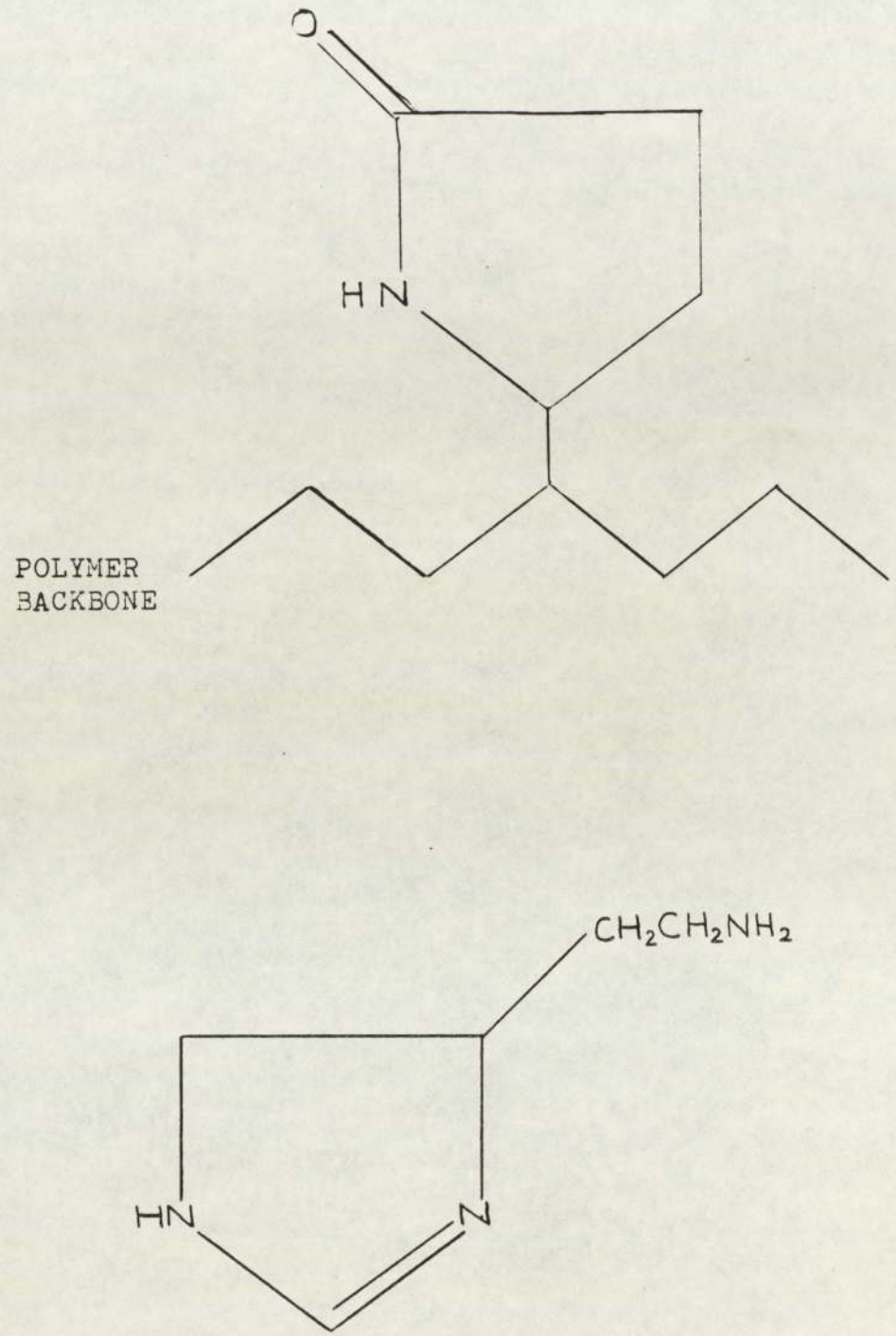


Fig 4.2 Structural similarity between the monomeric units of polyvinylpyrollidone (top) and histamine (bottom).

CHAPTER 5

SUBSTRATE-DEPENDENCY OF ACID SECRETION BY ISOLATED PARIETAL CELLS.

5.1. INTRODUCTION.

5.1.1. The Use of Isolated Parietal Cells to Investigate the Substrate-Dependency of Acid Secretion.

In this section it is the intention to answer the question, "Which substrates are best able to support acid secretion by isolated parietal cells?" To investigate how different types of substrate may contribute to the energy supply of the acid-secreting parietal cell, it was decided to test how effective the substrate was alone, and at a physiological concentration, in supporting acid secretion (aminopyrine accumulation - 3.2.1). It was also thought necessary to test whether the ability of the parietal cells to use different substrates was in any way dependent on the levels of endogenous substrate. Kasbekar (1976) and Hersey (1981) used tissues depleted of endogenous substrate to study the substrate-dependency of acid secretion when the influence of endogenously-derived energy was removed. Finally, supraphysiological concentrations of the substrates will be used to examine the possibility that the inability of any substrate to support acid secretion, when presented at a physiological concentration, was due merely to limited availability.

The choice of substrates to be tested was determined firstly from the results for arteriovenous difference measurements *in vivo* (Chapter 2), which particularly affected the combination of substrates used for the experiments described in Table 5.1 and Figs. 5.1 & 5.2, and secondly, by the results of others (see 5.1.2) on the substrate-dependency of acid secretion *in vitro*. Butyrate, virtually absent from arterial blood and consequently unlikely to be an important substrate

in vivo, was nevertheless tested at a concentration of 0.25mM to provide comparisons with others who have demonstrated the effectiveness of non-physiological concentrations of butyrate in supporting acid secretion in vitro.

From the results, a picture of the metabolic fuels most likely to provide energy for acid secretion could then be proposed, in conjunction with data including studies in vivo, here and from measurements by others of the activities of key enzymes and products of the metabolism of radiolabelled substrates.

5.1.2. Previous Investigations on the Substrate-Dependency of Acid Secretion.

The first observations on the substrate-dependency of acid secretion (Davenport & Chavre; 1951a, 1951b), using the mouse stomach in vitro, showed that increasing concentrations of glucose and acetoacetate were effective in supporting increasing rates of acid secretion.

Since then, a limited amount of work has been performed using amphibian and mammalian tissues. In frog gastric mucosa in vitro, short-and medium-chain fatty acids and ketones stimulate acid secretion whereas the same concentrations (10mM) of carbohydrates and amino acids are ineffective (Alonso et al., 1967); β-oxidation of fatty acids plays a major role in the acid-secretory process (Harris et al., 1975); butyrate (1mM) gave higher rates of acid secretion than glucose (1mM) or pyruvate (1mM) (Hersey, 1977) and

Chacin et al. (1980) found that acid-secreting mucosa utilised octanoate (1mM) more rapidly than glucose (1mM) or pyruvate (1mM). Although supraphysiological concentrations of substrates were used in the above experiments, there appears to be a concensus that fatty acids rather than glucose-derived fuels may be better able to support gastric acid secretion in amphibians.

On the other hand, in the field of the substrate-dependency of mammalian gastric acid secretion, there have been conflicting results. Hersey (1977) showed that rabbit gastric glands exhibited preferential utilisation of butyrate (1mM) over glucose (1mM) or pyruvate (1mM). Rabbit gastric glands, depleted of endogenous substrates, recovered their response to histamine more effectively in the presence of pyruvate (10mM), lactate (10mM), butyrate (10mM), or D-3-hydroxybutyrate (10mM) than in the presence of glucose (10mM)(Hersey, 1981). In piglet gastric mucosa, glucose (10mM), pyruvate (10mM) and acetate (10mM) were more effective than other short-chain fatty acids such as butyrate (10mM) in supporting histamine-stimuated acid secretion (Forte et al., 1980). Glucose, glutamine and isoleucine were metabolised by guinea-pig gastric glands and the rate of oxidation of these substrates increased by 15 - 30% when the glands were stimulated with histamine, (Saladino et al., 1982). By measuring the levels of glycolytic, citric acid cycle and other intermediates in samples of parietal-cell-enriched, freeze-dried sections of non-secreting and secreting dog gastric mucosa, removed *in vivo*, Sarau et al. (1977) proposed that the major energy source for acid secretion was derived from increased citric acid

cycle activity and that glycolysis and fatty acid oxidation increased to provide mitochondrial substrate. A more detailed consideration of the relationship between energy provision and gastric acid secretion has been described (Chapter 1).

The work in this chapter was undertaken, (i) to attempt to resolve the contradictions mentioned above, (ii) because most of the previous investigations had used solely non-physiological concentrations of substrates, (iii) to make the first assessment, in any mammal, as to whether amino acids or long-chain fatty acids could support acid secretion and (iv) because virtually no previous measurements had been made on the substrate-dependency of acid secretion in an animal which, like man, is omnivorous.

5.2. METHODS.

5.2.1. Aminopyrine Accumulation Tests in the Presence of Individual Substrates.

Cells were suspended for the aminopyrine test (3.2.1) in 1.5ml of Krebs-Ringer bicarbonate medium containing 30mg dialysed fatty-acid-free bovine serum albumin/ml (A.2.2) which was added to a siliconised glass scintillation vial, containing small volumes of test agents, and incubated for 30 minutes. Each substrate was prepared as a concentrated stock solution freshly each week, and was added in a volume of 30µl to the incubation vessel, with the exception of oleate which was first complexed with bovine serum albumin (A.2.7).

Stock solutions of substrates were also prepared for the experiments with supraphysiological concentrations of

substrates with the exception of oleate which, because of the high viscosity of the stock solution (0.2M) and consequent difficulty in forming a complex with bovine serum albumin, was omitted. Apportioning a diluted cell suspension and concentrated test agents to the vials, rather than adding a concentrated cell suspension to 1.5ml of incubation medium containing diluted test agents, avoided the problems of cell clumping and the resultant difficulty of pipetting equivalent numbers of cells to each vial.

5.2.2. Depletion of Parietal Cell Endogenous Fuel Stores by Preincubation.

To bring about at least a partial depletion of the endogenous stores of substrate in the parietal cell, isolated cells from two rats were incubated in 20ml of Krebs-Ringer bicarbonate medium, containing 30mg dialysed fatty-acid-free albumin/ml, for 20 minutes at 37^{o}C in a shaking water bath (120 cycles/min.) with continuous gassing (O_2/CO_2;95/5). The cell suspension was then centrifuged at 15^{o}C (100g, 10min.), the pellet was resuspended in an appropriate medium, and the cells were added to vials containing substrates and secretagogues. Thus, the cells were exposed to substrate-free conditions for a total of 30 minutes, after which time continued incubation in substrate-free conditions had a deleterious effect on the cells' subsequent aminopyrine response to secretagogues (see 5.3). In the experiments testing for this effect of increasing the substrate-depletion time (Fig. 5.2), cells were not centrifuged after the preincubation period, but instead

aliquots were transferred immediately to vials containing exogenous substrates.

5.2.3. Measurement of Substrates in Incubation Medium.

In one experiment the amounts of some of the substrates, being tested for their ability to support acid secretion, were measured in the incubation media after exposure of the cells to the substrate and other test agents for 30 minutes. After aliquots of cell suspensions had been centrifuged (Beckman Microfuge) samples of the supernatants were removed and assayed spectrophotometrically or colourimetrically as described in A.3.1-A.3.10.

5.2.4. Analysis and Presentation of Results.

Due to the considerable variation between batches of cells in the aminopyrine accumulation in response to secretagogues (see non-transformed data, Tables 5.2 & 5.5) it was necessary to separate this "batch" variation from that due to the individual treatments (substrates). The data were therefore subjected to a two-way analysis of variance (A6.4). If Tukey's test of additivity (Snedecor & Cochran, 1967) demonstrated a significant contribution of non-additivity to the error sum of squares, then a logarithnic transformation of the data (Snedecor & Cochran, 1967) was performed before the analysis of variance. Comparisons between individual treatments were made using the Newman-Keul's multiple range test (Zar, 1974). Results are presented in two ways: firstly, untransformed (Tables 5.2 & 5.5) to demonstrate the variation between cell batches and secondly, normalised (Tables 5.3 & 5.6). Normalisation of the results from

each experiment was performed by expressing the aminopyrine accumulation ratios seen with each substrate with respect to that seen in the presence of glucose, which was assigned a value of 100. An example of the procedure for statistical analysis of one section of data is now presented. The statistal analyses always refer to the non-transformed data.

(i) Two way analysis of variance of data shown in Table 5.5.

Variation	df	Sum square	mean square	F
Runs	4	3449.6	862.4	5.051
Treatments	10	14883.7	1488.4	8.718
Error	40	6829.1	170.7	
Total	54	25162.4		
Non additivity	1	5082.4	5082.4	113.4*
Remainder	39	1746.7	44.8	

*F = 113.4 (1,39), P<0.01
Tukey's test for additivity significant, therefore conduct ANOVAR on $\log_{10}$ transformed data.

(ii) Analysis on transformed data:-

Variation	df	Sum square	mean square	F
Runs	4	0.6853	0.1713	5.3667 (4,40) **
Treatments	10	5.1198	0.5120	16.0383 (10,40) ****
Error	40	1.2769	0.0319	
Total	54	7.0820		

*** Effect of runs P<0.01
**** Effect of treatments P<0.001

(iii) Newman-Keul's multiple range test (Zar, 1974).

Treatment:	None	Glu	Leu	Acetoacetate	Val
Sample mean:	0.6596	0.784	0.9462	1.0544	1.1528
Rank:	1	2	3	4	5

Treatment:	Acetate	Butyrate	Glucose	D-3-HB	Lactate	Ile
Sample mean:	1.1672	1.1854	1.2886	1.372	1.668	1.6682
Rank:	6	7	8	9	10	11

$$\text{Standard error} = \frac{\text{Error mean square}}{\text{no. of runs}} = 0.0799$$

Example: compare Isoleucine vs. rest.

Comparison	Difference	q*	p	conclusion.
11 v. 1	1.0086	12.623	11	P<0.01
11 v. 2	0.8842	11.066	10	P<0.01
11 v. 3	0.7220	9.0363	9	P<0.01
11 v. 4	0.6138	7.6821	8	P<0.01
11 v. 5	0.5154	6.451	7	P<0.01
11 v. 6	0.5010	6.2703	6	P<0.01
11 v. 7	0.4828	6.0425	5	P<0.01
11 v. 8	0.3796	4.7510	4	P<0.01
11 v. 9	0.2962	3.7070	3	P<0.05
11 v. 10	0.0002	0.0025	2	P>0.05

$$*q = \frac{\text{Difference}}{\text{S.E.}}$$

This value is compared with a table of q values for a given error degrees of freedom (40, here) and p value (number of means in the range of means being tested e.g. Ile vs. Butyrate, p=5)

5.3 RESULTS.

5.3.1 The Effectiveness of Secretagogues and Exogenous Substrates in Stimulating Acid Secretion.

Acid secretion was measured, by aminopyrine accumulation in isolated parietal cells, in the presence and absence of potential exogenous substrates (glucose, 10mM; D-3-hydroxybutyrate, 1mM; glutamine, 1mM) and with a combination of secretagogues (histamine, 0.5mM; IMX, 0.1mM; carbachol, 0.1mM) which probably caused near-maximal aminopyrine accumulation (Schepp et al., 1983a). In normal cells, acid secretion was stimulated above control levels by secretagogues in the absence of exogenous substrates (Table 5.1, compare condition A with condition B). This difference was not due to the absence of substrates from B since aminopyrine ratios similar to condition A (1.720 $\pm$ 0.577 (5)) were found if cells were incubated in the absence of secretagogues and substrates. The addition of exogenous substrates to cells stimulated by secretagogues produced a significant increase above the level of secretion seen in their absence (Table 5.1, compare condition B with condition C). Another set of experiments (Table 5.1) also showed that, in the presence of exogenous substrates and secretagogues, similar aminopyrine ratios were obtained whether or not cells were first preincubated for 30 minutes in the absence of exogenous substrates. However, as might be expected, the aminopyrine ratio obtained in the presence of secretagogues but in the absence of exogenous substrates (Table 5.1, condition B), was lower if preincubation was performed to partially deplete endogenous substrates.

In order to test whether the reduced aminopyrine

Table 5.1. The effect of substrates and secretagogues on aminopyrine accumulation by normal and substrate-depleted cells. Results (means ± S.E.M.) are for 5 batches of normal cells and 4 batches of substrate-depleted cells. The substrates were glucose (10mM), D-3-hydroxybutyrate(1mM), and glutamine (1mM). The secretagogues were histamine (0.5mM), IMX (0.1mM), and carbachol (0.1mM).

*P<0.01, **P<0.001 for comparison of condition B with condition A and ‡‡ P<0.001 for comparison of condition C with condition B (Newman-Kuel's multiple range test after two-way analysis of variance).

Incubation Conditions.	Aminopyrine Accumulation Ratio normal cells	substrate-depleted cells.
A. Substrates only	1.9 ± 0.09	1.5 ± 0.18
B. Secretagogues only	9.1 ± 2.50*	4.8 ± 1.60**
C. Substrates + secretagogues	21.0 ± 2.40‡‡	22.0 ± 9.7‡‡

accumulation in the absence of exogenous substrates was due directly to an insufficient supply of energy for the acid-secretory process or was caused by a general breakdown of cell integrity, experiments were performed in which cells were stimulated to secrete acid, in the presence of exogenous substrates, after being subjected to a range of times of exposure to substrate-free conditions. The presence or absence of secretagogues, during the preincubation period, did not appear to affect the subsequent responses (Fig. 5.1, two-way analysis of variance ; $F = 0.034$, $P>0.05$) and so the data shown in Fig. 5.2 were obtained using cells preincubated in the presence of secretagogues. Preincubation of cells for up to 30 minutes with secretagogues but without substrates appeared to have no deleterious effect on the cells' subsequent ability to secrete acid (Fig. 5.2). Therefore, the effect of substrates (Table 5.1 condition C) seemed to be directly related to the provision of energy for acid secretion, since cells incubated without exogenous substrates for 30 minutes (Table 5.1, condition B) do not suffer any loss of viability as judged by their subsequent aminopyrine response to the addition of substrates.

5.3.2. The Effectiveness of Physiological Concentrations of Substrates in Supporting Acid Secretion by Parietal Cells.

Table 5.3 shows the aminopyrine accumulation ratios, normalised with respect to glucose, obtained when cells were stimulated by secretagogues in the presence of substrates at physiological concentrations (except butyrate, see 5.1.1). In both normal and substrate-depleted cells, all potential substrates produced aminopyrine accumulation ratios which

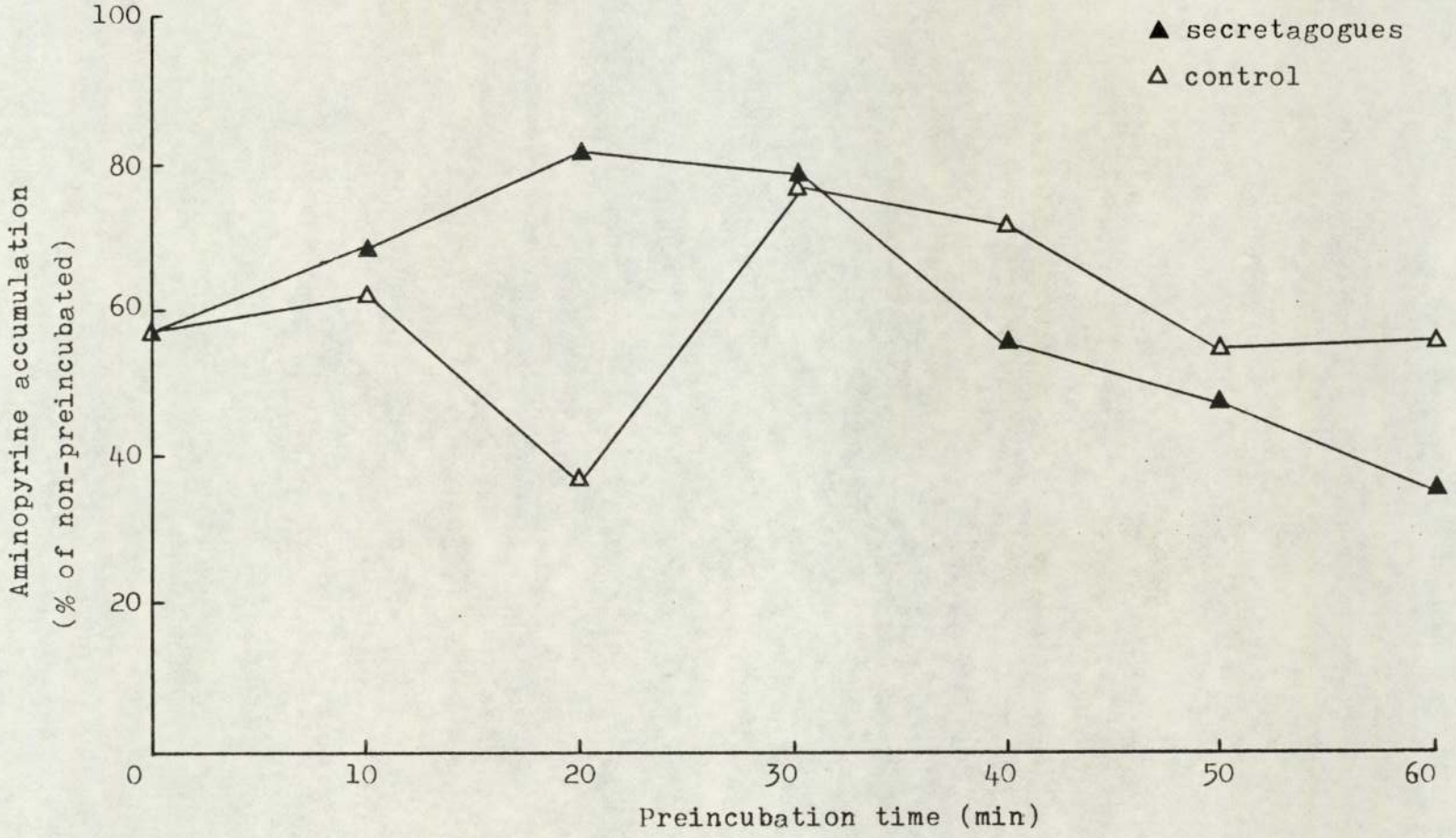


Fig 5.1 The effect of the presence of secretagogues during preincubation in the absence of exogenous substrates on the subsequent accumulation of aminopyrine by isolated parietal cells. Results, from a single experiment, were normalised by expressing the value relative to the aminopyrine accumulation ratio obtained without preincubation which was made 100. The substrates and secretagogues were as in Table 5.1.

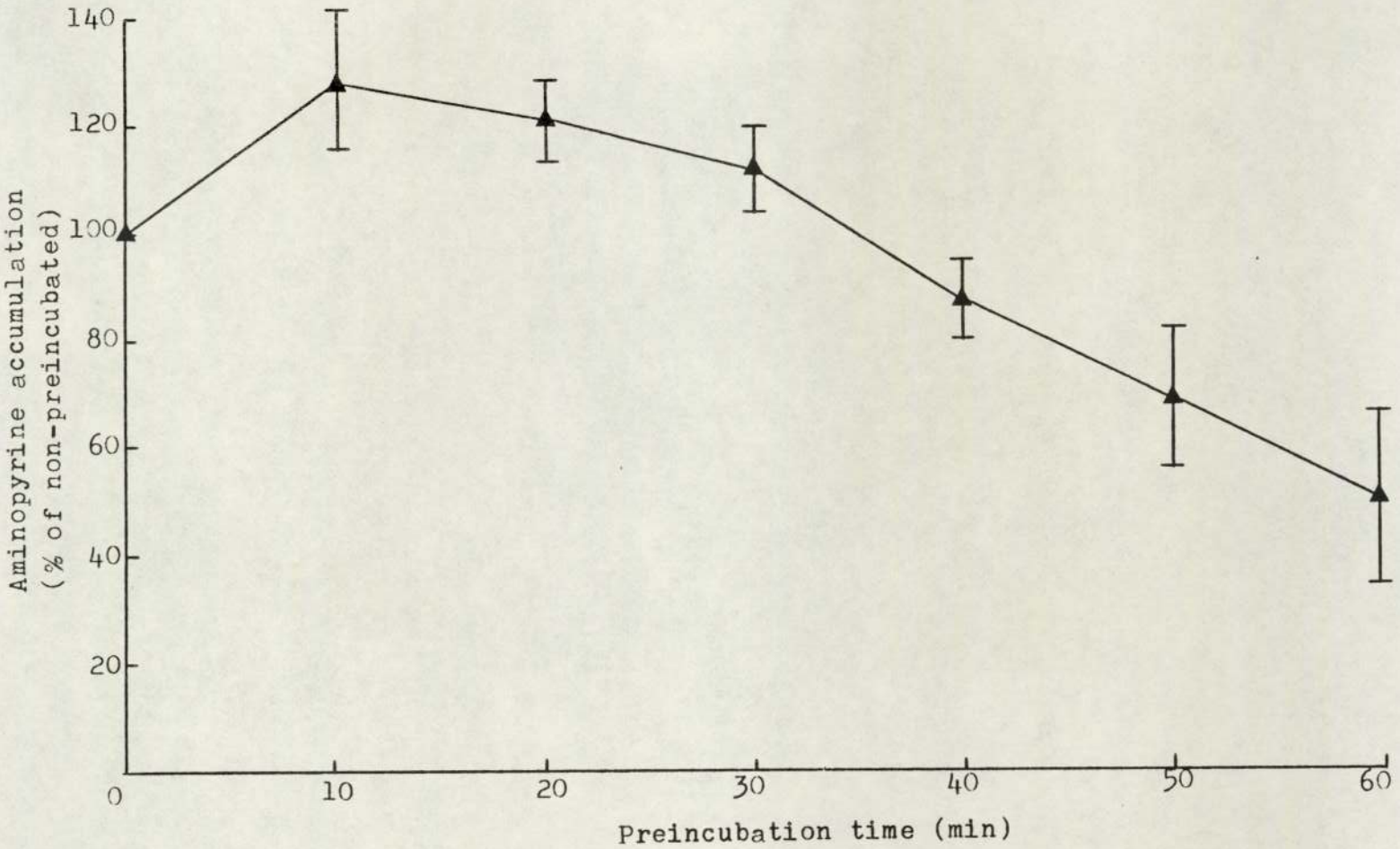


Fig 5.2 The effect of the time of preincubation in the absence of exogenous substrates on the subsequent accumulation of aminopyrine by isolated parietal cells. Bars represent the means±SEM from 4 batches of cells stimulated with secretagogues in the presence of substrates (Table 5.1) for 30 minutes after various preincubation times in the absence of exogenous substrates. The values were normalised by expressing the data for each batch of cells relative to the aminopyrine accumulation ratio obtained without preincubation which was made 100.

Table 5.2. The effect of physiological concentrations of potential substrates on accumulation of aminopyrine by isolated parietal cells (non-transformed data).

A. Normal Cells:- Data are presented as the individual aminopyrine accumulation ratios for each substrate from 5 experiments.

Substrate	Aminopyrine accumulation ratio					
	1	2	3	4	5	mean
Glucose	31.120	19.503	18.643	18.761	27.284	23.062
Oleate	29.122	15.263	16.758	12.633	17.003	18.136
Butyrate	25.574	17.374	13.404	13.428	12.738	16.504
Lactate	28.190	12.374	18.734	10.995	11.329	16.324
D-3-hydroxybutyrate	29.615	13.216	15.685	9.102	10.556	15.635
Isoleucine	26.740	12.318	16.641	7.052	9.707	14.492
Acetoacetate	26.788	9.091	11.119	9.275	8.771	13.009
Valine	18.132	8.420	12.746	7.074	10.751	11.425
Acetate	15.153	8.266	11.105	7.234	8.922	10.136
Leucine	11.904	5.601	10.219	5.610	9.529	8.573
Glutamine	14.963	5.618	7.254	4.718	6.044	7.719
None	17.786	5.974	9.524	5.505	6.821	9.122

Table 5.2 continued.

B. Substrate-depleted cells:- Data are presented as the individual aminopyrine ratios for each substrate from 4 experiments.

Substrate	Aminopyrine Accumulation Ratio				
	1	2	3	4	mean
Glucose	6.984	19.292	10.642	40.974	19.473
Oleate	4.982	14.632	6.601	26.436	13.163
Butyrate	5.896	11.888	5.078	22.013	11.219
Lactate	5.280	10.382	6.662	22.000	11.081
D-3-hydroxybutyrate	5.570	11.603	4.182	15.484	9.255
Isoleucine	4.966	11.206	4.589	16.612	9.343
Acetoacetate	4.243	7.066	3.929	16.438	7.919
Valine	3.790	5.974	4.081	12.472	6.564
Acetate	3.582	6.296	3.030	9.119	5.507
Leucine	3.364	6.069	2.977	10.644	5.764
Glutamine	2.206	5.246	2.299	7.507	4.314
None	2.633	4.622	2.647	9.424	4.832

Table 5.3. The effect of physiological concentrations of potential substrates on accumulation of aminopyrine by isolated parietal cells. Results (means ± SEM) are from 5 batches of normal cells and 4 batches of substrate depleted cells and have been normalised by expressing the data for each batch of cells relative to the aminopyrine accumulation ratio obtained with glucose, which was made 100. The non-transformed data are presented in Table 5.2. The concentrations of secretagogues are shown in Table 5.1.

*P<0.05, **P<0.01 ; significant difference from result with no exogenous substrates. ŧ P<0.05, ŧŧ P<0.01 ; significant difference from result with glucose. (Newman-Keul's multiple range test).

Substrate (mM)	Normalised aminopyrine accumulation ratio	
	normal cells	substrate-depleted cells
Glucose (5)	100*	100**
Oleate (0.6)	78±6**ŧ	68±3**ŧŧ
Butyrate (0.25)	72±7**ŧ	62±8**ŧŧ
Lactate (0.6)	71±11**ŧŧ	61±5**ŧŧ
D-3-hydroxybutyrate	67±10**ŧŧ	55±10**ŧŧ
Isoleucine (0.068)	62±11**ŧŧ	53±7**ŧŧ
Acetoacetate (0.35)	55±9*ŧŧ	43±6**ŧŧ
Valine (0.117)	49±6*ŧŧ	38±5**ŧŧ
Acetate (0.25)	44±5 ŧŧ	34±6 ŧŧ
Leucine (0.095)	37±5 ŧŧ	33±5 ŧŧ
Glutamine (0.5)	33±5 ŧŧ	25±3 ŧŧ
None	39±6 ŧŧ	27±3 ŧŧ

were significantly lower than those observed with glucose (Newman-Keul's test; $P<0.05$). In addition, all potential substrates, with the exceptions of acetate, leucine and glutamine, significantly increased accumulation of aminopyrine above the values obtained in the absence of substrate in normal and substrate-depleted cells (Newman Keul's test; $P<0.05$). Finally, the apparent order of effectiveness of the substrates was the same for normal or substrate-depleted cells, and depletion of endogenous substrates did not reveal any more substances as substrates than the experiments with normal cells.

Measurement of the amounts of some of the substrates in the medium after incubation (Table 5.4) showed that aminopyrine accumulation was unlikely to be limited by exhaustion of the particular substrate being tested.

5.3.3. The Effectiveness of Supraphysiological Concentrations of Substrates in Supporting Acid Secretion by Parietal Cells.

To test whether the inability of substrates to support aminopyrine accumulation at a level similar to that supported by glucose, was due to a limitation on substrate availability caused by insufficient extracellular concentration, the experiments were repeated with the concentration of substrates (glucose excepted) raised above normal physiological levels. Table 5.6 shows that only lactate and isoleucine, when present at 5mM, support levels of aminopyrine accumulation significantly higher than those produced with glucose (Newman-Keul's test, $P<0.05$). All other substrates were equally as effective as glucose in supporting aminopyrine accumulation, except leucine

Table 5.4. The utilisation of potential substrates by acid secreting parietal cells. Data represent the change in concentration of the substrate or metabolite after 30 minutes incubation. Negative values indicate net removal and positive values net output of the substrate.

Substrate	Concentration		%change
	Initial	Final	
Glucose	5.00	4.9408	-1.2
Lactate	0.60	0.5608	-6.5
Oleate	0.60	0.6911	+15.2
D-3-hydroxybutyrate	0.55	0.5615	+2.1
Acetoacetate	0.35	0.3416	-2.4
Glutamine	0.50	0.4220	-15.6

Table 5.5. The effect of supraphysiological concentrations of some potential substrates on accumulation of aminopyrine by isolated parietal cells (non-transformed data).

Data are presented as the individual aminopyrine ratios for each substrate from 5 experiments.

Substrate	Aminopyrine Accumulation Ratio.					
	1	2	3	4	5	mean
Glucose	8.406	16.575	12.292	24.995	65.029	25.459
Lactate	39.505	49.080	16.180	71.014	98.135	54.783
Isoleucine	35.530	57.000	20.746	52.267	99.808	53.070
D-3-hydroxybutyrate	26.273	19.890	14.439	35.945	26.701	24.650
Butyrate	18.940	15.490	11.282	15.702	16.302	15.543
Acetate	20.380	14.260	12.671	8.111	22.988	15.682
Acetoacetate	18.180	6.640	10.729	12.085	11.989	11.835
Valine	9.544	16.250	9.025	19.677	21.084	15.116
Leucine	7.349	11.890	5.060	10.231	11.906	9.287
Glutamine	4.679	5.570	4.280	10.151	7.357	6.407
None	2.873	5.000	6.450	8.904	2.415	5.128

Table 5.6. The effect of supraphysiological concentrations of some potential substrates on accumulation of aminopyrine by isolated parietal cells. Results (means ± SEM) are for 5 batches of cells and have been normalised as described in Table 5.3. The non-transformed data are presented in Table 5.5 and the concentrations of secretagogues shown in Table 5.1.

*P<0.05, **P<0.01 ; significant difference from result with no exogenous substrate. ‡ P<0.05, ‡‡ P<0.01 ; significant difference from result with glucose (Newman-Keul's multiple range test after two way-analysis of variance).

Substrate(mM)	Normalised Aminopyrine Accumulation Ratio
Glucose (5)	100*
Lactate (5)	266 ± 61**‡‡
Isoleucine (5)	260 ± 53**‡‡
D-3-hydroxybutyrate (2)	147 ± 45**
Butyrate (5)	100 ± 34**
Acetate (5)	100 ± 38**
Acetoacetate (2)	82 ± 35**
Valine (5)	79 ± 14**
Leucine (5)	52 ± 12*‡
Glutamine (5)	35 ± 7 ‡‡
None	31 ± 8 ‡‡

Table 5.7. Differential effects of potential substrates, presented at supraphysiological concentrations, on the accumulation of aminopyrine by isolated parietal cells. Results are presented as the differences among individual treatments (** $P<0.01$, * $P<0.05$, NS $P>0.05$).
The aminopyrine ratios from each experiment (Table 5.5) underwent $\log_{10}$ transformation (Tukey's test for additivity significant, $P<0.01$) and were then subjected to analysis by a Newman-Keul's multiple range test.

	Isoleucine	Lactate	D-3-hydroxybutyrate	Glucose	Butyrate	Acetate	Valine	Acetoacetate	Leucine	Glutamine	None
Isoleucine		NS	*	**	**	**	**	**	**	**	**
Lactate	NS		*	**	**	**	**	**	**	**	**
D-3-hydroxybutyrate	*	*		NS	NS	NS	NS	NS	*	**	**
Glucose	**	**	NS		NS	NS	NS	NS	*	**	**
Butyrate	**	**	NS	NS		NS	NS	NS	NS	*	**
Acetate	**	**	NS	NS	NS		NS	NS	NS	*	**
Valine	**	**	NS	NS	NS	NS		NS	NS	*	**
Acetoacetate	**	**	NS	NS	NS	NS	NS		NS	NS	**
Leucine	**	**	*	*	NS	NS	NS	NS		NS	*
Glutamine	**	**	**	**	*	*	*	NS	NS		NS
None	**	**	**	**	**	**	**	**	*	NS	

and glutamine. Only glutamine was not able to produce a level of aminopyrine accumulation significantly higher than that observed in the absence of exogenous substrate. Other differences between substrates, presented in Table 5.7, may be summarised as follows; (i) isoleucine and lactate were better than all other substrates and the no-substrate condition, (ii) valine, acetate, butyrate, glucose and D-3-hydroxybutyrate were all better than glutamine and the no-substrate condition and (iii) glucose and D-3-hydroxybutyrate were better than leucine.

5.4. PRELIMINARY DISCUSSION.

A full discussion of the results in this chapter together with those from Chapter 2 will be given in Chapter 6, where factors influencing the metabolism of individual substrates will be considered in detail.

5.4.1. Limitations on the Ability of Some Potential Substrates to Support Acid Secretion.

In this chapter, the assumption has been made that aminopyrine accumulation ratios accurately reflect acid secretion and that the effect of substrates on aminopyrine ratios is caused by their ability to provide energy for acid secretion. Although not unreasonable (see 3.1.2.4), these assumptions should nevertheless be borne in mind.

There are several reasons why some substrates should be more effective in supporting acid secretion than others. Exhaustion of the substrate supply could lead to an attenuated response. However, the results presented in Table 5.4 indicated that exhaustion of oleate, lactate, acetoacetate, D-3-hydroxybutyrate and glutamine was not

the reason for the inability of these substrates, when presented at physiological concentrations, to match the effectiveness of glucose. Transport of substrates into the parietal cell may control their availability for energy provision. Some substrates, such as amino acids and glucose require a carrier-mediated transport mechanism, whereas others enter the cell by diffusion. Thus, limitation of the entry of some substrates could be due to an intrinsically low activity transport system (low V_{max}) or alternatively, a transport system with a $K_{0.5}$ (substrate concentration giving half-maximal rate) well above the physiological substrate concentration, or a combination of the two. Metabolism of substrates may lead to products which are cytotoxic at high concentrations. Utilisation of glutamine leads to NH_3 production, which is toxic at high concentrations, and calculations based on the utilisation of glutamine, in one experiment, showed that the maximum concentration of NH_3 would have been 0.19mM, assuming all NH_3 from deamination of glutamine was released into the medium. As this is higher than normal plasma levels (~0.04mM) ammonia toxicity might have been a factor in the limited ability of glutamine to support acid secretion.

Finally, if access of substrate to the cell does not limit its metabolism, the pathway metabolising the substrate might be inhibited or of intrinsically low activity. Because of these various possibilities, no attempt has been made to make a detailed comparison of the ability of substrates, at physiological concentrations, to support acid secretion.

5.4.2. Comparison with Previous Studies on the Substrate-Dependency of Acid Secretion.

Overall, the results obtained in this chapter, show that although glucose was the most effective supporter of acid secretion when substrates were presented at physiological concentrations, it clearly did not support maximal rates of acid secretion alone. Lactate and isoleucine, at supraphysiological concentrations, were more effective than 5mM-glucose, a maximally effective concentration (G. P. Shaw, personal communication).

Also, there is no evidence that the ability of glucose to support acid secretion was increased by 100nM-insulin (G. P. Shaw, personal communication). Hersey (1981) showed supraphysiological concentrations of lactate to be more effective than glucose in supporting acid secretion by rabbit gastric glands, but butyrate was also found to be better than glucose, in contrast to the results here, where butyrate, is no better than a physiological concentration of glucose. Forte et al. (1980) produced data which suggested glucose was a better substrate than lipid-derived fuels although those particular studies were performed with whole gastric mucosa, where permeability of the tissue may have been limiting. In addition, there is the possibility that differences exist among species and that substrate-dependency is related to developmental status. Thus, it may not be valid to make comparisons among the mature rat (omnivorous), the mature rabbit (herbivorous) and the new-born piglet (omnivorous). However, the data presented here, may to some extent, resolve the controversy arising from the work of Hersey (1981) and Forte et al. (1980) in that, at physiological

concentrations, glucose is the better substrate, not lipid-derived fuels. The data also show, for the first time, that amino acids and a long-chain fatty acid can provide energy to support acid secretion. Although Saladino et al. (1982) have demonstrated, with guinea pig gastric glands, that metabolism of glucose, glutamine and isoleucine was stimulated by activators of acid secretion, the possibility exists that these substrates were not being utilised by parietal cells, since no unequivocal index of the acid-secretory response, such as aminopyrine accumulation, was used. However, the results with glucose and isoleucine would concur with these studies if these substrates were indeed being used by guinea pig parietal cells.

Although there is always the possibility that some aspect of the isolation procedure has had a deleterious effect on the parietal cells' ability to maximally metabolise certain substrates, a physiological concentration of glucose cannot apparently support maximal acid secretion, and the question; "How does the parietal cell derive sufficient energy to support maximal rates of acid secretion <u>in vivo</u>?", therefore requires consideration. It seems likely that maximal amounts of energy must be derived from the metabolism of other substrates in addition to glucose. Alternatively, metabolism of substrates other than glucose may provide intermediates which enable glucose metabolism to be increased to levels from which sufficient energy could be derived. Experiments investigating the effects of substrates in combination with glucose in supporting acid secretion by isolated parietal cells may provide answers

to these questions. Indeed, preliminary results (G. P. Shaw, personal communication) suggest that aminopyrine accumulation by stimulated parietal cells incubated with glucose (5mM), D-3-hydroxybutyrate (0.55mM), lactate (0.6mM) and isoleucine (0.07mM) was higher than when only glucose was present.

5.5. SUMMARY.

1. Parietal cells possess stores of endogenous substrate which can be utilised to provide energy for acid secretion. Incubation without exogenous substrates for 30 minutes does not impair the viability of isolated parietal cells.

2. Addition of exogenous substrates to parietal cells in the presence of secretagogues stimulates acid secretion. This effect of substrates seems to be directly related to the provision of energy for acid secretion.

3. The following can support acid secretion when added alone and at physiological concentrations to normal or substrate-depleted cells:- glucose, oleate, lactate D-3-hydroxybutyrate, isoleucine, acetoacetate and valine.

4. At higher, non physiological concentrations, acetate, butyrate, and leucine can also support acid secretion. Glutamine cannot support acid secretion at either concentration.

5. At physiological concentrations, glucose is the best substrate but high non-physiological concentrations of isoleucine or lactate support significantly higher

rates of acid secretion than the maximally-effective concentration of glucose.

6. It was concluded that the most likely reason for the inability of glucose to support maximal rates of acid secretion, was that, *in vivo*, the parietal cell derives energy from more than one substrate. Because of this is should be possible to increase aminopyrine accumulation by parietal cells above that obtained with glucose alone, if glucose was present in combination with other apparently effective substrates, such as isoleucine and lactate.

CHAPTER 6

SUBSTRATE DEPENDENCY OF ACID SECRETION - DISCUSSION.

CHAPTER 6. SUBSTRATE DEPENDENCY OF ACID SECRETION - DISCUSSION.

In this section the results obtained and the conclusions drawn from the studies in Chapters 2 and 5 will be considered together with a more detailed description of the degradative pathways of the most likely substrates to provide energy for acid secretion. From this, possible reasons will be proposed to account for the inability of some substrates to support the acid-secretory rates observed with other substrates. A description of the factors which limit utilisation of certain substrates has been presented (5.4.1). Thus, utilisation may be limited by the availability of the substrate or alternatively, the enzymes required for oxidation of the substrate may be inhibited or of intrinsically low activity in the parietal cell. Finally, conclusions will be drawn as to the most likely substrates providing energy for acid secretion by parietal cells *in vivo*.

6.1 METABOLISM OF GLUCOSE.

Both sections of work here on arteriovenous difference measurements *in vivo* and isolated parietal cells *in vitro* strongly suggest that glucose is not only able to provide energy for acid secretion *in vitro*, but probably does so *in vivo*. Data, supporting this have been derived from the measurements of the activity of hexokinase (EC 2.7.1.1) necessary for glucose oxidation, which is present with an activity in the gastric mucosa similar to other regions of the rat gastrointestinal tract (Hanson & Carrington, 1981), although its activity in parietal cells remains

to be established. Glucose has the potential to provide comparatively high quantities of energy from its metabolism (via glycolysis) through the TCA cycle. However, it appears, at least *in vitro*, that the flux through this degradative pathway cannot be increased sufficiently to provide the amount of energy, in the form of ATP, required by the maximally-secreting parietal cell (see 5.4.2). Metabolism of glucose could be limited at three sites, (i) transport into the cell, (ii) phosphofructokinase (EC 2.7.1.11) and (iii) pyruvate dehydrogenase (EC 1.2.4.1, EC2.3.1.12, EC 1.6.4.3).

With isolated rat parietal cells, increasing the concentration of glucose above 2mM produces no further change in the rate of acid secretion (G. P. Shaw, personal communication). Thus, it is unlikely that the $K_{0.5}$ of the glucose transport system is sufficiently high for glucose transport, if rate limiting, to be affected by changes in glucose concentration within the physiological range. However, the glucose transport system may have a limited number of binding sites for glucose (low V_{max}) thereby effectively limiting the rate of oxidation of glucose at the point of entry into the cell.

Regulation of non-equilibrium enzymes of the glycolytic pathway, especially phosphofructokinase, by allosteric inhibitors would normally be considered as further points of control. However, the main allosteric inhibitors of phosphofructokinase are ATP and citrate. In the acid-secreting parietal cell, ATP is being hydrolysed by the $H^+ + K^+$ ATPase and therefore should not accumulate. Elevation of citrate from oxidation of endogenous fatty

acids might also cause inhibition of phosphofructokinase, but since the levels of endogenous fat are clearly low (in the absence of exogenous substrate, the acid-secretory response to secretagogues was low; Table 5.1), it seems unlikely that a substantial inhibition of glycolytic activity occurs in this way. Also, unlikely, for the same reason, is inhibition of pyruvate dehydrogenase by acetyl-CoA derived from metabolism of endogenous fat stores.

An intrinsically low activity of the glycolytic pathway in parietal cells would also limit glucose metabolism and this possibility is supported by the fact that acid secretion is abolished by anoxia (Hersey, 1974). Even moderate activity of the glycolytic pathway should generate enough ATP to support an appreciable acid-secretory rate.

6.2. METABOLISM OF LACTATE.

The parietal cell appears able to derive sufficient energy from oxidation of lactate, to drive high rates of acid secretion since an exogenous lactate concentration of 5mM supports an acid-secretory rate approximately 2.6-fold higher than that seen with the maximally effective concentration of glucose (Chapter 5). However, it is questionable how much lactate contributes to the provision of energy to the parietal cell, for *in vitro*, a physiological concentration of lactate (0.6mM) was less effective than glucose, and *in vivo*, arteriovenous difference measurements demonstrated a net uptake of lactate only when the concentration in arterial blood was greater than 1.3mM. Nevertheless, concentrations of lactate in this range occur during

exercise (Newsholme & Leech, 1983) and at resting physiological concentrations, it is possible that lactate uptake by parietal cells *in vivo* is masked by an output of lactate by other cells in the stomach wall.

Lactate probably moves down a concentration gradient into the cell where it is converted to pyruvate by lactate dehydrogenase (EC 1.1.1.27). Conversion of pyruvate to acetylCoA by pyruvate dehydrogenase is a flux-generating step (a reaction in a metabolic pathway which limits the flux through the pathway) and so lactate oxidation is subject to regulation. A possible reason why high concentrations of lactate are better able to support acid secretion than glucose is that activation of pyruvate dehydrogenase by increased pyruvate levels (pyruvate inhibits the enzyme pyruvate dehydrogenase kinase, which converts pyruvate dehydrogenase to its active, phosphorylated form). Alternatively, as mentioned above, glucose oxidation could be effectively limited in the glycolytic pathway before formation of pyruvate.

6.3. METABOLISM OF FATTY ACIDS.

As the concentrations of short-chain fatty acids, such as acetate, propionate and butyrate are low in the blood, it is unlikely that they are an important source of energy for acid secretion *in vivo*, at least in mammals. However, the parietal cell clearly has the capacity to derive enough energy from acetate and butyrate to support acid secretion above resting levels *in vitro*, if the external concentration is not limiting. At supraphysiological concentrations their presence produces aminopyrine ratios not different from the maximally effective glucose

concentration. Oxidation of fatty acids requires their prior activation in the cell to the -CoA derivative. The enzymes involved are acetyl-CoA synthetase (EC 6.2.1.1) for acetate, and medium-chain acyl-CoA synthetase (EC 6.2.1.2) for butyrate. Since at low concentrations, butyrate is a substrate for acid secretion but acetate is not (Table 5.1), it is possible that only medium-chain acyl-CoA synthetase is present in parietal cells. If this is the case, then acetate may become a substrate for this enzyme if it is presented at high concentration (5mM).

The short-chain fatty acids are, however, less effective that 5mM-lactate in supporting acid secretion and therefore either their rates of conversion to acetyl-CoA must be restricted or, alternatively, the rate at which acetyl-CoA is metabolised in the TCA cycle is limited, when fatty acid substrates are presented alone, by the availability of oxaloacetate, (see Fig. 6.8).

The energy-providing potential of long-chain fatty acids such as oleate is, on a molar basis, several-fold higher than from a typical carbohydrate source such as glucose, Complete oxidation of the 18-carbon fatty acid oleate, provides 9 molecules of acetyl-CoA and consequently far greater formation of ATP than from glucose. However, <u>in vivo</u>, the concentration of glucose in the blood (5mM) is greater than non-esterified fatty acids (0.6mM), most of which are bound to albumin, and when physiological concentrations of the two substrates are presented to isolated parietal cells, glucose supports a higher level of acid secretion. Thus, the presumably very low concentration of unbound non-esterified fatty acids may limit their ability to act as a substrate.

Alternatively, oxidation of long-chain fatty acids could be controlled at the carnitine palmitoyltransferase (EC 2.3.1.21) site, but since oleate (0.6mM) is as effective a supporter of acid secretion as butyrate (0.25mM) whose metabolism by-passes this reaction, regulation at this point seems unlikely.

Arteriovenous difference measurements provided no conclusive information as to whether fatty acids are important in providing metabolic energy to acid-secreting parietal cells, *in vivo*, but it is clear, from the work on isolated cells, that enough energy could be derived from fatty acids to support levels of acid secretion above the resting rate.

6.4 METABOLISM OF KETONE BODIES.

Arteriovenous difference measurements for ketone bodies (Chapter 2) suggested that D-3-hydroxybutyrate was an important substrate for the provision of energy for acid secretion. With isolated parietal cells (Chapter 5), both ketone bodies were as effective as glucose when present at 2mM but less effective than glucose when present at the physiological concentrations seen after an overnight fast: acetoacetate (0.35mM) and D-3-hydroxybutyrate (0.55mM). This suggests that the availability of ketone bodies to the parietal cell is dependent on their concentration in the medium. Thus, entry of ketone bodies into the cell presumably occurs via a passive mediated transport system, which may limit the availability of ketone bodies for oxidation. The maximally effective concentration of D-3-hydroxybutyrate in stimulating acid secretion in

isolated parietal cells is 2-3mM and the physiological concentration after an overnight fast (0.55mM) produces only ~30% of the maximum rate (G.P. Shaw, personal communication; Fig 6.1). This concurs with the results achieved, here *in vivo* where, at least under acid-secreting conditions, the uptake of D-3-hydroxybutyrate by the stomach wall was related to its concentration in the arterial blood (see Fig. 2.10).

The activities of enzymes necessary for the oxidation of ketone bodies, 3-oxoacid CoA-transferase (EC 2.8.3.5) and D-3-hydroxybutyrate dehydrogenase (EC 1.1.1.30), have been measured in the gastric mucosa of the rat and found to be high compared with other tissues (Hanson & Carrington, 1981; MacGill et al., 1984). The two ketone bodies, at high concentrations, were not as effective as supraphysiological concentrations of either isoleucine or lactate in supporting acid secretion, and their metabolism may be regulated at the point of conversion to acetyl-CoA or the oxidation of acetyl-CoA via the TCA cycle may be limited by the availability of oxaloacetate.

The physiological importance of ketone bodies as providers of energy for acid secretion is probably partly dependent on nutritional status. Thus, during starvation, even for only short periods, the importance of maintaining blood glucose levels probably leads to increased utilisation of ketone bodies. Concentrations of ketone bodies in the blood are raised during starvation and the amount of energy obtainable through their oxidation is probably sufficient to maintain quite high rates of acid secretion. This is not to say, however, that ketone

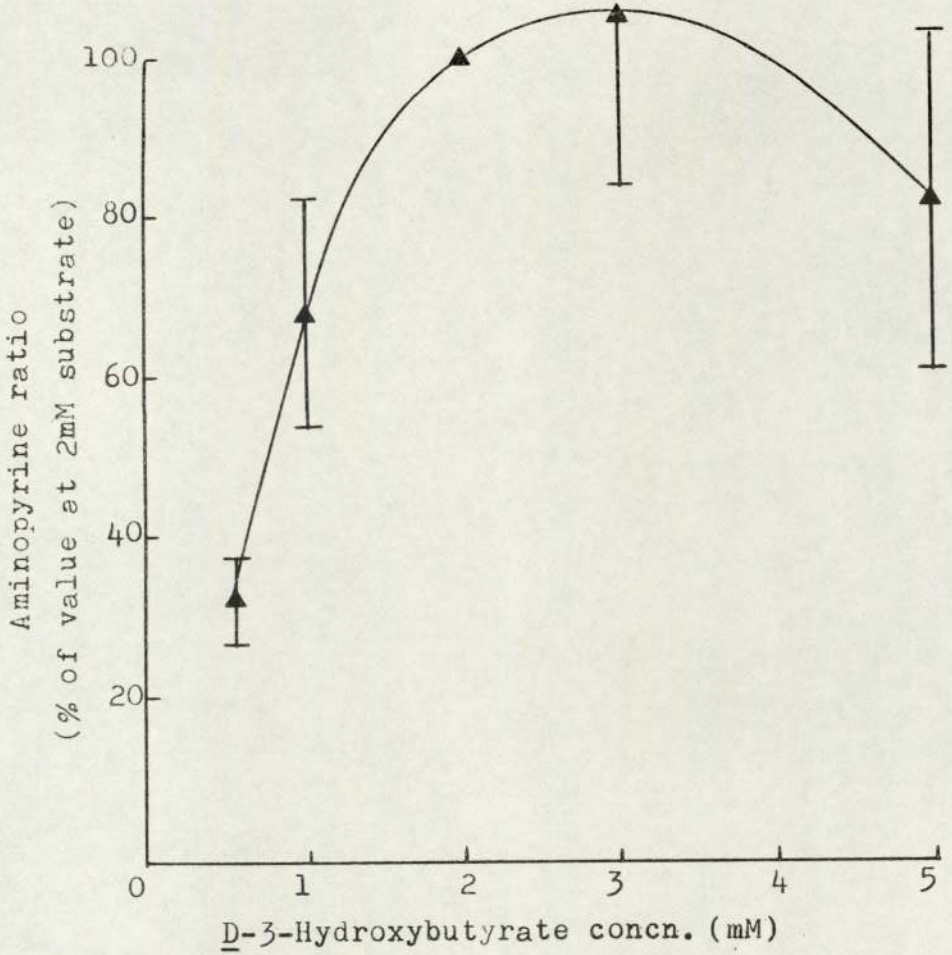


Fig 6.1 The relationship between the concentration of D-3-hydroxybutyrate and the aminopyrine accumulation by parietal cells. Results (means±SEM) from four experiments, were normalised with respect to the aminopyrine ratio obtained with 2mM-D-3-hydroxybutyrate which was made 100.

(work of G.P.Shaw)

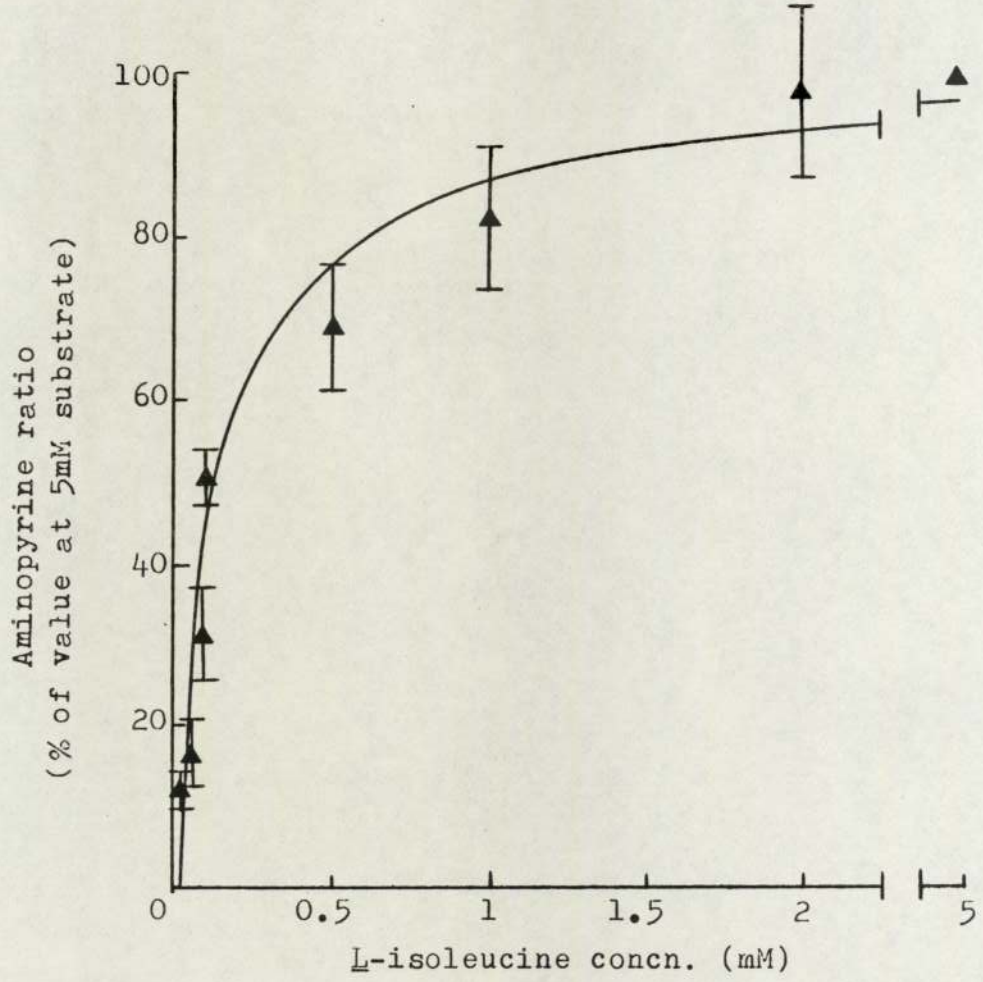


Fig 6.2 The relationship between the concentration of L-isoleucine and the aminopyrine accumulation by parietal cells. Data(means±SEM) from four experiments, were normalised with respect to the aminopyrine ratio obtained with 5mM-L-isoleucine which was made 100.

(work of G.P.Shaw)

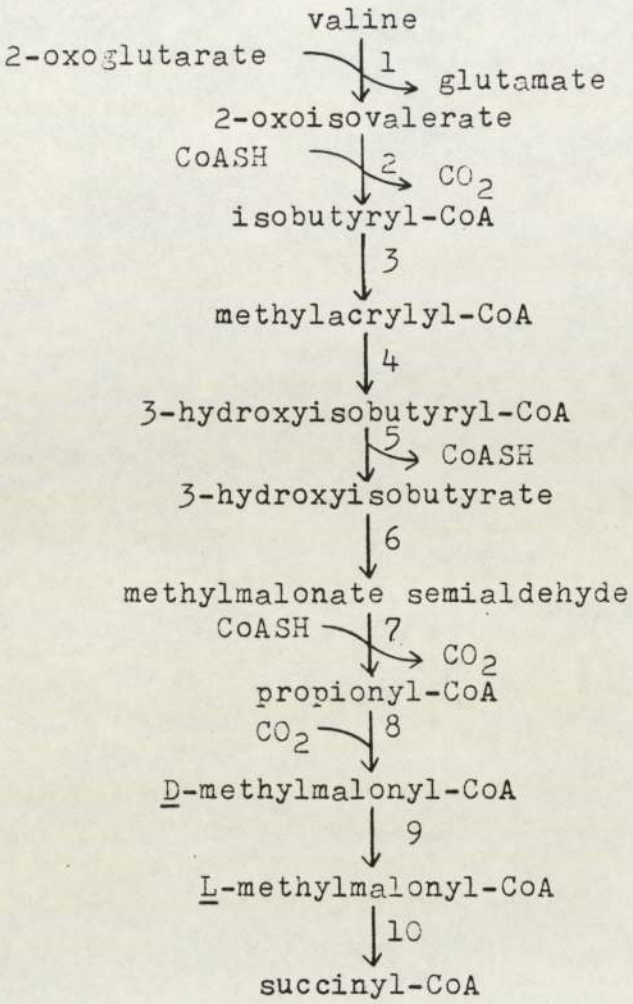
bodies are only important as a metabolic fuel during starvation. They may well, as the results in Chapter 2 suggest, contribute significantly to the gross energy demand of a maximally acid-secreting stomach, after an overnight fast.

6.5. METABOLISM OF BRANCHED-CHAIN AMINO ACIDS.

The finding that branched-chain amino acids were removed from the blood by the stomach wall *in vivo* suggested that they had a possible role as substrates for acid secretion (Chapter 2) and further supporting evidence was obtained with isolated parietal cells (Chapter 5). However, it seems that the three amino acids are not equally effective in supporting acid secretion. Thus, leucine is not a substrate at physiological concentration (0.095mM), and indeed supports acid-secretory rates only slightly higher than in the absence of exogenous substrates, when presented at a supraphysiological concentration (5mM). Valine and isoleucine are both substrates at physiological concentrations and whereas valine can equal the effectiveness of the maximally effective glucose concentration if presented at a high concentration (5mM), metabolism of isoleucine appears able to provide enough energy to support a rate of acid secretion significantly higher than that seen with glucose, if its concentration in the medium is raised above physiological levels. Indeed, isoleucine supports acid secretion by isolated parietal cells at a rate which is related to its concentration in the medium (G. P. Shaw, Personal communication; Fig. 6.2).

Why, then, are there apparent differences among the abilities of branched-chain amino acids to act as metabolic substrates for the provision of energy for acid secretion? The first site at which differences may arise is at the level of transport into the cell. There are several different carrier proteins for different classes of amino acid, but it seems that branched-chain amino acids are all transported by the same carrier, System L (Guidotti et al., 1978). It may be that the kinetics of the transporter system are different for the three amino acids so that the system has a much higher affinity and possibly capacity for isoleucine than for leucine. Hence, very little leucine is taken up at physiological concentrations and therefore it is unable to act as a substrate. The abilities of isoleucine and valine to act as substrates at physiological concentration appear to be limited by their availability.

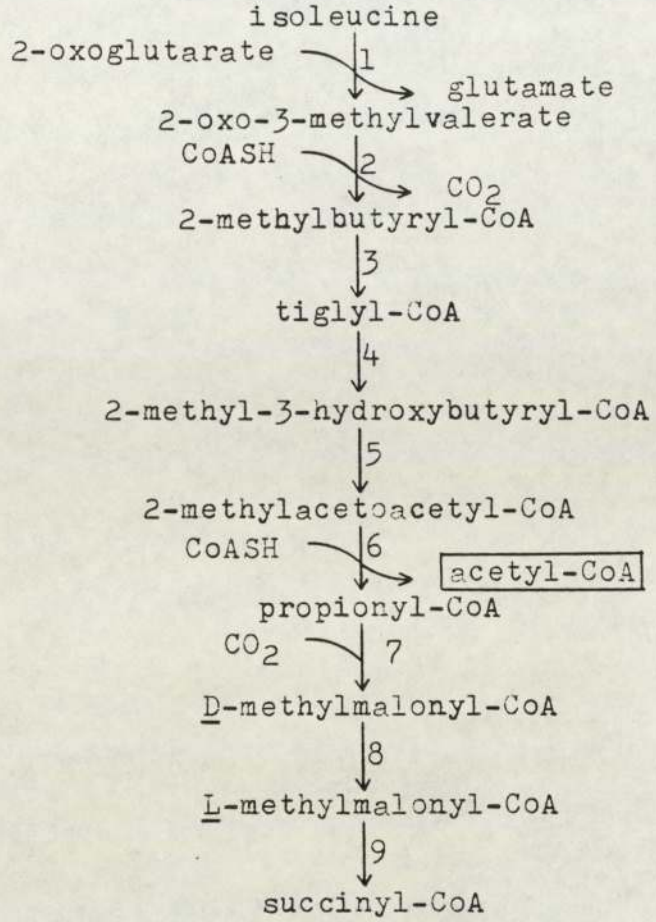
Branched-chain amino acids are metabolised by different pathways. It is interesting to note that metabolism of valine and isoleucine (Figs. 6.3 and 6.4) produce succinyl-CoA, whereas metabolism of leucine produces acetoacetate (Fig. 6.5). Therefore, the site of entry of products of valine and isoleucine metabolism into the TCA cycle is via succinyl-CoA whereas leucine provides acetyl-CoA from oxidation of acetoacetate (Fig. 6.6). Isoleucine appears to be a more effective substrate than valine and this could result from several of the enzymes of the pathway of isoleucine oxidation being common with the pathway of β-oxidation of fatty acids, whereas the equivalent enzymes of the valine degradative pathway could be less



1 valine aminotransferase (EC 2.6.1.32)
2 2-oxoisovalerate 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 3-hydroxyisobutyryl-CoA hydrolase (EC 3.2.1.4)
6 3-hydroxyisobutyrate dehydrogenase (EC 1.1.1.31)
7 methylmalonate 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 involved in the β-oxidation of fatty acids

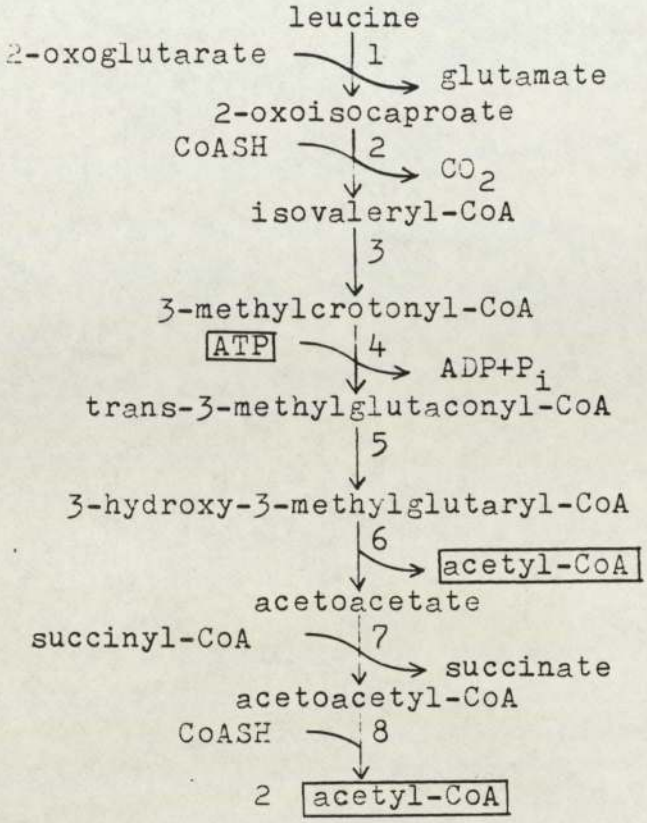
Fig 6.3 Potential pathway for the metabolism of valine by the rat stomach.



1 branched-chain amino acid aminotransferase (EC 2.6.1.42)
2 2-oxoisovalerate 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 3-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 involved in the β-oxidation of fatty acids

Fig 6.4 Potential pathway for the metabolism of isoleucine by the rat stomach.



1 leucine aminotransferase (EC 2.6.1.6)

2 2-oxoisovalerate 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)

7 3-oxoacid-CoA transferase (EC 2.8.3.5)

8 acetyl-CoA acetyltransferase (EC 2.3.1.9)

* enzymes involved in the oxidation of fatty acids

Fig 6.5 Potential pathway for the metabolism of leucine by the rat stomach.

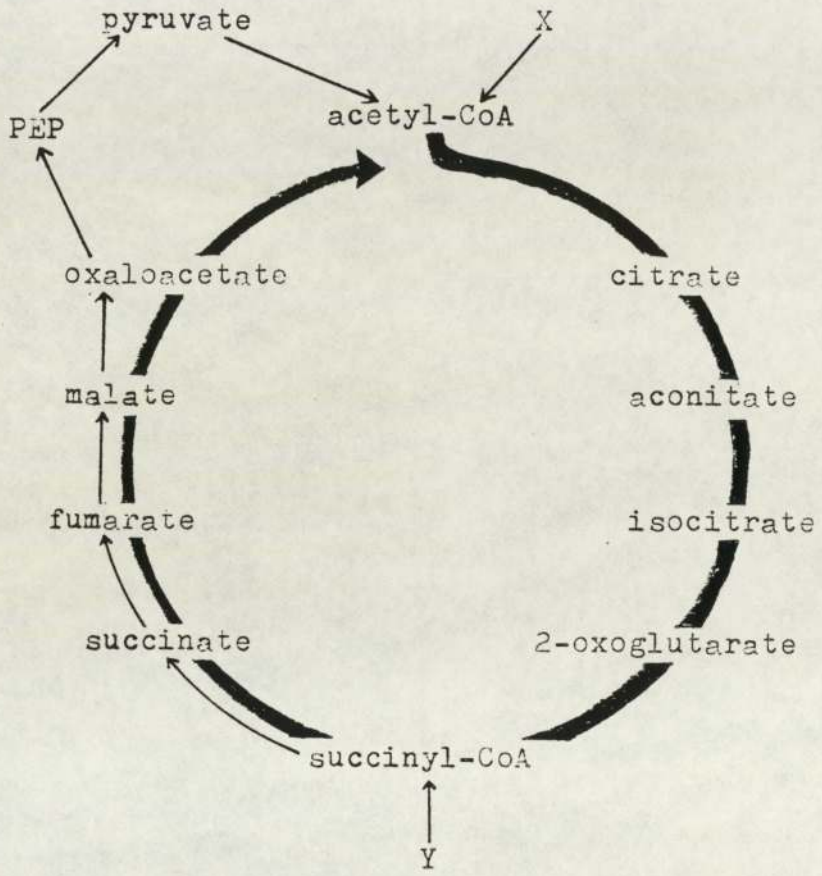


Fig 6.6 Oxidation of branched-chain amino acids by the tricarboxylic acid cycle.

X point of entry of products of leucine and isoleucine metabolism

Y point of entry of products of isoleucine and valine metabolism

small arrows represent the pathway for conversion of succinyl-CoA to acetyl-CoA

active. For an alternative explanation, see section 6.7.

The reasons why leucine, even at high concentration, is a relatively poor substrate for acid secretion must lie within a low activity of its degradative pathway as far as acetoacetate, or saturation of the transport system providing its entry into the cell.

Although there are apparent differences in the effectiveness of the three branched-chain amino acids in supporting acid secretion by isolated parietal cells, arteriovenous difference measurements indicated that all three were taken up equally by the stomach wall. The 2-oxoacids produced by transamination of valine and leucine may be released into the blood *in vivo* as occurs in muscle (Livesy & Lund, 1980) to a greater extent than the 2-oxoacid derived from isoleucine.

Overall, isoleucine and valine probably contribute some energy to the maximally acid-secreting parietal cell, *in vivo*, but it is questionable whether leucine has a similar role.

6.6. METABOLISM OF GLUTAMINE

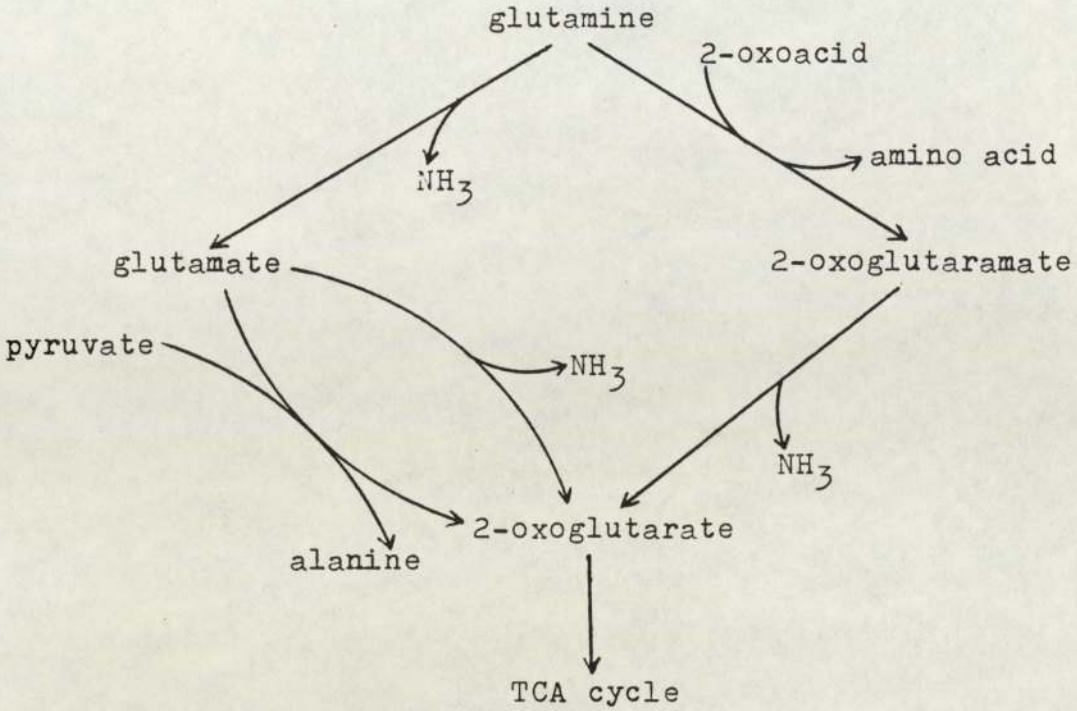
The inability of glutamine, even when presented at a supraphysiological concentration, to support acid secretion by isolated parietal cells (Chapter 5) was unexpected since it was taken up by the stomach wall *in vivo* (Chapter 2). Thus, it would appear that glutamine may not serve as a metabolic fuel for acid secretion *in vivo*, and its uptake was caused by a different cell type. Alternatively glutamine may suppress endogenous substrate metabolism in parietal cells and the rate of its own metabolism might be insufficient to stimulate acid secretion above

the level in the absence of exogenous substrate. Thus, in pancreatic islets, glutamine (1mM) does not stimulate insulin secretion, for although it is metabolised, it suppresses endogenous fatty acid metabolism by as much as 50% (Malaisse-Lagae et al., 1982).

The apparent lack of oxidation of glutamine may result from restrictions on its entry into the cell or from limitations associated with its degradative pathways (Fig. 6.7). Active transport of glutamine into some cells occurs through transport system N (Kilberg et al., 1980), a sodium-dependent system which may not be present in parietal cells. The major degradative pathway beginning with deamidation of glutamine requires glutaminase (EC 3.5.1.2) whose activity in the gastric mucosa was shown by Pinkus & Windmueller (1977) to be much lower than in other regions of the rat gastro intestinal tract, such as the small intestine, and the activity of the enzyme in parietal cells seems to be about 10% of that in jejunal epithelial cells (G.P. Shaw, Personal communication).

Another possible explanation is that glutamine is not a metabolic fuel in the stomach at all, but is required in a different capacity. Glutamine is required by rapidly dividing cells to provide amide nitrogen for purine nucleotide synthesis (Krebs, 1980) and since there is a rapid turnover of, notably epithelial mucous cells in the stomach, glutamine uptake may be indicative of its role in this process.

Overall, the experiments reported here, together with the relatively low activity of glutaminase in parietal cells, do not support a major role for glutamine as an



1 glutaminase (EC 3.5.1.2)
2 alanine aminotransferase (EC 2.6.1.2)
3 glutamine-oxoacid aminotransferase (EC 2.6.1.15)
4 ω-amidase (EC 3.5.1.3)
5 glutamate dehydrogenase (EC 1.4.1.3)

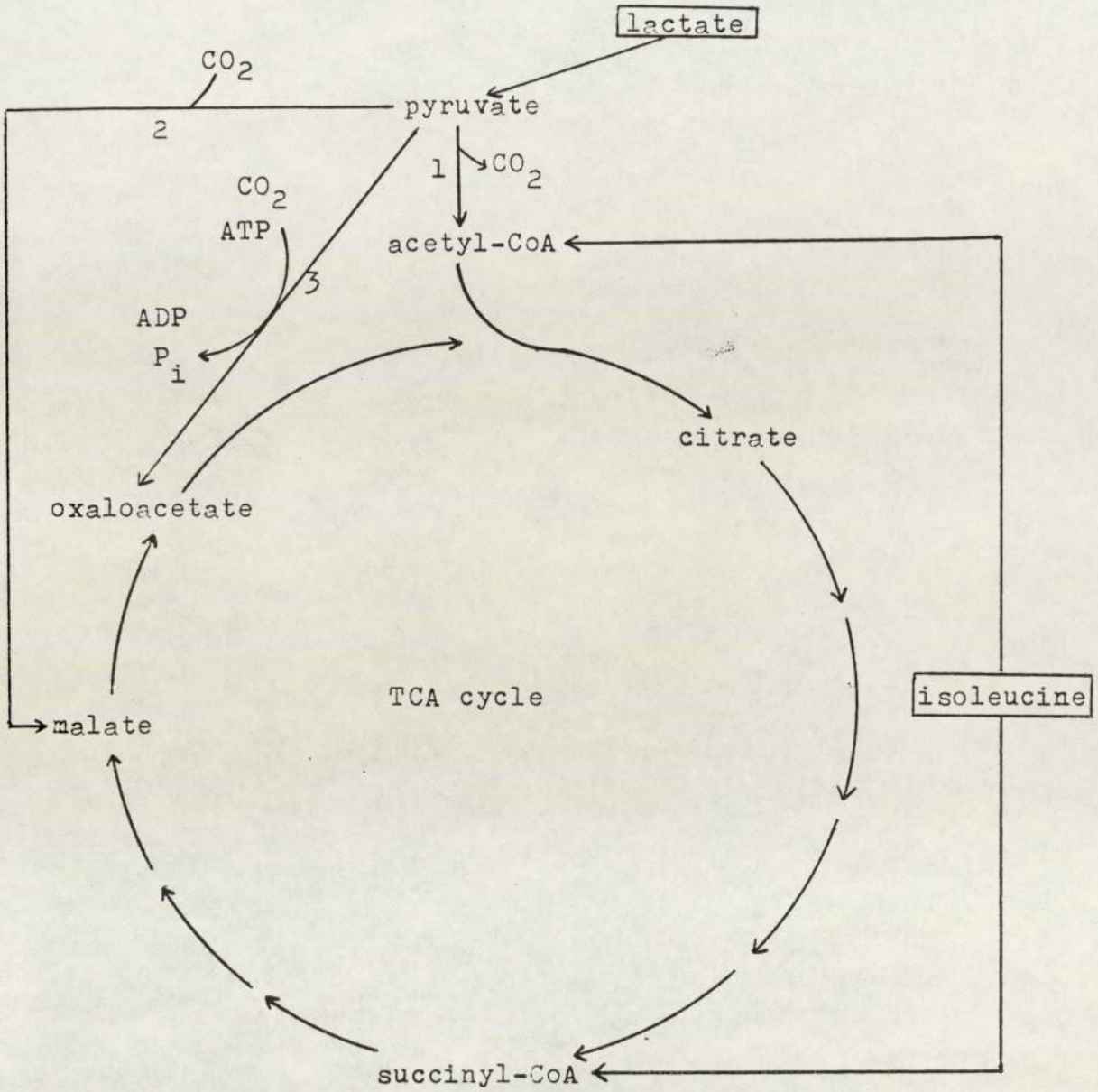
Fig 6.7 Alternative pathways for the metabolism of glutamine. 2-oxoglutarate enters the TCA cycle and is converted to acetyl-CoA (see Fig 6.6).

energy-providing substrate for acid secretion. Why glutamine is completely unable to raise aminopyrine accumulation in stimulated cells above control levels is, however, uncertain.

6.7. CONCLUSIONS.

Overall, it appears that a wide range of substrates are able to support acid-secretory rates above resting levels in isolated parietal cells. For the first time, it has been shown that certain amino acids can act as substrates as well as at least one long-chain fatty acid. Alone, and at a physiological concentration, glucose appears to be the best substrate, but it is apparently unable to support maximal rates of acid secretion, possibly because of an intrinsically low activity of the glycolytic pathway in parietal cells. The fact that lactate and isoleucine, when presented at supraphysiological concentrations, are better substrates than lipid-derived fuels may be due to the fact that both of these substrates can provide oxaloacetate as well as acetyl-CoA via their metabolism and so induce improved oxidation of the acetyl-CoA through the TCA cycle (Fig. 6.8).

Since glucose alone apparently cannot generate sufficient energy via its metabolism, to support maximum acid secretion, the parietal cell *in vivo* probably has to increase the metabolism of several substrates to obtain the required energy. Therefore the theory of an increase in the metabolism of one particular substrate or class of substrates, induced by acid secretagogues, would appear unlikely. Increased levels of TCA cycle intermediates in canine parietal cells stimulated to



1 pyruvate dehydrogenase (EC 1.2.4.1,EC 2.3.1.12,EC 1.6.4.3)

2 "malic enzyme" (malate dehydrogenase,EC 1.1.1.40)

3 pyruvate carboxylase (EC 6.4.1.1)

Fig 6.8 Possible pathways by which isoleucine and lactate may provide oxaloacetate to enable increased oxidation of acetyl-CoA by the TCA cycle.

secrete acid (Sarau et al., 1977) strongly suggested that the primary source of energy for acid secretion is ATP derived from mitochondrial metabolism and rabbit gastric glands, made permeable to ATP by electric shock, recover their ability to accumulate aminopyrine when 5mM-ATP is added to the suspension (Berglindh et al., 1980a). The results in this study suggest that glucose, ketone bodies and possibly branched-chain amino acids are metabolised at high rates by parietal cells in vivo. However, further experimentation must be carried out before one can say unequivocally how important the metabolism of any one substrate is to the energy requirements of acid secretion in vivo. The activities of many key enzymes have not yet been established in parietal cells, and such data might elucidate why some substrates, such as leucine appear to be poor substrates. Further studies on the substrate-dependency of acid secretion by isolated parietal cells using combinations of substrates would possibly demonstrate how the metabolism of one substrate may influence that of another and the identification of metabolic products using radiolabelled substrates may lead to a better understanding of parietal cell metabolism. Finally, the whole area of the relationship between the control of substrate metabolism and the control of acid secretion remains to be explored.

6.8. SUMMARY.

1. 5mM-glucose alone cannot provide enough energy to support acid secretion, at the rates found with some other substrates, possibly because the maximal capacity of the glycolytic pathway in parietal cells

to metabolise glucose is limited.

2. There is evidence that the ability of acetate, butyrate, lactate, acetoacetate, D-3-hydroxybutyrate isoleucine, leucine, and valine to act as substrates is dependent upon the exogenous substrate concentration and that substrate availability is therefore a factor limiting their use *in vivo*.

3. Differences in the activities of the pathways degrading isoleucine, valine and leucine are probably an important factor affecting their different abilities to support acid secretion.

4. The reason why metabolism of glutamine is insufficient to stimulate aminopyrine accumulation by parietal cells remains to be established.

5. Isoleucine and lactate are the best substrates when exogenous concentration is not a limiting factor, possibly because their metabolism provides the means of oxidising acetyl-CoA at high rates by providing oxaloacetate to the TCA cycle.

6. Combining data from studies *in vivo* and *in vitro* suggests that metabolism of the following may be of importance in providing energy for acid secretion *in vivo*; glucose, D-3-hydroxybutyrate and isoleucine.

CHAPTER 7.

THE EFFECT OF TETRADECANOYLPHORBOL-13-ACETATE (TPA) ON ACID SECRETION BY ISOLATED PARIETAL CELLS.

7.1. INTRODUCTION.

This section is concerned with an investigation of one aspect of the intracellular events associated with stimulus-secretion coupling in parietal cells. Protein phosphorylation may be involved in the regulation of gastric acid secretion since deactivation of the KCl symport in vesicles isolated from parietal cells correlates with the dephosphorylation of a vesicular protein in the 80kda range (Wolosin & Forte, 1982). Phosphorylation by calcium-sensitive, phospholipid-dependent protein kinase (protein kinase C) of a 40kda protein in platelets (Castagna et al., 1982) and a 29kda protein on the insulin secretory granule membrane (Brocklehurst & Hutton, 1983) seems to be implicated in the secretory mechanisms in these tissues. It was therefore of interest to investigate the putative role of protein kinase C in signal transduction in isolated parietal cells.

Protein kinase C is a calcium- and phospholipid-dependent enzyme which is activated by diacylglycerol (Kishimoto et al., 1980). Kinetic analysis indicates that a small amount of diacylglycerol dramatically increases the apparent affinity of protein kinase C for Ca^{2+}, which would fully activate the enzyme without any change in normal cellular Ca^{2+} levels, the most active diacylglycerols being those possessing at least one unsaturated fatty acid irrespective of the chain-length of the other fatty acyl moiety. In the activation of protein kinase C, phosphatidylserine is indispensable, other phospholipids showing positive or negative cooperativity with phosphatidylserine (Kaibuchi et al., 1981). Despite its activation by Ca^{2+}, the enzyme is

independent of calmodulin (Mori et al., 1980).

Protein kinase C consists of a single polypeptide chain (mol. wt. 77,000 da) which appears to be composed of two functionally different domains, separable by Ca^{2+}-dependent thiol proteases (Kishimoto et al., 1983). One is a hydrophobic domain which may bind to membranes; the other is hydrophilic and carries the catalytically active centre, and, when separated from the hydrophobic domain, is fully active without Ca^{2+}, phospholipid and diacylglycerol.

In vitro, protein kinase C has a broad substrate-specificity and phosphorylates seryl, threonyl, but not tyrosyl residues of many endogenous proteins in several tissues (Nishizuka, 1984). However, the nature of the physiological substrates of protein kinase C in most tissues is still poorly understood.

Work on other tissues suggests that possible intracellular events following cholinergic stimulation of the parietal cell could be as in Fig. 7.1. Thus, carbachol, by inducing breakdown of polyphosphoinositides (Berridge, 1984), may generate a bifurcating signal, with inositoltrisphosphate causing liberation of intracellular calcium and diacylglycerol activating protein kinase C. Activation of muscarinic cholinergic receptors can also stimulate an influx of Ca^{2+} into the cell, but the causal relationship of this process to the breakdown of polyphosphoinositides remains to be established (Berridge, 1984). Stimulation of acid secretion in parietal cells by acetylcholine could thus involve activation of protein kinase C.

Protein kinase C has a wide tissue distribution (Kuo et al., 1980), but its presence in rat parietal cells remains

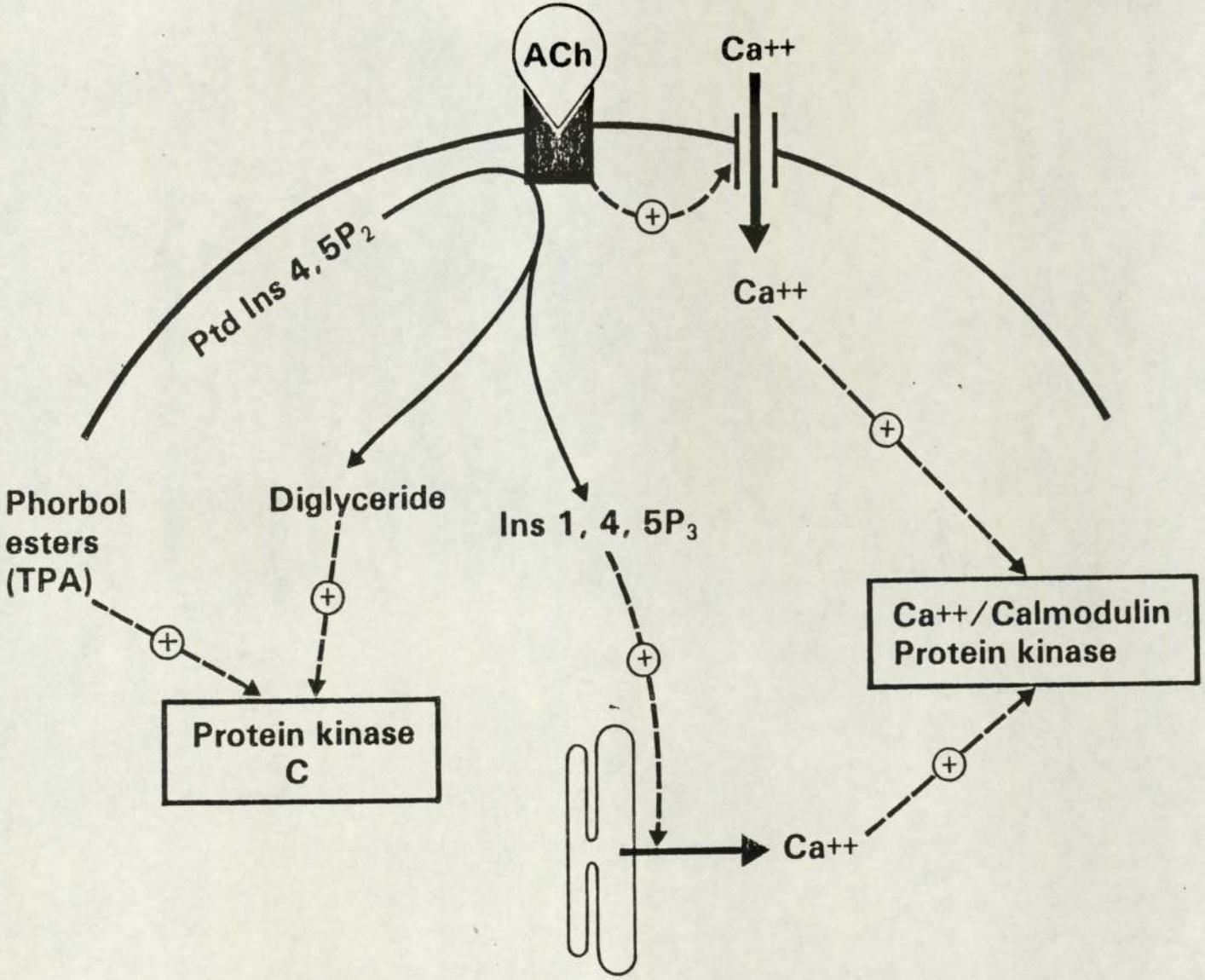


Fig 7.1 Some intracellular events which could follow cholinergic stimulation of the parietal cell.

to be established. The enzyme may be assayed directly in cell homogenates (see Chapter 8) or evidence supporting its existence may be obtained indirectly. Tumour-promoting phorbol esters, such as 12-O-tetradecanoylphorbol-13-acetate (TPA), induce protein secretion from pancreatic acini (Gunther, 1981), insulin secretion from pancreatic B-cells (Yamamoto et al., 1982) and 5-hydroxytryptamine secretion from platelets (Castagna et al., 1982). Phorbol esters seem to mimick the natural second messenger, diacylglycerol, and thereby activate protein kinase C. Indeed, TPA contains a diacylglycerol-like moiety in its structure (Fig. 7.2). There is an approximate correlation between the ability of individual phorbol esters to promote tumours and activate protein kinase C (Castagna et al., 1982; Yamanishi et al., 1983) and TPA, like diacylglycerol, dramatically increases the affinity of the enzyme for Ca^{2+} to the 10^{-7}M range, whereby full activation can take place without elevation of intracellular Ca^{2+} above resting levels (Yamanishi et al., 1983). Kraft & Anderson (1983) state that TPA causes translocation of cytosolic protein kinase C to the cell membrane, where it appears to be activated (Kraft et al., 1982).

Thus, if protein kinase C is present in parietal cells and is associated with signal transduction, phorbol esters ought, themselves, to promote acid secretion or alternatively influence the response of parietal cells to secretagogues. Therefore, in this section, the effect of the potent tumour-promoter, TPA, on acid secretion by parietal cells was investigated. A problem with this approach is that tumour-promoters, such as TPA, may influence cell responsiveness via pathways not involving protein kinase

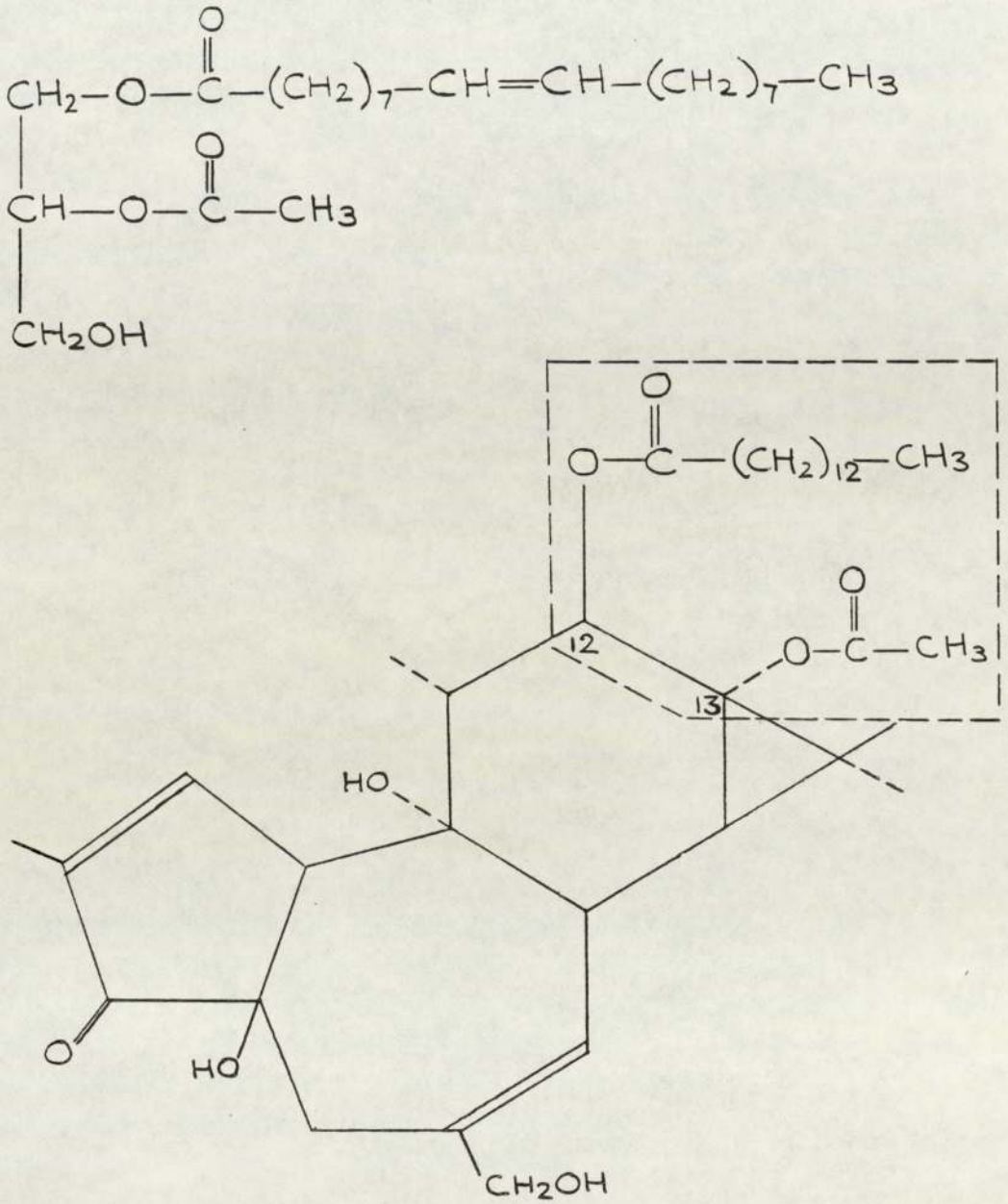


Fig 7.2 Structures of synthetic diacylglycerol (1-oleyl-2-acetyl glycerol ;top) and tumour-promoting phorbol ester (12-O-tetradecanoylphorbol-13-acetate,TPA;bottom). TPA contains a diacylglycerol-like moiety (dotted area) within its structure.

C-activation and these alternatives are considered in the discussion to this chapter.

7.2. METHODS.

7.2.1. The Effect of TPA on Acid Secretion.

Cells were isolated as described in section 3.4.1 and fractionated to enrich the proportion of parietal cells if required (4.2.1). Acid secretion was measured by the accumulation of aminopyrine (3.2.1). Usually, cells were incubated in medium B (3.4.1), along with test agents for 30 minutes after which radioactivity was determined in the cells and medium (3.2.1). TPA is not water soluble and was therefore added to the cell suspension as a concentrated solution in dimethylsulphoxide (DMSO). A stock solution of 10mg TPA/ml DMSO was prepared and an appropriate volume (usually 2-5µl) was added to a solution of rapidly stirring medium C (4.2.1). A small volume of this mixture was then added to the cell suspension. The concentration of DMSO in all vials, including those not containing TPA, was 0.01%(v/v).

7.2.2. The Effect of TPA on Glucose Oxidation.

The oxidation of glucose by parietal cells was determined in the presence and absence of TPA. Oxidation of $[^{14}C]$-glucose was measured according to the method described in 3.2.3. 2ml of purified parietal cells (5 x 10^5cells/ml) were incubated with $[U\text{-}^{14}C]$-glucose (0.5µCi/ml) and TPA (32nM, added as described in 7.2.1) in medium B. The $[^{14}C]$ counts trapped on the filter paper in the centre well of the flask were determined and the results expressed as the amount of glucose converted to CO_2/min/mg.dry weight

of cells.

7.2.3. Analysis and Presentation of Results.

Because of the variation between batches of cells in aminopyrine accumulation in response to secretagogues (e.g. aminopyrine ratio in response to histamine + IMX : batch 1 32.99; batch 4, 130.652; Table 7.2), it was necessary to separate this "batch" variation from that due to the individual treatments. In this chapter the data were therefore subjected to a factorial analysis of variance. For example, this analysis was used for the data presented in Table 7.2 to separate variation due to "batch", to TPA, to secretagogues and to the interaction between TPA and secretagogues. The following example shows a summary of the factorial analysis of variance as applied to the data presented in Table 7.2.

Variation	df	Sum sq.	mean sq.	F
Total	23	33377.6		
Treatment combinations	5	22545.4	4509.09	8.2987***
Secretagogues	2	9944.9	4972.43	9.1515**
Control vs. TPA	1	8577.6	8577.56	15.7865**
Interaction	2	4023.0	2011.50	3.7021*
Blocks (runs)	3	2682.0	893.99	1.6454
Error	15	8150.2	543.35	

***P<0.001 **P<0.005 *P<0.05

Conclusions: significant effects of secretagogues, TPA, the interaction between TPA and secretagogues and the overall treatment combination. No significant effect of cell "batch".

In this particular example the significant effect of TPA may be further defined by testing its effect on each secretagogue by means of a t-test,

	Histamine + IMX	Carbachol	dbcAMP
Control	84.898	12.548	78.700
TPA	20.020	9.632	33.060
difference	64.878	2.916	45.640

The standard error is calculated from the error mean square:

$$SE = \frac{2(\text{error mean sq})}{\text{no. of blocks}} = 16.482$$

Therefore, the t values (difference/SE, with 15 degrees of freedom) for each secretagogue were as follows,

$$\text{Histamine + IMX} = \frac{64.878}{16.482} = 3.9363 \; ; \; P<0.002$$

$$\text{Carbachol} = \frac{2.916}{16.482} = 0.1769 \; ; \; P>0.05$$

$$\text{dbcAMP} = \frac{45.640}{16.482} = 2.7691 \; ; \; P<0.02$$

Thus, TPA significantly affects aminopyrine accumulation in response to histamine + IMX and dbcAMP, but not carbachol.

7.3. RESULTS AND PRELIMINARY DISCUSSION.

7.3.1. The Effect of TPA on Acid Secretion.

TPA (32nM) inhibited the aminopyrine accumulation by parietal cells stimulated with histamine + IMX ($P<0.002$) and with dibutyrylcyclicAMP ($P<0.02$), but no significant inhibition of carbachol-stimulated aminopyrine accumulation was revealed by this analysis (Table 7.1).

An inhibition of aminopyrine accumulation (acid secretion) by TPA may have been caused by a non-specific action of the phorbol ester on an aspect of the structural or general functional integrity of the parietal cell. However, 30 minutes incubation with TPA (32nM) did not appear to

Table 7.1. The effect of TPA (32nM) on aminopyrine accumulation in parietal cells stimulated by secretagogues. Cells were incubated for 30 min. All vials contained 0.01%(v/v) DMSO. Results are expressed as means ± SEM (n = 4) and the significance of the effects of TPA was determined by factorial analysis of variance *P<0.02 ; **P<0.002 . The aminopyrine ratio in the absence of secretagogues and TPA was 2.9 ± 0.006(4).

Secretagogues	Aminopyrine ratio		Effect of TPA on aminopyrine ratio (% of control)
	Control	TPA	
Histamine (0.5mM) + IMX (0.1mM)	85 ± 24	20 ± 7**	23
Carbachol (0.1mM)	13 ± 1	10 ± 1	77
DibutyrylcAMP (1mM)	79 ± 16	33 ± 6*	42

Table 7.2. The effect of TPA (32nM) on aminopyrine accumulation in parietal cells stimulated by secretagogues - untransformed data.

Secretagogues	Aminopyrine ratio							
	Control				TPA			
Cell batch	1	2	3	4	1	2	3	4
Histamine + IMX	32.990	55.788	120.164	130.652	11.453	15.802	39.908	12.918
Carbachol	14.978	13.854	11.912	9.446	12.512	9.422	8.696	7.898
DibutyrylcAMP	52.600	54.668	88.091	119.440	38.850	37.438	37.716	16.238

affect the integrity of the plasma membrane as assessed by the exclusion of trypan blue (line 1, Table 7.3) nor the overall metabolism of resting parietal cells as assessed by glucose oxidation (line 2, Table 7.3). TPA also appeared to have no effect on the aminopyrine accumulation of unstimulated parietal cells (line 3, Table 7.3). These three findings suggest that the action of TPA on acid secretion by parietal cells did not occur as a result of non-specific effects.

Half-maximal inhibition of aminopyrine accumulation stimulated by histamine (0.5mM) and IMX (0.1mM) was achieved with 2.7nM-TPA (Fig. 7.3). The acid-secretory response was not totally inhibited by high concentrations of TPA, the maximally effective concentration (10^{-6}M) causing a reduction in aminopyrine accumulation to 25% of that in the absence of TPA. Because TPA-induced inhibition of histamine-stimulated acid secretion occurred in a population of gastric cells containing, on average, only about 20% parietal cells, the possibility existed that TPA was acting on another cell type causing release of an agent which inhibited acid secretion by parietal cells. For example, TPA might have stimulated D-cells to release somatostatin, which inhibits acid secretion (see section 1.1.4). However, Fig. 7.3, shows that the inhibition by TPA was also seen with purified cells (~80% parietal cells). Thus, unless copurification of the cell-type releasing an inhibitor of acid secretion had occurred (see Chapter 4, Discussion), TPA must be acting directly on the parietal cell. The dose-response curve with purified cells appeared similar in shape to that with unpurified cells, which also

Table 7.3. The effect of TPA (32nM) on parietal cell viability assessed by trypan blue exclusion and oxidation of [^{14}C] glucose, and on the aminopyrine accumulation by unstimulated cells. Control experiments contained DMSO at the same concentration (0.01% v/v) as experiments containing TPA.

Test	Control	TPA(32nM)
Trypan blue exclusion (%cells excluding dye)	95.9±0.4(3)	95.9±1.7(4)
Glucose oxidation (pmol glucose converted to CO_2/min/ mg.dry wt.)	214 ± 7(5)	206 ± 8(4)
Aminopyrine accumulation (aminopyrine ratio in absence of secretagogues)*	2.310	2.414

*single experiment.

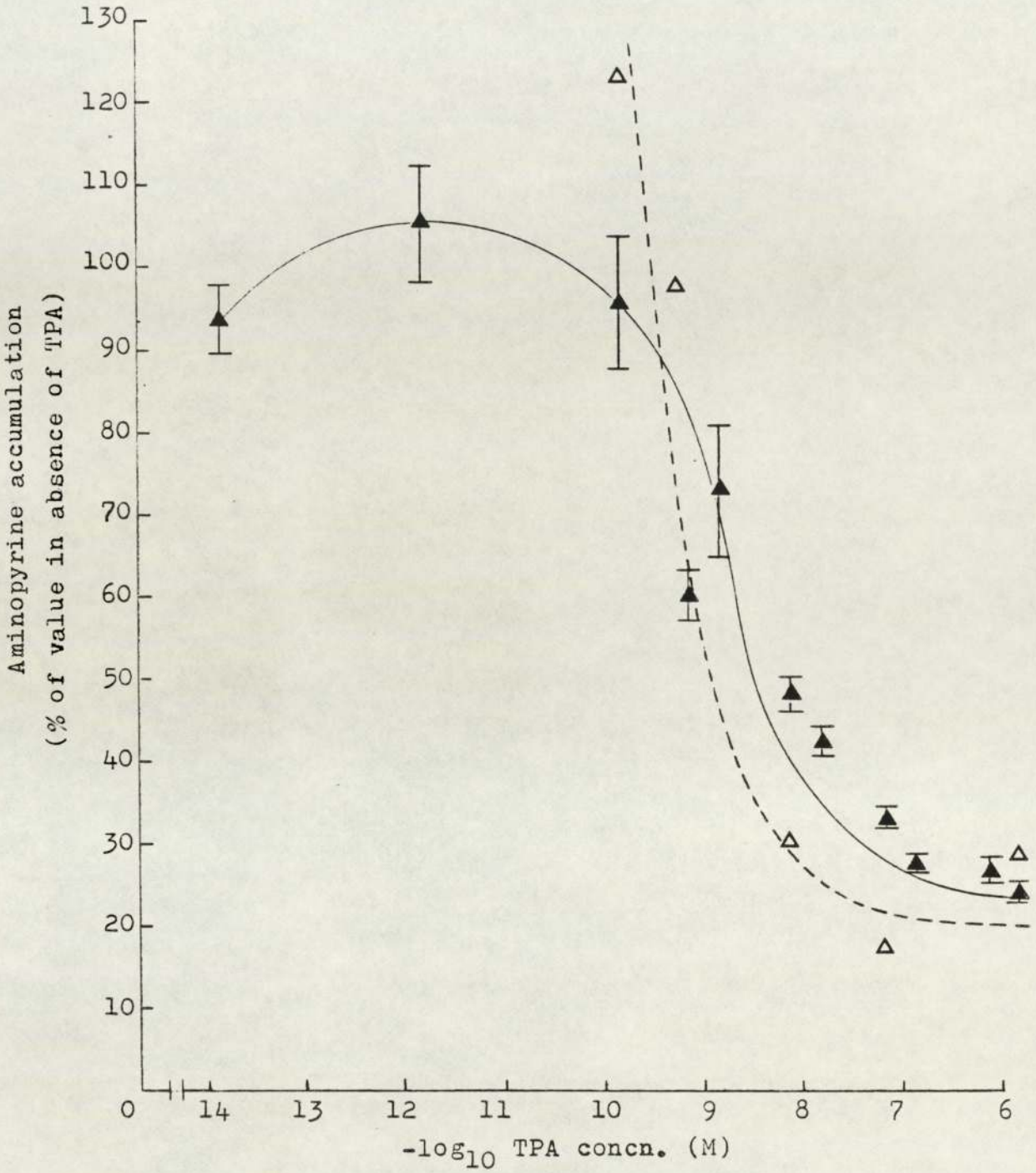


Fig 7.3 The effect of TPA on the aminopyrine accumulation by unenriched (▲——▲) and purified (Δ---Δ) parietal cells stimulated with histamine (0.5mM) and IMX (0.1mM). Values were normalised by expressing the aminopyrine accumulation seen at each concentration of TPA with respect to that seen in the absence of TPA which was made 100. For unenriched cells results are means ± SEM (n=3-6) and for purified cells the points represent the average of results from two different cell batches.

supports the idea that TPA was acting directly on the parietal cells.

Inhibition by TPA of carbachol-stimulated acid secretion could derive from a direct interaction with the carbachol stimulatory process, or through antagonism of the effect of histamine which potentiates the effect of carbachol (Chapter 3) (histamine could be released by histamine-containing cells present in the medium). TPA inhibited aminopyrine accumulation in response to carbachol (Fig. 7.4) whether or not the histamine H_2-receptor antagonist, cimetidine, was present. Factorial analysis of variance indicated a significant effect of TPA concentration ($F=7.409$, $P<0.001$) and cimetidine ($F=11.548$, $P<0.01$) on carbachol (0.1mM)-stimulated aminopyrine accumulation. However, no significant interaction between the cimetidine and TPA effects was found, and therefore it is unlikely that TPA inhibits aminopyrine accumulation, in response to carbachol, by inhibiting an endogenous histamine-associated stimulation. In a separate experiment, cimetidine, at the concentration used above (0.1mM), completely inhibited acid secretion in response to histamine (0.5mM) + IMX (aminopyrine ratio with histamine + IMX, 22.069; with histamine + IMX + cimetidine, 2.802), indicating that 0.1mM-cimetidine would inhibit any endogenous histamine stimulation.

TPA was, however, a less potent inhibitor of aminopyrine accumulation when carbachol, rather than histamine + IMX, was the secretagogue (cf Figs. 7.3 & 7.4). 2.7mM - TPA (the concentration producing half-maximal inhibition of histamine-stimulated aminopyrine accumulation) caused, on average, less than a 5% reduction of aminopyrine

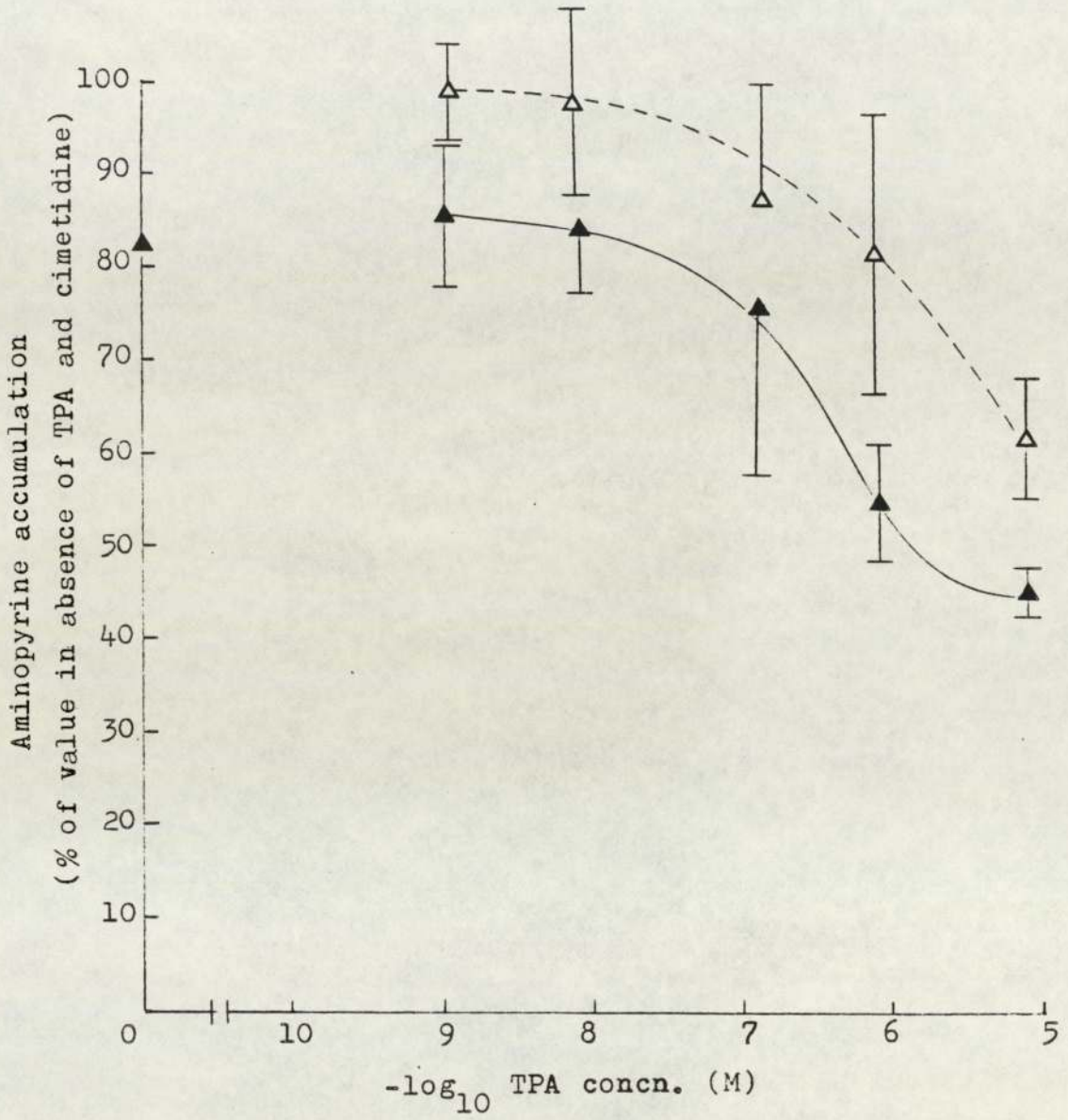


Fig 7.4 The effect of TPA on the aminopyrine accumulation by unenriched parietal cells stimulated by carbachol (0.1mM) in the presence (▲—▲) or absence (△--△) of cimetidine (0.1mM). Values were normalised by expressing the aminopyrine accumulation seen at each concentration of TPA with respect to that seen in the absence of TPA which was made 100, and represent the means ± SEM from three experiments.

accumulation stimulated by 0.1mM carbachol alone. The efficacy was also probably less since 10^{-5}M TPA reduced the aminopyrine accumulation to 60% of that obtained in the absence of TPA, although higher concentrations could conceivably have been more effective.

7.3.2. The Effect of Inhibitors of Prostaglandin/Leukotriene Synthesis on the Inhibition of Histamine-Stimulated Acid Secretion by TPA.

TPA could have caused inhibition of histamine-stimulated acid secretion by stimulation of endogenous prostaglandin synthesis via activation of phospholipase A_2 (Yamamoto et al., 1982 and see Fig. 7.5 and Discussion). Therefore, the inhibitory action of TPA against acid secretion, in response to histamine + IMX, was challenged separately by four agents which, either directly or indirectly, inhibit prostaglandin synthesis. Mepacrine and p-bromophenacylbromide, which are putative inhibitors of phospholipase A_2, both completely inhibited acid secretion in the absence of TPA, possibly due to non-specific effects on overall cell integrity (Table 7.4). However, indomethacin, an inhibitor of cyclo-oxygenase, and nordihydroguaiaretic acid (NDGA), an inhibitor of cyclo-oxygenase and lipoxygenase, did not appear to have gross effects on the response of the cells to histamine + IMX and their effect on the inhibitory action of TPA was tested. Neither indomethacin nor NDGA appeared to prevent the inhibition of aminopyrine accumulation by TPA (Table 7.5). Global two-way analysis of variance indicated no significant effect of indomethacin or NDGA at any level of stimulus and so it would appear

Table 7.4. The effect of four inhibitors of prostaglandin synthesis on the inhibition by TPA (32nM) of aminopyrine accumulation in response to histamine (0.5mM) + IMX (0.1mM) Results are for a single batch of cells.

Inhibitor (concn.used)	Site of inhibition	Aminopyrine ratio		
		Control	Hist+IMX	Hist+IMX+TPA
None	-----	2.347	58.185	17.912
Mepacrine (100μM)	phospholipase A_2	1.168	1.764	1.022
p-bromophenacylbromide (100μM)	phospholipase A_2	0.695	0.483	0.700
Indomethacin (10μM)	cyclo-oxygenase	1.686	75.070	12.322
NDGA (100μM)	cyclo-oxygenase/lipoxygenase	2.068	59.468	7.422

Table 7.5 The effect of indomethacin (10μm) and NDGA (100μm) on the inhibition by TPA (32nM) of aminopyrine accumulation in response to histamine (0.5mM) and IMX (0.1mM).

	Aminopyrine Ratio									
Drug →	None					Indomethacin				
Stimulus cell batch→	1	2	3	4	mean ± SEM	1	2	3	4	mean ± SEM
Control	2.347	2.284	3.362	2.726	2.680±0.247	1.686	2.255	3.503	3.010	2.614±0.402
Hist+IMX	58.185	42.572	67.580	77.440	61.444±7.418	75.070	38.816	64.302	74.966	63.288±8.540
Hist+IMX+TPA	17.912	13.222	19.738	18.918	17.448±1.457	12.322	9.482	18.507	14.888	13.800±1.919

	Aminopyrine Ratio				
Drug →	NDGA				
Stimulus cellbatch→	1	2	3	4	mean ± SEM
Control	2.068	1.580	2.614	3.250	2.378±0.359
Hist+IMX	59.468	33.381	46.497	62.582	50.482±6.680
Hist+IMX+TPA	7.422	9.247	14.368	13.420	11.114±1.659

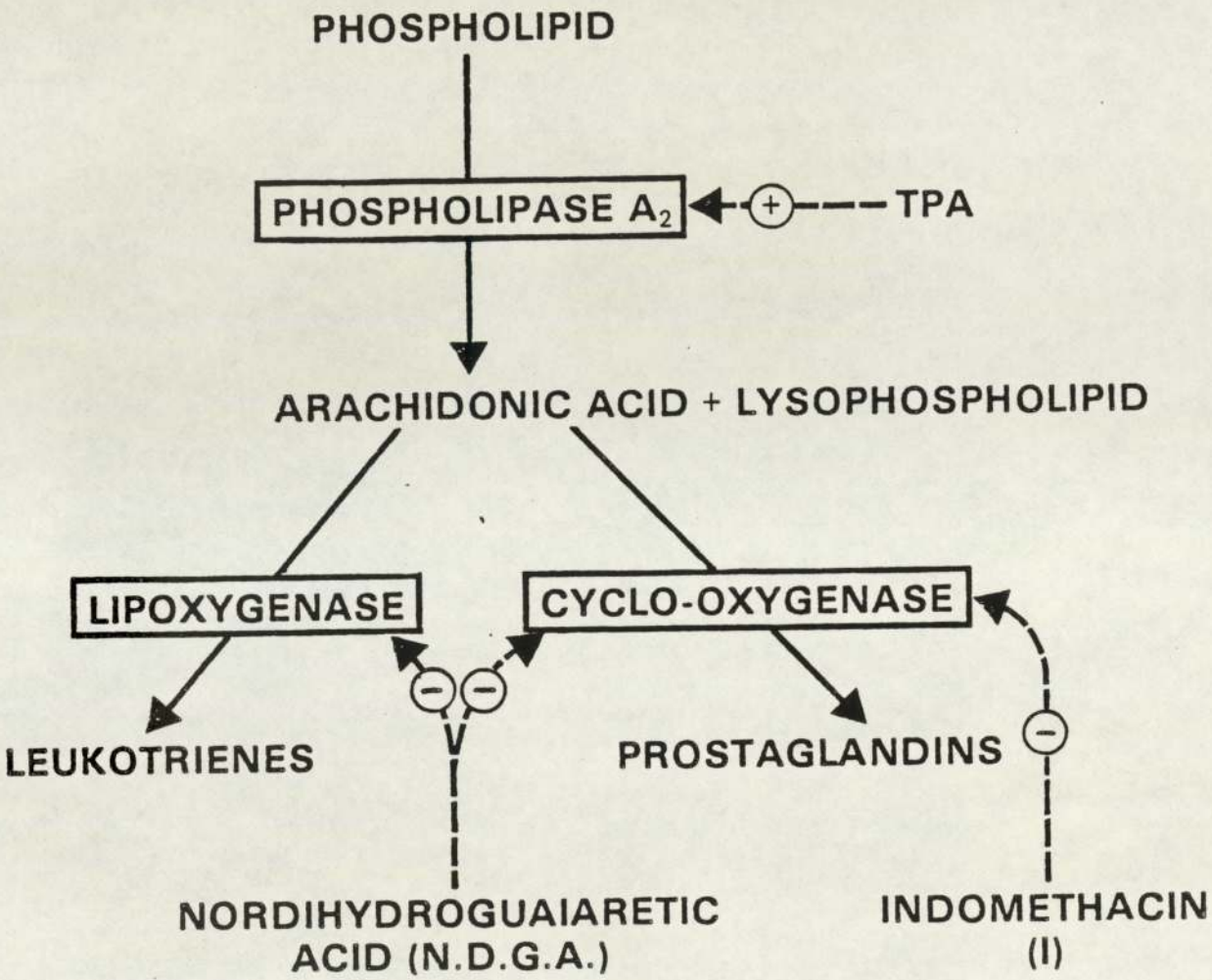


Fig 7.5 Potential mechanism for the inhibition of acid secretion by TPA.

unlikely that TPA causes inhibition of acid secretion by stimulating the production of prostaglandins. However, it would appear that NDGA may enhance the action of TPA since aminopyrine accumulation in response to histamine, IMX and TPA in the presence and absence of NDGA was significantly different (Student's t-test; $P<0.05$, see first and last column of means, line 3, Table 7.5). Indeed, leukotrienes, which originate from the action of lipoxygenase on arachidonic acid (Fig. 7.5), may stimulate acid secretion to a small extent (Magous et al., 1983), and inhibition of their production by NDGA could explain this effect.

7.4. GENERAL DISCUSSION.

As described in the introduction to this chapter, the reason for testing the effect of phorbol esters on acid secretion by parietal cells, was to derive information concerning the intracellular events mediating the secretory response to secretagogues, and in particular to investigate whether protein kinase C might play a role in the regulation of acid secretion. In conducting such experiments it is necessary to remember that phorbol esters may affect cells in ways not connected with protein kinase C-activation, particularly at higher concentrations, where TPA may act as a membrane fusigen or perturber (Kaibuchi et al., 1983; Yamanishi et al., 1983). The data presented in this chapter provide good evidence that the potent inhibition of histamine-stimulated acid secretion is not caused by non-specific effects of the phorbol ester. Thus, glucose oxidation (a measure of the cells' metabolic integrity) and trypan blue

exclusion (an index of the intactness of the cell membranes) were unaffected by incubation with TPA (32nM), nor was basal aminopyrine accumulation altered (Table 7.3; cf the effects of p-bromophenacylbromide, Table 7.4). The extremely low concentration of TPA required to cause half-maximal inhibition of acid secretion stimulated by histamine + IMX (2.7nM) also points strongly to a specific mode of action.

The inhibition by TPA of aminopyrine accumulation stimulated by carbachol was not due to inhibition of an endogenous histamine component of the observed stimulation, and a major reduction in aminopyrine accumulation was only observed in the presence of high concentrations of TPA (8μM), when deleterious effects on the overall structural and functional integrity of the parietal cell may well have occurred.

Assuming, then that TPA does not disrupt cell integrity at low concentrations, how does it cause inhibition of the acid-secretory response to histamine?

Histamine activates adenylate cyclase and increases the cAMP concentration within parietal cells (Soll & Wollin, 1979), but since TPA also significantly affected acid secretion stimulated by the stable analogue of cAMP, dibutyrylcAMP, it is unlikely that antagonism of histamine-stimulation occurs at the sites of control of cellular cAMP levels, such as the H_2-receptor or adenylate cyclase.

Three lines of evidence indicate that TPA did not cause inhibition of histamine stimulation by acting on D-cells which release a potent inhibitor of acid secretion, somatostatin (Chew, 1983). Firstly, somatostatin, unlike TPA, does not inhibit acid secretion induced by dibutyryl-cAMP (Chew, 1983) and secondly, maximal reduction in histamine

stimulated aminopyrine accumulation in rat parietal cells by somatostatin was to 56% of control values (Schepp et al., 1983a) compared with a maximal reduction by TPA to 25% of control aminopyrine accumulation. Thirdly, a similarly potent and efficacious inhibition by TPA was observed when the proportion of parietal cells in the suspension was enriched, presumably with a concomitant reduction in the proportion of D-cells (Fig 7.3).

Prostaglandins also inhibit acid secretion stimulated by histamine (Soll & Whittle, 1981) and it was conceivable that TPA was acting by stimulating endogenous prostaglandin synthesis. TPA appears to promote insulin secretion in pancreatic islets in part by activation of phospholipase A_2 and the generation of lipoxygenase products (Yamamoto et al., 1982). However, as discussed above (7.3), in parietal cells, increased prostaglandin synthesis does not appear to be the cause of the inhibition of histamine-stimulated acid secretion by TPA.

TPA increases the intracellular pH of Swiss 3T3 cells (Burns & Rozengurt, 1983) and it has been proposed (Berridge 1984) that diacylglycerol, whose action is thought to be mimicked by TPA (see Introduction), activates a $Na^+ + H^+$ exchange process in such cells. If TPA were to activate such an exchange in parietal cells, it is possible that the increase in intracellular pH and/or Na^+ concentration may perturb some aspect of the histamine-associated stimulatory process. Indeed, intracellular Na^+ appears to inhibit acid formation in gastric glands stimulated by high extracellular K^+ (Koelz et al., 1981).

Since phorbol esters activate protein kinase C in many cell types (see Introduction) and because protein kinase C

activity is found in most tissues (Kuo et al., 1980), there is a strong possibility that TPA may trigger the inhibitory effect of acid secretion in parietal cells via interaction with this enzyme.

If TPA is acting by substituting for diacylglycerol, then activation of protein kinase C in parietal cells must inhibit the cell's secretory functioning, in contrast to other cells, where activation of protein kinase C is linked with stimulation of secretion (Yamamoto et al., 1982; Castagna et al., 1982). Either stimulation of parietal cells by carbachol (see Fig 7.1) does not lead to activation of protein kinase C, because diacylglycerol levels are not raised, or activation of protein kinase C by carbachol represents an inhibitory 'arm' of the overall carbachol effect. Thus carbachol might stimulate acid secretion by raising intracellular Ca^{2+}concentration resulting in activation of Ca^{2+}/calmodulin-dependent protein kinases (Fig. 7.1) and also inhibit the histamine-stimulated secretion by activating protein kinase C which may then phosphorylate and activate an inhibitory protein such as a phosphatase. If such a mechanism were to occur <u>in vivo</u>, the inhibitory 'arm' might only be a transient phenomenon whereby maximal acid secretion was prevented before membrane rearrangements (see 1.1.3.1) took place, and this inhibition would be gradually overridden as stimulation continued. One essential requirement to support the hypothesis that the action of TPA is mediated by protein kinase C is the demonstration that protein kinase C activity is present in rat parietal cells. An attempt to ascertain this is described in the next chapter.

CHAPTER 8.

STUDIES TO DETERMINE THE EXISTENCE OF CALCIUM-SENSITIVE, PHOSPHOLIPID-DEPENDENT PROTEIN KINASE (PROTEIN KINASE C) IN PARIETAL CELLS.

8.1. INTRODUCTION.

One of the main conclusions to be drawn from the work described in Chapter 7 was that TPA might be acting via protein kinase C to cause inhibition of acid secretion by parietal cells. This section is concerned with an attempt to directly assay this enzyme in purified parietal cells.

Normally, the detection of protein kinase C activity is based simply upon the enzyme's ability to transfer a phosphate residue from $[\gamma\text{-}^{32}P]$-ATP to a protein into which an adequate amount of phosphate may be incorporated, such as calf thymus histone-H1 (Corbin & Reimann, 1974). Incorporation of phosphate should be enhanced by calcium and diacylglycerol in the presence of phospholipid, thus distinguishing protein kinase C activity from other protein kinase activities, such as cAMP-dependent or Ca^{2+}/calmodulin-dependent protein kinases. The radioactively labelled protein is trapped by precipitation and, after separation from $[\gamma\text{-}^{32}P]$-ATP, the $[^{32}P]$ incorporation is determined.

8.2. METHODS.

8.2.1. Preparation of Calcium-containing and Calcium-free Buffers.

The following three buffers were prepared:-

Buffer A : 25mM-Tris, 6.2mM-magnesium acetate, 24.8mM-EGTA (pH 7.40, 30°C)

Buffer B : 25mM-Tris, 6.2mM-magnesium acetate, 25.296mM-$CaCl_2$ (pH 7.40, 30°C)

Buffer C : 25mM-Tris, 6.2mM-Magnesium acetate (pH 7.40, 30°C)

Buffers containing the desired concentration of $CaCl_2$ were prepared by adding 5ml of Buffer A to the requisite

amount of Buffer B calculated by computer program (J. M. Lord, personal communication) according to the method of Severson et al., (1974). Thus, a buffer containing 50μM-Ca^{2+} was prepared by adding 5ml of Buffer A to 4.951ml of Buffer B and 0.049ml of Buffer C.

TPA, histone-H1 and [γ-^{32}P]-ATP were dissolved directly in either 50μM-Ca^{2+} buffer or Ca^{2+}-free buffer. Phosphatidylethanolamine supplied as a solution of 10mg/ml chloroform:methanol (95:5) was dispersed by drying 12.4μl of the stock solution under N_2 and sonicating (100W, 5min) in 1ml of buffer on ice. The suspensions were stored deep-frozen (-20°C) and resonicated before use.

8.2.2. Assay of Protein Kinase C.

The assay was performed on homogenates prepared from the spleen, oxyntic mucosa and purified parietal cells from the rat. The spleen was removed from a rat anaesthetised with sodium pentobarbitone(60mg/kg body wt), rinsed in ice-cold saline, blotted dry and weighed. Nine volumes of ice-cold homogenisation buffer, pH 7.4 containing (mM) Tris-HCl, 20; EDTA, 2; 2-mercaptoethanol, 50 were added and the spleen was homogenised (Ultra-Turrax, Janke & Kunkel, Staufen i. Breisgau, West Germany). A 2% (w/v) homogenate was finally prepared by adding a further four volumes of homogenisation buffer. Oxyntic mucosa was removed as described in 3.3.1 and a 10% homogenate was prepared by adding nine volumes of homogenisation buffer to the tissue, followed by homogenisation (Ultra-Turrax). Parietal cells were isolated (3.4.1) and purified (4.2.1) before suspension in 1ml of homogenisation buffer and sonication (A350G, Ultrasonics Ltd., Shipley, W. Yorks, U.K)

on ice for a total of 25 sec. (100W, 5X 5sec., cooling on ice between sonications). Homogenates were then centrifuged (MSE 50 centrifuge, see Table 8.1 for conditions) at 4°C and the supernatants retained for assay of protein kinase C.

12µl of the supernatant was added to 40µl assay buffer (pH 7.4) containing (mM), tris-HCl (20), Mg acetate (5) $CaCl_2$(0.05) with phosphatidylethanolamine (1.2µg/ml), histone-H1 (200µg/ml) and TPA (0.02µg/ml) . The constituents of the assay mixture are based on those of Tanigawa et al., (1982) except that diolein was replaced with TPA, because of the difficulty of dispersing diolein in buffer in the same way as phospholipid (see 8.1.1) and because TPA was implicated in activation of protein kinase C in parietal cells (Chapter 7). In some assays, TPA, phospholipid and calcium were omitted, the latter being replaced with EGTA (20mM).

The mixture was preincubated at 30°C for 2 min. and the reaction initiated by adding 10µl of $[\gamma\text{-}^{32}P]$-ATP to give 40µCi/ml. After 2 min., duplicate 20µl samples were removed and dispensed onto 1.5cm squares of filter paper (Whatman 31ET). In later experiments, the filter papers were presoaked in a solution of 1mM-ATP and 1mM-Mg acetate and dried before use (this dramatically reduced the radioactivity trapped in the absence of homogenate). The papers were immediately transferred to a wire-mesh basket immersed in ice-cold, trichloroacetic acid (5%w/v). After 15 min., the basket containing the filter papers was transferred to a tank of fresh ice-cold trichloroacetic acid. The filter papers were washed a further two times in 5% trichloroacetic acid at room temperature and then washed for 15min. in industrial methylated spirits ethanol:methanol

(95:5) . Finally, the filter papers were dried rapidly and placed in the bottom of 20ml. scintillation vials to which 10ml of scintillation fluid [toluene containing 0.4% (w/v) 2-(4'-tert-butylphenyl)-5-(4"-biphenylyl)-1,3,4-oxadiazole] was added. The radioactivity sequestered on the filter papers was then determined as described in A.5.2.

8.3. RESULTS AND DISCUSSION.

Table 8.1 shows protein kinase activities under various assay conditions in three tissues. In spleen, an appreciable amount of the calcium-stimulated activity was not dependent on phospholipid or TPA, if the assay was performed on the supernatant from a centrifugation of the homogenate at 30,000g for 20min. This result was probably due tc phcspholipid associated with membranes present in the 30,000g supernatant, since centrifugation of the spleen homogenate at 100,000g for 60 min,. which should sediment membranous particles, revealed a substantial protein kinase activity (i.e. the effect of Ca^{2+} was dependent upon the presence of phospholipid and TPA). Thus, the spleen contains a substantial activity of protein kinase C as found by Kuo et al., (1980).

The activity of protein kinase C in the oxyntic mucosa was approximately one tenth of that in the spleen (Table 8.1) but no such activity could be detected with a homogenate prepared from a cell fraction containing 87.6% parietal cells (Table 8.1). With this homogenate, addition of Ca^{2+} (50μM) actually reduced incorporation of [^{32}P]-phosphate into histone below levels obtained in the absence of Ca^{2+} (Tables 8.1.&.8.2). The presence of benzamidine (10mM) in the homogenisation buffer to prevent modification

Table 8.1 Evidence for the existence of protein kinase C activity in rat spleen, oxyntic mucosa and purified parietal cells. The results are from single experiments.

Assay conditions	^{32}P incorporation into Histone-H1 (cpm/min/mg.wet weight tissue)			
	spleen 30,000g supernatant	spleen 100,000g supernatant	oxyntic mucosa 100,000g supernatant	purified parietal cells, 100,000g supernatant
EGTA	7575	---	751	1469
EGTA+TPA+phospholipid	10187	---	---	1414
Ca^{2+}	24715	6426	657	816
Ca^{2+}+TPA+phospholipid	25867	15420	1659	797

Table 8.2 The effect of inhibitors of proteases (benzamidine, 10mM) and protein phosphatases (fluoride, 50mM; pyrophosphate, 15mM) on the assay of protein kinase C activity in purified parietal cells. Results are from a single experiment.

Assay conditions	^{32}P incorporation into histone-H1 (cpm/min/mg.wet weight tissue)	
	Control	F^-+ PPi
EGTA	3804	1866
Ca^{2+}	237	424
Ca^{2+}+phospholipid+TPA	278	348

of homogenate enzymes by proteases, and the protein phosphatase inhibitors, fluoride (50mM) and pyrophosphate (15mM) in the assay buffer, did not prevent this effect of Ca^{2+}, or provide any evidence supporting the presence of protein kinase C.

Overall, the work in this chapter has, so far, demonstrated a small, but significant, activity of protein kinase C in the oxyntic mucosa. However, the exact location(s) of this activity remains to be established. Preliminary experiments have failed to demonstrate activity in purified rat parietal cells and further studies are clearly required, perhaps involving DEAE-cellulose fractionation of the parietal cell cytosolic extract. A report of the presence of protein kinase C activity in purified rabbit parietal cells has recently been published (Chew, 1984).

FINAL COMMENTS

The first major aim of this study was to answer the question, "Which substrates are best able to provide, via their metaboiism, the quantities of energy required by the acid-secreting parietal cell?" It was evident from studies *in vivo* and *in vitro* that metabolism of glucose, D-3-hydroxybutyrate and isoleucine, was probably of importance in the process of energy provision. The parietal cell appears unable to derive sufficient energy from the metabolism of any one substrate present at the normal physiological concentration, and therefore *in vivo*, energy is probably provided by a combination of substrates. There was no evidence that the pattern of substrate utilisation changed upon stimulation of acid secretion. These studies have been performed with rats and therefore the results may be of relevance to the situation in man, since both rat and man are omnivorous.

The second aim of this work was to initiate an investigation of the intracellular events involved in stimulus transduction in parietal cells. The apparently specific inhibition of histamine-stimulated acid secretion, by a phorbol ester, 12-O-tetradecanoylphorbol-13-acetate (TPA), suggested that protein kinase C may be involved in the mechanism of acid secretion in an inhibitory capacity.

The following publications have resulted from this work,

Anderson, N.G. & Hanson, P.J. (1983) Substrate utilization by the stomach of control- and pentagastrin-stimulated rats *in vivo* Trans. Biochem. Soc. 11, 398-399.

Anderson, N.G. & Hanson, P.J. (1983) Metabolism of amino acids by rat stomach *in vivo*. Trans. Biochem. Soc. 11, 297-298.

Anderson, N.G. & Hanson, P.J. (1983) Arteriovenous differences for amino acids across control and acid-secreting rat stomach *in vivo*. Biochem. J. **210**, 451-455.

Anderson, N.G. & Hanson, P.J. (1983) Substrate utilization by rat stomach *in vivo*. Biochem J. **212**, 875-879.

Anderson, N.G. & Hanson, P.J. (1984) Inhibition of gastric acid secretion by a phorbol ester : effect of 12-0-tetradecanoylphorbol-13-acetate on aminopyrine accumulation by rat parietal cells. Biochem. Biophys. Res. Commun. **121**, 566-572.

MacGill, A.K., Anderson, N.G., Trotman, C.N.A., Carrington, J.M. & Hanson, P.J. D-3-hydroxybutyrate dehydrogenase and metabolism of ketone bodies by rat stomach. Trans. Biochem. Soc. (in press 1984).

Anderson, N.G. Shaw, G.P., & Hanson, P.J. Substrate dependency of gastric acid secretion : effect of metabolic substrates on aminopyrine accumulation by rat parietal cells. Trans. Biochem. Soc. (in press 1984).

Anderson, N.G. & Hanson, P.J. Inhibition of aminopyrine accumulation in isolated rat parietal cells by the tumour promotor 12-0-tetradecanoylphorbol-13-acetate. Trans. Biochem. Soc. (in press 1984).

APPENDICES

A.1. SOURCE OF REAGENTS.

Reagent	Supplier
A. General chemicals and biochemicals.	
Adenosine-5'-diphosphate, disodium salt (ADP)	Boeringer
Adenosine-5'-triphosphate, disodium salt (ATP)	Boeringer
Benzamidine hydrochloride	BDH
Bovine serum albumin (BSA), fraction V	Miles Labs.
Dextran, mol.wt. 40,000 da	Sigma
4-dimethylaminoantipyrine (aminopyrine)	Sigma
Dimethylsulphoxide (DMSO), sp.gr.1.1g/ml	Sigma
sym-diphenylcarbazide	Sigma
DL-dithiothreitol	Sigma
Ethylenediaminetetraacetic acid (EDTA)	Hopkin & Williams
Ethyleneglycol-bis-(β-aminoethyl ether) N, N'-tetraacetic acid (EGTA)	Sigma
Fatty-acid-free bovine serum albumin	Sigma
Glucose	BDH
Glutamic acid	Sigma
L-glutamine	Sigma
Glycerol	BDH
Glycine	Sigma
Histone, type V-S from calf thymus	Sigma
Hydrazine hydrate, sp.gr. 1.03g/ml	BDH
DL-3-Hydroxybutyrate, disodium salt	Boeringer
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

Reagent	Supplier
Lithium acetoacetate	Sigma
2-mercaptoethanol sp.gr.1.12g/ml.	BDH
NAD^+, grade II	Boeringer
NADH, disodium salt	Boeringer
$NADP^+$, disodium salt	Boeringer
Nitro-blue tetrazolium, grade III	Sigma
2-oxoglutarate, disodium salt	Boeringer
palmitic acid	Sigma
Percoll, for density gradient centrifugation, sp.gr. 1.13g/ml	Pharmacia
Phenolphthalein	BDH
Sodium acetate trihydrate	BDH
Sodium-n-butyrate	BDH
Sodium oleate	Sigma
Sodium pyruvate	Sigma
Succinic acid, disodium salt	Sigma
5-sulphosalicylic acid	BDH
Trichloroacetic acid	Sigma
Triethanolamine, sp.gr. 1.125g/ml	BDH
Tris(hydroxymethyl)methylamine	BDH
Trypan blue, mw 961da	BDH
Trypsin inhibitor, lyophilised from soybean	Sigma
Universal indicator	BDH
L-valine	Sigma

B. Enzymes.

Collagenase	Type IV, from *Clostridium histolyticum*	Sigma
Glucose-6-phosphate dehydrogenase, grade II from yeast		Boeringer
Glutamate dehydrogenase, from beef liver		Boeringer

Reagent	Supplier
Glutaminase, grade V, lyophilised from Escherischia coli	Sigma
Glycerokinase, from Candida mycoderma	Boeringer
Glycerol-3-phosphate dehydrogenase, from rabbit muscle	Boeringer
Hexokinase, from yeast	Boeringer
3-Hydroxybutyrate dehydrogenase, grade II, from Rhodopseudomonas spheroides	Boeringer
Pronase, 70,000 PUK/g	BDH
Pyruvate kinase, from rabbit muscle	Boeringer

C. Scintillation counting

"Aquasol"	New England Nuclear
2-(4'-tert-butylphenyl)-5-(4"-biphenylyl) 1,3,4-oxadiazole, Scintran grade	BDH
"Econofluor"	New England Nuclear
"Protosol"	New England Nuclear
Toluene, scintran grade	BDH

D. Radiochemicals.

Adenosine-5'-[γ-^{32}P] triphosphate, triethylammonium salt	Amersham International
Aminopyrine, dimethylamine-^{14}C	New England Nuclear
[1,2-^{3}H] -polyethylene glycol, average mol.wt.4000da	Amersham International

E. Drugs and secretagogues

4-bromophenacylbromide	Sigma
carbamylcholine chloride (carbachol)	Sigma
Cimetidine	Sigma
DibutyrylcyclicAMP, sodium salt	Sigma

Reagent	Supplier
Histamine dihydrochloride	Sigma
Indomethacin	Sigma
3-isobutyl-1-methylxanthine (IMX)	Sigma
Nordihydroguaiaretic acid (NDGA)	Sigma
Quinacrine hydrochloride (mepacrine)	Sigma
Sodium heparin, freeze dried, 17-U/mg	BDH
12-O-tetradecanoylphorbol-13-acetate (TPA)	Sigma

A.2. COMPOSITIONS OF MEDIA.

A.2.1. Krebs-Ringer bicarbonate buffer.

The composition of the Krebs-Ringer bicarbonate medium was (mM): NaCl (120), $NaHCO_3$ (25), KCl (4.5), $MgSO_4$ (1.0), Na_2HPO_4 (1.8), NaH_2PO_4 (0.2) and $CaCl_2$ (1.25).

A.2.2. Preparation of Krebs-Ringer bicarbonate medium containing fatty-acid-free bovine serum albumin.

The medium with which the investigation of the substrate-dependency of acid secretion by isolated parietal cells was carried out was Krebs-Ringer bicarbonate buffer containing 30g fatty-acid-free albumin/litre. Dialysis of albumin was performed to ensure that it was free of potential substrates, and to produce a free-calcium concentration of 1.25mM in the Krebs-Ringer bicarbonate albumin medium as albumin binds calcium. Usually 3g of fatty-acid-free albumin was dissolved in 30ml of Krebs-Ringer bicarbonate medium gassed with O_2:CO_2 (95:5) and placed in a ¾" diameter dialysis bag. The albumin was dialysed against 3 x 200ml of Krebs-Ringer bicarbonate buffer in a stoppered measuring cylinder for 48h at 4°C. After dialysis the medium was made up to 100ml with Krebs-Ringer bicarbonate medium, filtered through sterile disposable filters (0.45um: Schleicher and Schull, Dassel, W.Germany) and stored in aliquots at -20°C.

A.2.3. Composition of Minimum Essential Medium Eagle (modified) with Earle's salts.

The medium was purchased from Flow Laboratories, Ayr, U.K and contained the following constituents (mM): Arginine (0.73), Cystine (0.23), Histidine (0.27), Isoleucine

(0.40), Leucine (0.40), Lysine (0.50), Methionine (0.09), Phenylalanine (0.20), Threonine (0.40), Tryptophan (0.05), Tyrosine (0.25), Valine (0.40), $CaCl_2$ (1.80), KCl (5.37), $MgSO_4$ (0.81), NaCl (116.4), $NaHCO_3$ (23.8), NaH_2PO_4 (1.01) and glucose (5.56). A range of vitamins and cofactors were also present in small quantities as well as 17g phenol red/litre.

A.2.4. Composition of Hanks' Balanced Salts Medium.

The medium was purchased from Flow Laboratories, Irvine, U.K and was composed of the following (mM):- arginine (0.73), cysteine (0.23), histidine (0.27), isoleucine (0.4), leucine (0.4), lysine (0.4), methionine (0.09) phenylalanine (0.2), threonine (0.4), tryptophan (0.05), tyrosine (0.25), valine (0.4), $CaCl_2$ (1.25), KCl (5.37), KH_2PO_4 (0.44), $MgSO_4$ (0.81), NaCl (137), $NaHCO_3$ (4.2), NaH_2PO_4 (10.1), Na_2HPO_4 (0.33), and glucose (5.56). A range of vitamins and cofactors were also present in small quantities as well as 17g phenol red/litre.

A.2.5. Composition of RPMI 1640 medium.

The medium was purchased from Flow Laboratories, Irvine, U.K and was composed of the following (mM):- arginine (1.16), asparagine (0.43), aspartic acid (0.15), cysteine (0.48), glutamate (0.14), glutathione (0.003), glycine (0.14), histidine (0.1), isoleucine (0.4), leucine (0.4), lysine (0.27), methionine (0.1), phenylalanine (0.1), proline (0.17), serine (0.29), threonine (0.17), tryptophan (0.025), tyrosine (0.14), valine (0.17), $Ca(NO_3)_2$ (0.42), KCl (5.37), $MgSO_4$ (0.40), NaCl (103), $NaHCO_3$ (23.8), Na_2HPO_4 (5.56), and glucose (11.2). A range of vitamins and cofactors were also present in

small quantities as well as 5g phenol red/litre.

A.2.6. Preparation of sodium acetoacetate.

A 2M solution of ethyl acetoacetate was hydrolysed with sodium hydroxide (2M) in a volume of 10ml for 1h at 40°C. The solution was then freeze-dried and the product assayed for acetoacetate (A.3.5). On two occasions analysis showed the yield of sodium acetoacetate to be 64% of the theoretical yield.

A.2.7. Preparation of an oleate-albumin complex.

Sodium oleate is only sparingly soluble in water. In order to present it to parietal cells in an accessible form, it had first to be complexed with the albumin present in the incubation medium. This was achieved by adding an aliquot of warmed sodium oleate stock solution (65.3mM) at 50°C via a warmed pipette to a solution of rapidly stirring incubation medium containing 30g albumin/litre. The final concentration of oleate in the albumin-oleate solution was 32.65mM.

A.3. SPECTROPHOTOMETRIC ASSAYS OF SUBSTRATES AND METABOLITES.

Absorbance measurements were made using a Pye Unicam SP30UV spectrophotometer with glass cuvettes (path length 1cm) at room temperature. In all enzymatic assays, the absorbance by NADH or NADPH of light at 340nm (extinction coefficient = 6.3) was used to estimate the amount of NADH or NADPH present, at the beginning and end of a reaction. The appearance or disappearance of NADH or NADPH is stoichiometrically related to the amount of substrate present in a reaction which has gone to completion.

e.g. L-(+)-Lactate + NAD^+ $\rightleftharpoons$ pyruvate + NADH + H^+

In this example the amount of L-(+)-lactate present is stoichiometrically related to the appearance of NADH. To ensure the oxidation of all the L-(+)-lactate present, the reaction equilibrium must be displaced in the direction of NADH formation by removing the products of the reaction. In this reaction, protons are trapped by using a buffer with an alkaline pH, and pyruvate is removed by forming a hydrazone with the hydrazine also present in the buffer. NAD^+ is also present in excess. Calculations were based on the formula:-

$$A = \varepsilon_{340} c\ d$$

where
- A the observed absorbance change
- ε_{340} extinction coefficient of NADH at 340nm.
- c concentration (mM)
- d path length (cm)

Each result was calculated using the formula below:-

$$C = \frac{A}{6.3} \times \frac{\text{volume in cuvette(ml)}}{\text{volume of sample added (ml)}} \times xy$$

C = concentration of substrate or metabolite in blood
A = observed change in absorbance
$\mathcal{X}$ = dilution factor due to deproteinisation
y = dilution factor due to neutralisation

All determinations were made in duplicate with reference to a blank containing all components of the assay system except the substrate being measured. Preparation of substrate samples for analysis is described in 2.2.2. In enzymic assays the reaction was initiated by adding the appropriate enzyme. All assays were first verified by establishing that a linear relationship existed between absorbance and concentration of substrate and that recovery of a known amount of substrate added to whole blood or plasma before deproteinisation was in the region of 95-100%.

A.3.1. Measurement of D-Glucose.

Determination was made essentially according to Bergmeyer et al. (1974). The assay mixture contained, (mM): triethanolamine (260), Mg SO_4 (3.52), ATP (0.41), and NADPH (0.51). To initiate the reaction, glucose-6-phosphate dehydrogenase (EC 1.1.1.49), and hexokinase (EC 2.7.1.1) were added simultaneously giving activities of 0.79U of each enzyme/ml. The change in absorbance at 340nm was determined after 10 minutes.

A.3.2. Measurement of L-(+)-Lactate.

L-(+)-lactate was determined essentially according to the method of Gutmann & Wahlefeld (1974). The concentrations (mM) of the components in the assay mixture were, hydrazine (371), glycine (470), and NAD^+ (1.34) in a final volume of 2.52ml, pH9.0. Lactate dehydrogenase (EC 1.1.1.27) was

added to give an activity of 12U/ml and the resulting change in absorbance at 340nm was measured after 40 minutes.

A.3.3. Measurement of Pyruvate.

Determination was made essentially according to Czok & Lamprecht (1974). The assay mixture contained (mM):- triethanolamine (131), EDTA (1.64) and NADH (0.09) in a final volume of 3.05ml, pH 7.6. The addition of lactate dehydrogenase (EC 1.1.1.27) gave an activity of 1 U/ml and the change in absorbance at 340nm was measured after 5 minutes.

A.3.4. Measurement of Glycerol.

Determination was made using the method of Wieland (1974). The assay mixture contained (mM): hydrazine (690), glycine (137), $MgCl_2$ (1.37), ATP (1.23), and NAD (0.49) in a final volume of 2.04ml, pH 9.8. Glycerophosphate dehydrogenase (EC 1.1.1.8) was added to give an activity of 4U/ml. and after checking that the absorbance had not changed, glycerokinase (EC 2.7.1.30) was added to initiate the reaction (activity = 8.5 U/ml). The change in absorbance at 340nm was determined after 30 minutes.

A.3.5. Measurement of Acetoacetate.

Determination was made according to Mellanby & Williamson (1974). The assay mixture contained (mM): phosphate buffer (33), NADH (0.2), in a final volume of 3.1ml, pH 6.8. D-3-hydroxybutyrate dehydrogenase (EC 1.1.1.30) was added to give an activity of 50mU/ml . The reaction end point was estimated by extrapolation of absorbance

measurements at 340nm made after 10,15 and 20 minutes. This was necessary because of the low activity of the D-3-hydroxybutyrate dehydrogenase preparation. Extrapolation was performed by extending the readings to an end point by assuming an hyperbolic reaction rate curve.

A.3.6. Measurement of D-3-hydroxybutyrate.

Determination was carried out using the method of Williamson & Mellanby (1974). The concentrations (mM) of the components of the assay mixture were, hydrazine (330) tris (22), EDTA (0.86) and NAD^+ (0.4) in a final volume of 3.1ml, pH 8.5. D-3-hydroxybutyrate dehydrogenase (EC 1.1.1.30) was added to give an activity of 50 mU/ml. As for the assay of acetoacetate, the end point of the reaction was estimated by extrapolation of absorbance measurements made after 40, 50, and 60 minutes.

A.3.7. Measurement of Ammonia.

Ammonia, as NH_4^+, was measured by the method of Kun & Kearney (1974). The final concentrations (mM) in the assay mixture were, tris (100), 2-oxoglutarate (10) and NADH (0.24) in a volume of 2.0ml, pH 8.0. The reaction was initiated by the addition of glutamate dehydrogenase (EC 1.4.1.3) to give 9 U/ml. The change in absorbance after 90 min. was measured in sealed cuvettes to prevent contamination by atmospheric ammonia.

A.3.8. Measurement of Glutamate.

Determination was carried out essentially according to the method of Bernt & Bergmeyer (1974). The assay mixture contained (mM): hydrazine (340), glycine (430), NAD^+

(0.9) and ADP (0.34) in a final volume of 2.1ml., pH 9.0. Glutamate dehydrogenase (EC 1.4.1.3) was added to give an activity of 5.71 U/ml. The change in absorbance at 340nm was measured after 60 minutes.

A.3.9. Measurement of Glutamine.

Glutamine was measured essentially according to Lund (1974) in which the amount of glutamate is measured (A.3.8) before and after conversion of glutamine to glutamate by glutaminase (EC 3.5.1.2). Digestion by glutaminase was carried out in 273mM-acetate buffer, pH 5.0 for 1h at 37°C using 0.41 U enzyme/ml. After hydrolysis, an aliquot of the digest was assayed for glutamate directly.

A.3.10. Measurement of Non-Esterified Fatty Acids.

Non-esterified fatty acids were measured in plasma samples by the method of Falholt et al. (1973) which is based on the formation of copper soaps of the free fatty acids, which are determined colourimetrically. 50µl samples were added to 1ml phosphate buffer, pH 6.4 and 6ml. chloroform: heptane:methanol (40:40:1.6) in a 20ml scintillation vial and shaken (160 cycles/min) for 3min. After standing for 15 min., the mixture was centrifuged (4000rpm, 10min). The aqueous phase was removed and 5ml. of the organic phase shaken (160 cycles/min), with 2ml of Copper-triethanolamine buffer, pH 8.1 for 5 min. After centrifugation (2800g,5min) 3ml. of the upper phase was mixed with 0.5ml of 0.4% (w/v) diphenylcarbazide solution containing 10mM triethanolamine. After 15 min., the absorbance was measured at 550nm. A standard curve was prepared using palmitic acid for each set of determinations.

A.4. AMINO ACID ANALYSIS.

Amino acids were measured in samples of plasma, which had been deproteinised with 3% (w/v) sulphosalicylic acid and stored at -20°C (2.2.2), on an amino acid analyser (Locarte, London). Samples (0.5ml) were loaded on to the top of a Locarte 'microbead' ion-exchange resin (length-200mm; diameter - 9mm) followed by 0.02M-HCl (0.5ml) then Buffer 1 (see below) containing 30% (v/v) methanol (3.0ml). The flow-rate was 1.0ml/min. and the column temperature 55°C, initially, and 65°C after 64 min. The following table shows the composition of the three running buffers and their running times.

Buffer	pH	Na^+ (M)	Citrate (M)	Borate (M)	Thiodi-glycerol (%v/v)	Polyoxy-ethylene. lauryl ether(%v/v)	Time (min)
1	3.17	0.255	0.1	---	0.5	0.07	30
2	4.25	0.210	0.1	---	0.3	0.07	40
3	9.45	0.420	0.12	0.033	0.8	0.07	66

The column eluate was mixed with buffered ninhydrin solution, heated (100°C, 10min) and the absorbance measured at 570nm and 440nm.

The column was regenerated at the end of the run with 0.2M NaOH containing polyoxyethylene laurylether (0.07%, v/v) for 20min, and equilibrated with buffer 1 for 60min.

A.5. SCINTILLATION COUNTING.

A.5.1. Dual-label counting.

In experiments where the aminopyrine ratio was determined both $[^{14}C]$ and $[^{3}H]$ had to be counted in the same sample. To separate the pulses of radioactivity and resultant scintillations produced from the two radionuclides, two counting channels are necessary. In the Packard Tri-carb 2600 Scintillation Counter, the limits of the low and high energy channels are set by the machine in accordance with the scintillation system and degree of quenching in each sample. This reduces the counting efficiency of the low energy nuclide, $[^{3}H]$, in the high energy channel to zero and the counting efficiency of the high energy nuclide, $[^{14}C]$, in the low energy channel to a minimum. To calculate the activity of each radionuclide present, the counting efficienty (cpm/dpm) of the two radionuclides in both channels are determined. Efficiency is calculated by the method of the external standard channels ratio (ESR) from a curve relating efficiency to ESR. The curve is generated by providing the machine with a series of differently quenched samples containing a known amount of radioactivity of $[^{14}C]$ and $[^{3}H]$. The resultant quench curve is then stored in the counter's memory.

For each sample cpm are converted to dpm via the following equations:-

$$Y = \frac{B}{E_4} \qquad X = \frac{A - E_3Y}{E_1}$$

Y = dpm of the higher energy radionuclide

B = total cpm of higher energy channel

E_4 = efficiency of higher energy radionuclide in higher energy channel

X = dpm of lower energy radionuclide

A = total cpm of lower energy channel

E_3 = efficiency of higher energy radionuclide in lower energy channel

E_1 = efficiency of lower energy radionuclide in lower energy channel

Using $[^{14}C]$ aminopyrine and $[^{3}H]$ PEG the following counting efficiencies were achieved:-

$[^{14}C]$ in high energy channel	64%
$[^{14}C]$ in low energy channel	5%
$[^{3}H]$ in low energy channel	29%

A.5.2. $[^{32}P]$ counting.

Homogeneous samples were counted, with efficiencies determined by ESR as in A.5.1. An efficiency of approximately 80% was achieved for determination of $[^{32}P]$ emmissions in the low energy channel.

$[^{32}P]$ was also determined in heterogeneous samples consisting of protein containing $[^{32}P]$ absorbed onto a solid-phase support of Whatman 31ET filter paper. The radioactive source remains on the paper support during counting and, therefore, the efficiency and reproducibility of the counting is dependent on several factors. The magnitude of the β-energy, the nature of the solid support, its orientation in the vial and the amount of sample dissolving in the scintillation solvent are the main factors involved. $[^{32}P]$ has a high β-energy (1.71 MeV) and so there is no problem with self-absorption

due to the structure of the paper. However the very high energy range of β-particles emitted from [^{32}P] samples may result in some emissions escaping from the sample through the bottom of the vial without causing a scintillation. This is made more likely because the filter papers lie on the bottom of the vial. The amount of sample dissolving in the scintillation solvent is minimal but cannot be determined easily. Because of the factors described, quench correction, in the same way as described for homogeneous samples, cannot be justified for heterogeneous samples, and the results are therefore given as cpm.

A.6. STATISTICS.

A.6.1. Student's t-test.

This analysis was used to determine whether two sample means, drawn from normal populations, were different from each other, for example in Chapter 7, was there a significant difference between the average cell viabilities under control conditions and after treatment with TPA? The calculation of t is based on the formula below and the calculated value of t is compared with a table of t values for a given degrees of freedom $(n_1 + n_2 - 2)$.

$$t = \frac{\bar{X}_1 - \bar{X}_2}{\frac{1}{n_1} + \frac{1}{n_2}} \quad \text{where} \quad = \frac{(n_1 - 1)S_1^2 + (n_2 - 1)S_2^2}{n_1 + n_2 - 2}$$

$\bar{X}_1, \bar{X}_2$ means ; S_1, S_2 standard deviations ; n_1, n_2 number of observations

A.6.2. Student's Paired t-test.

In some experiments, individual observations made to study the effect of two treatments could be paired as, for example, when comparing the arterial and venous substrate concentrations (Chapter 2) and determining whether the difference was significantly different from zero. Pairing was justified, in this instance, as the observations were made on the same animal in each experiment. In a paired t-test the calculation of t is based on the formula below and the calculated value of t is compared with a table of t values for a given degrees of freedom (n - 1).

$$t = \frac{\frac{\Sigma D}{n}}{\sqrt{\frac{D^2}{n-1} + \frac{(\Sigma D)^2}{n(n-1)}}}$$

D differences between the individual pairs of observations

n total number of paired observations.

A.6.3. Linear Regression Analysis.

This tests for a significant relationship between two variables. A relationship, in which one variable is dependent on the magnitude of the other, is termed a regression and may be analysed by calculating the regression coefficient, b. In this, the data points are compared for "closeness of fit" to the calculated slope of the best fit regression line by the method of least squares.

In the studies made with arteriovenous differences (Chapter 2), it was not possible to fully justify the analysis of a relationship by regression, as neither of the variables, arterial concentration and arteriovenous difference, could be said to be independent and predeterminable. In such experiments it is more valid to test the correlation between the two variables, which vary independently of each other. This is a measure of the intensity of the association between the two variables, which can be tested by calculating the correlation coefficient, r, via the formula below, and comparing this with a table of r values with n-2 degrees of freedom.

$$r = \frac{\Sigma xy}{\sqrt{\Sigma x^2 \Sigma y^2}}$$

Σx the deviation of each x value from the mean of x

Σy the deviation of each y value from the mean of y

Under certain circumstances it may be necessary to compare two correlation coefficients. For example, a

significant relationship between the arterial concentration and arteriovenous difference for a metabolite under control and acid-secreting conditions. Before applying the formula below, it is necessary to transform each r value, representing the correlation coefficient in the population sampled to a value, z, the inverse hyperbolic tangent of r (Zar, 1974),

$$z = 0.5 \ln \left(\frac{1 + r}{1 - r} \right)$$

A Z value for the comparison is then calculated, via the formula below, and compared with a table of Z values with n - 3 degrees of freedom.

$$Z = \frac{z_1 - z_2}{\sqrt{\frac{1}{n_1 - 3} + \frac{1}{n_2 - 3}}}$$

z_1, z_2 z values from each correlation ; n_1, n_2 number of observations

A.6.4. Analysis of Variance.

In some experiments it was necessary to analyse the effect of more than one factor on a particular variable. For example, in Chapter 5, the effect of different substrates on the accumulation of aminopyrine by parietal cells, together with the effect of different cell batches. A simple two-way analysis of variance tests for significant effects of both of these factors on the variable, and is represented in the following algebraic notation,

	replications (A)					
treatment(B)	1	2	3	4	sum	mean
1	AB1,1	AB1,2	AB1,3	AB1,4	B_1	B_1
2	AB2,1	AB2,2	AB2,3	AB2,4	B_2	B_2
3	AB3,1	AB3,2	AB3,3	AB3,4	B_3	B_3
4	AB4,1	AB4,2	AB4,3	AB4,4	B_4	B_4
5	AB5,1	AB5,2	AB5,3	AB5,4	B_5	B_5
6	AB6,1	AB6,2	AB6,3	AB6,4	B_6	B_6
7	AB7,1	AB7,2	AB7,3	AB7,4	B_7	B_7
8	AB8,1	AB8,2	AB8,3	AB8,4	B_8	B_8
9	AB9,1	AB9,2	AB9,3	AB9,4	B_9	B_9
10	AB10,1	AB10,2	AB10,3	AB10,4	B_{10}	B_{10}
11	AB11,1	AB11,2	AB11,3	AB11,4	B_{11}	B_{11}
12	AB12,1	AB12,2	AB12,3	AB12,4	B_{12}	B_{12}
sum	A_1	A_2	A_3	A_4	AB	
mean	A_1	A_2	A_3	A_4		AB

replications = cell batches ; treatments = different substrates

The sum of squares for each factor is calculated as follows:-

$$\text{Correction} = C = \frac{(AB)^2}{(4)(12)}$$

$$\text{Total} = AB_{1,1}{}^2 + AB_{1,2}{}^2 + \ldots + AB_{12,4}{}^2 - C$$

$$\text{Treatments} = b = \frac{B_1{}^2 + B_2{}^2 + \ldots + B_{12}{}^2}{4} - C$$

$$\text{Replications} = a = \frac{A_1{}^2 + A_2{}^2 + A_3{}^2 + A_4{}^2}{12} - C$$

$$\text{Residuals} = e = \text{Total} - (\text{Treatments} + \text{Replications})$$

The mean squares are calculated by dividing the sums of squares by the degrees of freedom for each factor. The mean squares for each factor are then divided by the residual mean square to give an F value, which is compared with a table of F values with the degrees of freedom associated with the factor in question and the residuals. These

calculations may be summarised by the following table,

source of variation	degrees of freedom	sum sq.	mean sq.
treatments	4 - 1 = 3	b	b/3
replications	12 - 1 = 11	a	a/11
residuals	47-(3 + 11)=33	e	e/33
total	(4)(12) -1=47	a+b+e	

This analysis may be adapted to test for the significance of more than one level of two factors, and these analyses are described together with the appropriate data (see Chapter 7).

calculations may be summarised by the following table,

source of variation	degrees of freedom	sum sq.	mean sq.
treatments	4 - 1 = 3	b	b/3
replications	12 - 1 = 11	a	a/11
residuals	47-(3 + 11)=33	e	e/33
total	(4)(12) -1=47	a+b+e	

This analysis may be adapted to test for the significance of more than one level of two factors, and these analyses are described together with the appropriate data (see Chapter 7).

A.7. THE AMINO ACID COMPOSITIONS OF TWO PROTEINS SECRETED BY THE GASTRIC MUCOSA.

A.7.1. Pepsinogen.

The following table represents the amino acid composition in terms of number of residues, of porcine pepsinogen as determined by Dayhoff (1975).

Amino acid	No. of residues	Amino acid	No. of residues
Alanine	16	Leucine	28
Arginine	4	Lysine	10
Aspartate	26	Methionine	4
Asparagine	14	Phenylalanine	12
*Asx	4	Proline	17
Cysteine	6	Serine	48
Glutamine	7	Threonine	25
Glutamate	19	Tryptophan	5
Glycine	34	Tyrosine	17
Histidine	3	Valine	22
Isoleucine	22	‡Glx	2

*aspartate or asparagine ‡glutamate or glutamine

A.7.2. Sulphated glycoproteins.

The following table shows the amino acid composition of one of the main fractions of sparingly soluble mucin from canine gastric mucosa (Kim & Horowitz, 1971). The data is expressed as μ moles amino acid per 100 umoles of amino acid residues.

Amino acid	occurrence	Amino acid	occurrence
Alanine	4.2	Tyrosine	1.3
Aspartate	4.2	Proline	12.6
Glutamate	6.2	Serine	17.8
Glycine	6.6	Threonine	22.3
Cysteine	2.1	Valine	9.8
Isoleucine	1.5	Arginine	2.1
Leucine	3.5	Histidine	2.4
Methionine	0.6	Lysine	2.1
Phenylalanine	1.5		

A.8. PHOTOGRAPHY.

Photographs were taken on a Carl-Zeiss Photomicroscope II incorporating a 35mm camera with automatic exposure control. 100 ASA black and white negative film and 100 ASA colour transparency films were used.

A.9. THIN LAYER CHROMATOGRAPHY OF [^{14}C]- AMINOPYRINE.

This method follows that of Sack & Spenney (1982). [^{14}C] aminopyrine (1 µl, 100,000dpm) and unlabelled aminopryine (1 µl, 10 µg) were applied to an aluminium-backed silica gel 60 TLC plate, which was placed in a tank containing butyl acetate : methanol : 85% formic acid (60:40:20). After chromatography, the solvent front was marked and the plate allowed to dry. Cochromatographed unlabelled aminopyrine was visualised in an iodine chamber by the development of a yellow colouration. Radioactivity was localised and quantified by cutting the plate into 1cm strips and scraping the silica gel coating into scintillation vials, containing 10ml Econofluor. In one experiment 92% of the counts applied to the plate were recovered and 13% of the total counts had an Rf value different from authentic aminopyrine.

	Rf
Authentic unlabelled aminopyrine	0.243
[^{14}C] aminopyrine	0.239
[^{14}C] impurity	0.659

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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