The isolation of gastric mucosal cells and a study of their isoenzyme patterns

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SUMMARY.

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Preliminary experiments on cell isolation from rat and pig gastric mucosa revealed that the chelating agent, ethylenediaminetetraacetic acid, or the enzyme collagenase were unsuitable as cell releasing agents for stomach tissue from these two species. Pronase was found to be singularly effective for dissociating the tissue and provided a sufficiently high yield of viable cells for further study.

A cell isolation procedure was subsequently developed using pronase and mild mechanical action to release all cell types from prepared pig fundic mucosa. Separation of the different cell types found in gastric mucosa was accomplished by centrifuging the mixed cell suspension in a discontinuous Ficoll density gradient.

Light microscopy, cell size estimations and a specialised staining technique were used to examine the cell suspensions obtained from the interphases of the gradient. These studies revealed that an upper fraction contained a relatively pure suspension of parietal cells. The majority of mucous secreting cells were obtained from the lower fractions. Homogenous preparations of surface mucous cells were obtained by abrading the surface epithelium of the mucosa. In a few experiments, concentration of the chief cell population was achieved by subjecting one of the lower fractions to a second gradient of higher Ficoll densities.

The cellular fractions and abrasion, together with samples of fundic and pyloric mucosa were examined for isoenzymes of lactate dehydrogenase, creatine phosphokinase and carbonic anyhydrase. Marked differences were observed when the lactate dehydrogenase isoenzyme patterns of the cellular fractions were compared, and the differences appeared to be related to the proportion of individual cell populations in the fractions. The suspensions of parietal cells exhibited a high level of lactate dehydrogenase-1 isoenzyme, whereas those containing predominantly mucous cells, and the abrasion sample revealed a preponderance of lactate dehydrogenase-3 and-4.

The only creatine phosphokinase isoenzyme activity detected was in samples of fundic mucosa, and no carbonic anhydrase activity could be found in any of the tissue and cell samples analysed.

The cell isolation method produced equally satisfactory results when applied to human gastric mucosa. The characteristic lactate dehydrogenase isoenzyme patterns found in the cellular fractions from pig stomach were also observed in samples obtained from human tissue.

Declaration.

I hereby declare that the whole of the work submitted in this thesis is the result of my own investigations except where reference is made to published literature and where assistance is acknowledged.

Candidate

Director of Studies.

Certificate.

I hereby declare that the work embodied in this thesis has not already been submitted in substance for any degree, and is not being concurrently submitted in candidature for any degree.

Signed _____

Date 17th December 1975.

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"For this is the nature of things, that the process of an objective experimental research finally reaches a certain stage of maturity where, I maintain, the true spirit of the subject emerges in its simplicity and shows itself to the Scientist in all its shining glamour".

Jan Ev. Purkyně. Czech. Physiologist 1840.

To my wife, Janice, in appreciation of her

sacrifices and constant devotion.

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1. THE MAMMALIAN STOMACH

1. THE MAMMALIAN STOMACH.

A basic understanding of the anatomy and physiology of the mammalian stomach is required before attempting an investigation of the cells isolated from this tissue. The descriptions that follow have been taken from the published work of Lillibridge (1964), Ito (1967) and Davenport (1971).

The stomach is a specialised segment of the alimentary tract between the oesophagus and small intestine. A common feature of the entire alimentary tract is its four consecutive layers : (1) mucosa or mucous membrane, (2) submucosa, (3) muscularis and (4) serosa, (Fig. 1c). Although the three outer layers are similar in diverse parts of the alimentary tract, the mucosa has distinctive structural and functional features.

1.1. The glandular mucosa.

This type of epithelium contains specialised cells which distinguish the stomach from the rest of the alimentary tract. The entire surface of the epithelium is lined by a layer of simple columnar cells, surface mucous cells. The surface has numerous tubular invaginations, the gastric pits or foveolae. One or more simple or branched tubular glands containing specialised secretory cells open into the bottom of the gastric pits. The epithelium rests on a delicate basement membrane and the underlying lamina propria is composed of connective tissue cells and fibres, as well as blood vessels, lymphatics, nerves and smooth muscle cells. The muscularis mucosa is an inner circular and outer longitudinal layer of smooth muscle and forms the border between the gastric mucosa and submucosa.

Three types of gastric glands are found in the glandular mucosa. They are the cardiac, pyloric and fundic glands.

1.1.1. Cardiac glands.

These are found in the region near the oesophageal orifice. The zone occupied by the cardiac glands varies from a narrow rim of a few mm. or less in the cat, dog or human stomach, to an extensive area in the pig gastric mucosa (Figs. 1a, 1b). The gastric pits are relatively short and joined by tortuous branched, tubular glands composed of cardiac mucous cells and a few argentaffin

cells. The mucous cells are histologically similar to mucous neck cells. Chief cells and parietal cells are absent from this area. The functional significance of the cardiac glands has not been well defined, although extraction of the hormone gastrin from the glands has been reported (Gregory and Tracy, 1961).

1.1.2. Pyloric glands.

These glands occupy a considerably larger area than that of the cardiac glands. Surface mucous cells line the deep gastric pits which occupy at least half of the total thickness of the epithelium. In the isthmus of the glands surface mucous cells are present, but the remainder of the simple branched tubular glands is formed by cells resembling mucous neck cells of the fundic glands. Although the major secretion from the pyloric glands is mucus, it has been shown in dog stomach that pepsinogen is also secreted (Grossman and Marks, 1960). More recently, Etherington and Taylor (1970), using agar gel electrophoresis, have separated several zones of proteolytic activity from human gastric mucosa. They observed that the pepsinogens extracted from pyloric and fundic mucosa exhibited differences in the number and intensity of zones. Gastrin is released from pyloric glandular mucosa and is partly responsible for the stimulation of pepsinogen secretion.

1.1.3. Fundic glands.

This type of gland occupies most of the gastric mucosa, but the amount varies in different animals. They are characterised by the presence of the parietal and the chief cells. In addition there are surface mucous, mucous neck and occasional argentaffin cells (Fig. 1d). Several glands may be associated with a foveola, and empty into a gastric pit. In the human stomach the glands are approximately 1.2 mm. long and 50 μm. wide. The foveola of the glands is usually straight, but the neck and base regions are tortuous. The foveola is lined entirely with surface mucous cells. The short portion of the gland beneath the foveola, the isthmus region, is composed predominantly of mucous cells although some parietal and occasional chief cell may be present. Parietal cells predominate in the neck region and are interspersed with some chief and mucous neck cells. The base of the glands is composed mainly of chief cells.

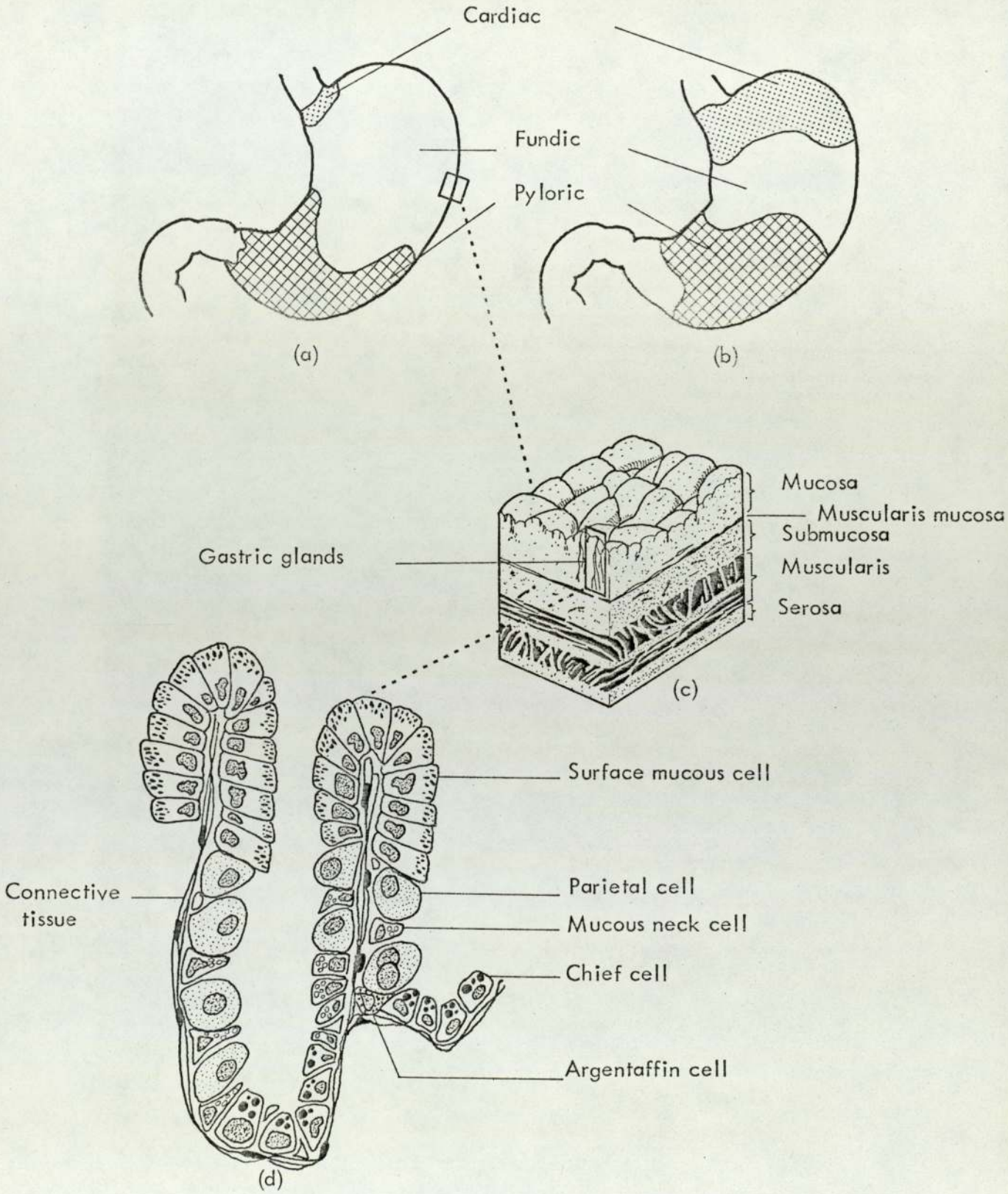

Fig. 1. Diagram of the glandular regions of pig (a) and human stomach (b).
(c) A view of the stomach wall and its four layers.
(d) A fundic gland.

The predominant organic secretion of the surface mucous cells is visible mucus. Desquamation frequently accompanies mucus secretion. It is secreted as a viscous gel and tenaciously covers the mucosa in the resting state. Surface mucous cells are distinguished by their location, columnar shape and content of dense mucous granules which often occupy much of the supranuclear cytoplasm (Fig. 2). Histochemical tests such as the periodic acid - Schiff (PAS) reaction readily demonstrate their mucopolysaccharide content. A fairly orderly progression of changes is apparent as the surface mucous cells are observed between the upper parts of the gastric pit and the isthmus region. The cells on the luminal surface are slender truncated pyramids measuring approximately 5 μm. by 25 μm. Towards the isthmus of the gland they become progressively shorter (about 9 μm.), and their mucous content is diminished.

The mucous neck cells are the second type of mucus secreting cell in the gastric mucosa. They secrete a soluble mucus, which is chemically and physically distinct from the secretion of the surface cells. The mucus is absent from the fasting gastric juice, but is secreted in response to parasympathomimetic or vagal excitation. These cells are found predominantly in the neck region and are readily distinguishable from the parietal cells but may be confused with the chief cells unless the PAS reaction is employed to demonstrate their mucopolysaccharide content. The mucous granules are larger and more spherical than those observed in the surface mucous cells, and often found in the paranuclear or basal cytoplasm (Fig. 3).

The chief cells are usually the predominant cell type at the base of the fundic glands, and measure approximately 7 μm. by 16 μm. The histological characteristic of these cells is the abundant basal ergastoplasm, which accounts for the cytoplasmic basophilia, and the numerous large apical zymogen granules measuring up to 3 μm. in diameter (Fig. 4). The mode of pepsinogen granule secretion appears to be quite similar to secretion of pancreatic zymogen granules. The inactive pepsinogen is converted to proteolytically active pepsin when it mixes with acid secreted in the gastric juice.

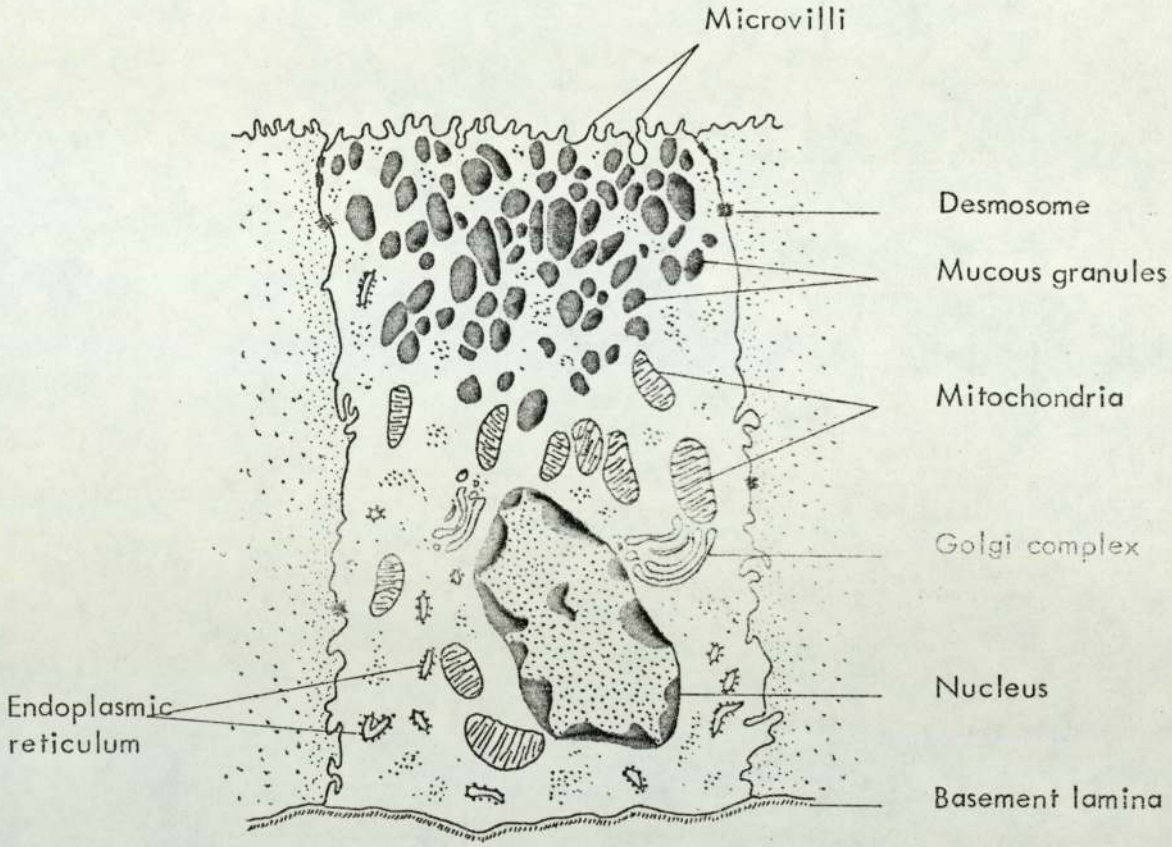

Fig. 2. Diagram of a surface mucous cell.

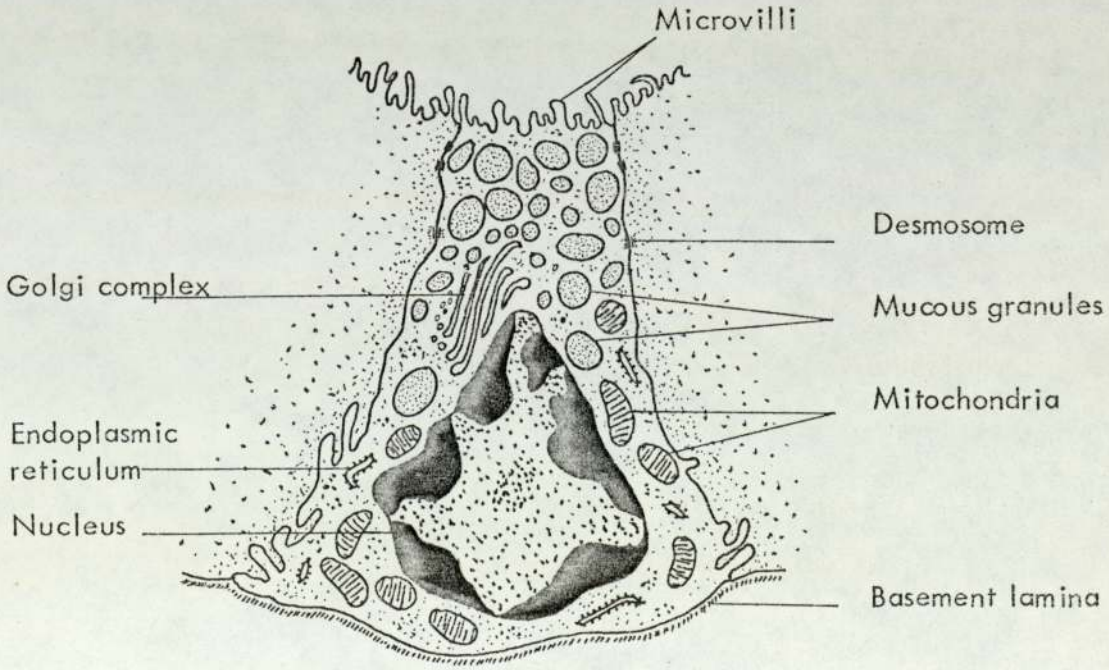

Fig. 3. Diagram of a mucous neck cell.

Parietal cells appear by conventional light microscopy as large (20 μm. to 35 μm.) oval cells which lie with a considerable portion of their surface against the basement membrane. These cells are numerous in the neck and isthmus region of the gastric glands but are also found in the base of some glands where chief cells are sparse or absent. The cytoplasm contains numerous rod-shaped and oval mitochondria, and stains vividly with acid dyes in routine histological stains. No secretory granules are found. Electron microscopical studies have revealed an unusual specialisation in the parietal cell in the form of an intracellular canaliculus. The canaliculus is bounded by a membrane which is continuous with the plasma membrane of the cell, and is formed into microvilli that project into the intracellular lumen (Fig. 5).

The probable association of hydrochloric acid production in the stomach with the parietal cells, prompted Rohrer et al. (1965) to study these cells during various stages of secretory activity in human gastric mucosa. After histamine stimulation, the intracellular canals in the parietal cells became more prominent and complex, in contrast to the cells in the non-secreting state. The morphological changes observed were thought to be due to the process of acid secretion. Simular observations in the parietal cell secretory canaliculi during secretion have been made by Sedar and Friedman (1961) in the dog and by Helander (1962) in the mouse. Vial and Orrego (1960), noted a large increase in the membrane area of the canaliculi of the cat parietal cell during acid secretion, and suggested the cytoplasmic vesicles as the source of plasma membrane.

The argentaffin cells of the gastric mucosa are found only infrequently and are poorly understood. They are lodged between the basal parts of the glandular epithelial cells and always have a broad base. Some have cytoplasmic extensions to the lumen. The cells have dense granules concentrated in the basal or infranuclear regions.

1.1.4. Cell renewal in gastric glandular mucosa.

Studies on cell renewal in the gastric mucosa have shown that it is one of the most rapidly proliferating epithelial tissues. Using autoradiographic techniques, Messier and Leblond (1960) observed mitotic activity in the two types of mucous cells

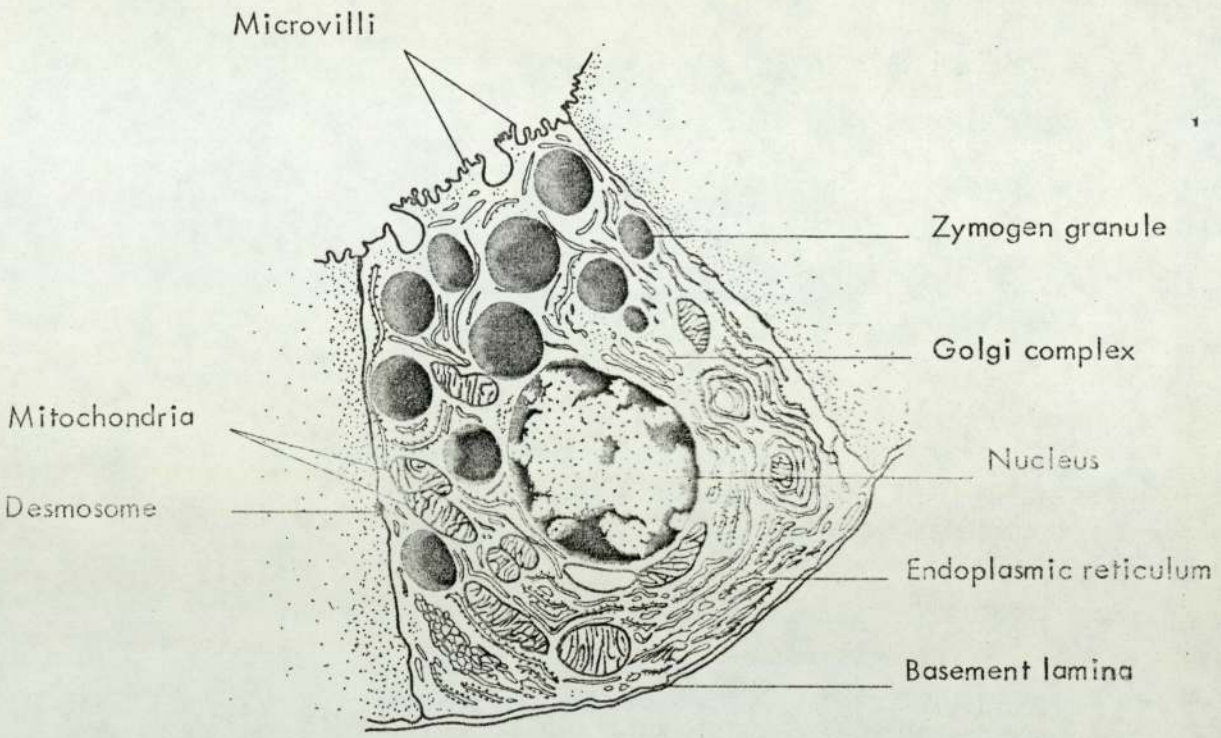

Fig. 4. Diagram of a chief cell

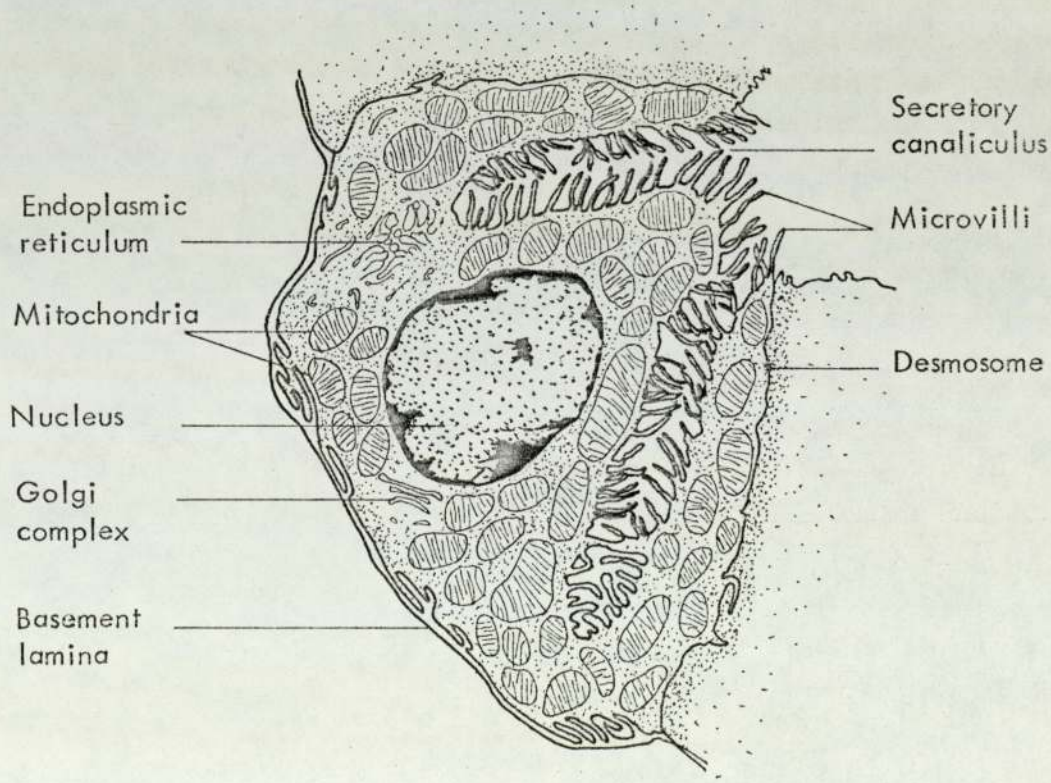

Fig. 5. Diagram of a parietal cell.

and demonstrated the movement of the labelled surface mucous cells from the isthmus to the gastric pits, and finally to the luminal surface to replace desquamated cells. At the same time the labelled mucous neck cells were restricted to the neck region of the glands. Hunt and Hunt (1962), verified the study of Messier and Leblond and also noted that several days after administration of tritiated thymidine, some of the labelled cells in the mucosa were parietal cells. They concluded that the parietal cells and possibly chief cells are replaced by less differentiated mucous neck cells. Lipkin et al. (1963) however, investigating cell proliferation kinetics in human stomachs found very few parietal or chief cells labelled with H^3-thymidine, and Lee (1972) observed no significant incorporation of H^3-thymidine into rabbit parietal cells, even after histamine stimulation.

It seems therefore, unlikely that the chief or parietal cells undergo division. Matsuyama and Suzuki (1970) grafted mucosa in the subcutaneous tissue of mice and demonstrated by ultrastructural classification that mature mucous, parietal, chief and argentaffin cells are differentiated from a multipotential precursor, the immature mucous neck cell.

1.1.5. Lamina propria.

The lamina propria occupies the area beneath the gastric epithelial cells and the muscularis mucosae (Fig. 1d). In the fundus and corpus regions the gastric glands are closely packed and there is little lamina propria in contrast to the cardiac and pyloric areas where it is more prominent. The number of cells present in the lamina propria is variable, but it is not uncommon to find lymphocytes and plasma cells as well as mast cells and eosinophils. The basement membrane underlies all gastric epithelial cells and is formed by the thin amorphous basement lamina and some associated collagen and reticulin fibres.

2. INTRODUCTION.

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2.1. The isolation of cells from animal tissues.

Numerous methods have been published for dissociating tissues into individual cells, either by mechanical disruption or by altering the intercellular attachments with chemical or enzymatic treatment. The diversity of intercellular binding materials (Rinaldini, 1958), and the presence of specialised intercellular attachments such as desmosomes (Fawcett, 1958; Farquhar and Palade, 1963; Tamarin and Sreebny, 1963), have not afforded the use of a single method equally applicable to all tissues.

The use of mechanical stress has been utilised to disperse cells. In an an attempt to prepare cell suspensions from gastric mucosa, BreMiller and Davenport (1961) chopped the tissue into fragments with razor blades then triturated the pieces by repeated squirting through a small syringe. Sjöstrand (1968) placed everted rat gut segments on a glass rod attached to a stirrer motor, and gently rubbed the mucosa with a piece of plexiglass as the tissue revolved, releasing sheets of intestinal mucosa cells. Fine metal sieves (Kaltenback, 1954; Mateyko and Kopec, 1963) and loose-fitting tissue homogenisers have also been employed to release cells in a variety of tissues (Berry, 1962; Jacob and Bhergava, 1962).

Controlled mechanical forces in the form of low amplitude, high frequency vibration has been found to yield suspensions of intact intestinal epithelial cells when used on its own (Levine and Weintraub, 1970; Moore et al., 1971), and in conjunction with the chelating agent, ethylenediaminetetra acetic acid (EDTA), by Harrison and Webster (1964).

The application of chemical or enzyme treatment for cell dispersion takes precedence over mechanical methods, although many procedures incorporate some form of additional mild mechanical action to enhance their effect.

Since it has been shown that divalent cations, notably calcium, play an important role in the maintenance and structure of the functional complexes between cells (Sedar and Forte, 1964), several investigators have employed chelating agents to 'loosen' the cellular attachments then separate the cells with mechanical treatment.

Sodium citrate (Stern and Jensen, 1966; Reiser and Christiansen, 1971), and EDTA (Kimmich, 1970; Evans et al., 1971) have been used to isolate intestinal epithelial cells in different animals. The dissociation of mouse liver by sodium tetrophenylboron (TPB), a potassium complexing agent, prompted Rappaport and Howze (1966) to suggest that potassium is involved in the adhesion of cells in liver. The use of citrate and TPB however, has been criticised by Kerkof et al. (1969), who found that citrate-dissociated rat liver cells failed to carry out normal ribosomal protein synthesis, and TPB inhibited enzyme activity by uncoupling energy transfer reactions in the mitochondria.

Since Rous and Jones (1916) used trypsin with avian and mammalian tissue, and later Moscona (1952) with embryonic tissue, many methods employing enzymes as dissociating agents have been developed. Although trypsin is still used on its own for cell dispersal in such tissues as adrenal glands (Sayers et al. 1971), and thyroid glands (Burk and Kowalski, 1971), it has been largely superseded by relatively specific proteases such as elastase, collagenase and hyaluronidase, or used in conjunction with one of these enzymes.

Cells have been successfully isolated from adipose tissue (Rodbell, 1964), adrenal glands (Kloppenborg et al., 1968) and gastric mucosa (Walder and Lunseth, 1963) using collagenase solutions. A mixture of collagenase and hyaluronidase was found to be particularly suitable for isolating liver parenchymal cells (Howard et al., 1967; Howard and Pesch, 1968; Berry and Friend, 1969). Cell suspensions prepared from mammalian tissue and tumours using collagenase and hyaluronidase, revealed no appreciable effect on the surface charge of the isolated cells, and were found to be suitable for measurement of the electrical charges of the surface membrane (Yamada and Ambrose, 1966).

An alternative method employed for isolating cells has been to perfuse physiological calcium-and-magnesium-free solutions into the blood vessels before removing the tissue, which is sliced or minced and placed in a solution containing an enzyme such as collagenase (Huang and Lin, 1965; Pisano et al., 1968; Lentz and Di Luzio, 1971), or lysozyme (Huang, 1965). Morphological studies of rat liver cells prepared by perfusion with chelating agents, however, have revealed cellular damage (Howard et al., 1967). Similar observations were made when a

combination of trypsin and EDTA were used to dissociate mouse liver (Rappaport and Howze, 1966), and embryonic chick thyroid (Hilfer and Hilfer, 1966). In the latter study, using ultrastructural classification, it was noted that when trypsinization was followed by EDTA treatment, swelling of intracellular compartments occurred, even using relatively short incubation times. These changes were attributed to the effects of EDTA.

A proteolytic enzyme with a broad substrate specificity was prepared from Streptomyces griseus by Nomoto et al. (1960). It became commcercially available in a partially purified form under the name 'Pronase', and was used by Gwatkin and Thomson (1964), to disperse cells from mouse embryos and found to be far superior to trypsin. Equally successful results were obtained with adult mouse lung and kidney. Pronase was subsequently used to isolate viable cells from Necturus gastric mucosa (Blum et al., 1971), in preference to hyaluronidase, collagenase, EDTA, trypsin and mechanical disruption. Its mucolytic property was found to be a particular advantage with this type of tissue.

The separation of different types of cells has been achieved by a variety of methods. Subjecting the same tissue to different treatments has sometimes yielded cells of differing functional activity. Garvey (1961) obtained reticulo-endothelial cells by digesting liver with trypsin and collagenase, and parenchymal cells by perfusion and subsequent mechanical disruption. Lentz and Di Luzio (1971) perfused samples of liver with collagenase, followed by trypsin digestion, or with a mxiture of collagenase and hyaluronidase, to obtain kupffer cells or parenchymal cells respectively.

Cell surface properties have been used to separate mixtures of cells, by counter-current distribution. Particles or cells are separated by a large number of extraction steps carried out in a liquid-liquid phase system, such as dextran and ethylene glycol (Albertsson, 1970), as a result of their different partition coefficients in the two liquids. This method was applied for the separation of erythrocytes and leucocytes in different species (Walter et al., 1969), erythrocytes of different ages (Walter and Selby, 1966) and the partial separation of colony forming units, granulocytes and antibody producing cells from cell suspensions of mouse spleen

(Brunette et al., 1968). Unfortunately, this method requires specialised apparatus which is not available in many research departments.

Electrophoretic separation of cells due to differing surface charge has been studied (Seaman and Ambrose, 1965; Schubert, 1973), but methods have not been developed sufficiently to collect the separated cells for further investigation.

The most practical technique available for cell separation has been the use of discontinuous density gradient centrifugation, in which cells separate according to their size and density. Various gradients have been employed for this purpose. Turner et al. (1967) used a bovine serum albumin gradient to separate mouse haemopoietic cells and separation of gastric mucosal cells has been achieved using sucrose (Lee, 1971) and Ficoll gradients (Walder and Lunseth, 1963; McDogual and DeCosse, 1970). Ficoll gradients have also been used for the separation of brain cells (Hamberger et al., 1971; Giorgi, 1971), old and young erythrocytes (Boyd et al., 1967), metabolically distinct liver cells (Castagna and Chauveau, 1963) and for purifying lymphocyte suspensions (Harris and Ukaejiofo, 1970).

Several methods have been reported for the isolation and separation of cells from the gastric mucosa of animal species. One of the first attempts was published by BreMiller and Davenport (1961), who used rabbit gastric mucosa. Gentle trituration of mucosal fragments in a citrate-sucrose-albumin medium was followed by filtration and low intensity centrifugation. Cell suspensions contained parietal cells contaminated with nuclei and cellular debris. Many of the preparations obtained were heavily contaminated and purification was not achieved by using sucrose density gradients, since they found the cells were damaged at high sucrose concentrations. The parietal cells were also contaminated by pepsinogen, and their preparations were regarded as being unsuitable for biochemical study.

A relatively pure suspension of parietal cells was prepared from rabbit gastric mucosa by Walder and Lunseth (1963), by digesting minced pieces of fundic mucosa in collagenase. The resultant cell suspensions were filtered, layered onto a Ficoll density gradient and centrifuged. Examination of the cell layers obtained, for

succinic dehydrogenase activity, and by haematoxylin and eosin and Zimmerman trichrome stain, revealed that one fraction contained between 30% and 50% parietal cells.

McDogual and DeCosse (1970) using the method described by Walder and Lunseth, for studying chloride movement in isolated parietal cells, claimed to have obtained suspensions containing more than 95% parietal cells. Morphological documentation of the purity of the cell suspensions was not presented.

Recently, Lee (1972) described a method to isolate and purify parietal cells from rabbit stomach which had been briefly perfused with 1% glutaraldehyde. The partially fixed mucosa was initially digested with trypsin and subsequently with collageanse. The parietal cells were found to be more resistant to the effects of the proteolytic enzyme than the other cell types, and were recovered and purified by centrifugation in a discontinuous sucrose gradient. Ultrastructural observation on the isolated cells revealed some degree of cell damage, particularly to the cell membrane.

Using a combination of pronase and collagenase to disperse cells from canine gastric mucosa (Croft and Ingelfinger, 1969), two cell suspensions were prepared containing cells which were 80% and 90% viable. Fundic mucosa, stripped free of muscularis mucosae and submucosa was minced and incubated with pronase. The suspension obtained was filtered and centrifuged and found to contain 15% parietal cells. The remaining particles of mucosa were subsequently dispersed with collagenase and the suspension obtained by this treatment contained 54% parietal cells. Cells were identified in stained preparations using the PAS technique. The cells were used to study oxygen consumption, electrolyte content and intracellular pH. Higher rates of oxygen consumption in the suspension containing 54% parietal cells was attributed to the presence of these cells.

Homogenous suspensions of viable gastric mucosal cells were obtained from Necturus stomachs by Blum et al. (1971) using pronase digestion. In the amphibian stomach a single cell type, the oxyntic cell is probably responsible for the production of hydrochloric acid and pepsinogen, in contrast to the two specialised cells in the mammalian stomach. Consequently, contamination in cell

suspensions prepared from this species is restricted to the mucous cells. A portion of fundic mucosa was fixed to the bottom of a dish and shaken with several changes of a pronase solution over a period of 2 hours. The initial pronase treatment released the surface epithelial cells, whilst further shaking in the presence of fresh pronase gradually produced a suspension of virtually pure oxyntic cells. Electron microscopic studies demonstrated that the cells were intact and retained good morphological characteristics, and electrical potential of the isolated cells was found to compare with that of intact mucosa. Respiration rates, electrolyte content, and cell viability by a dye exclusion technique were also used as evidence to indicate that the pronase method was adequate for isolation of viable gastric mucosal cells.

2.2. Isoenzyme studies.

The determination of isoenzyme patterns in tissues is dependent upon extraction procedures, and the type of tissue being extracted. Isoenzyme patterns reported from gross extraction of whole organs leave much to be desired since complex organs contain many different cell types with different functional roles, which probably have individual isoenzyme patterns.

Different modes of metabolism are reflected by changes in the pattern of lactate dehydrogenase (LDH) isoenzymes. The enzyme is a tetrameric molecule made up from two types of polypeptide sub-units (H and M), each synthesized in the cell under separate genetic control. The sub-units combine by random association to form five LDH isoenzymes : LDH-1 (H4); LDH-2 (H3M1); LDH-3 (H2M2); LDH-4 (H1M3); LDH-5 (M4).

Pfleiderer and Wachsmuth (1961) demonstrated that LDH-5 is more predominant in anaerobically metabolizing tissues such as human liver and skeletal muscle, whereas LDH-1 prevails in aerobically metabolizing tissues such as human brain and heart.

Evidence supporting the theory of a relationship between sub-unit composition of LDH and different types of tissue metabolism have been provided by a number of investigators in a variety of animal species (Cahn et al., 1962; Lindsay, 1963; Wilson et al., 1963; Dawson et al., 1964).

The predominance of LDH-1, which is strongly inhibited by excess pyruvate, is indicative of a system requiring a steady supply of energy from oxidative metabolism. LDH-5 however, is associated with glycolysis and high pyruvate concentrations (Dawson et al., 1964).

A further significant difference in metabolic roles exists between LDH-1 and LDH-5. Fritz (1965) has shown that intermediary metabolites of the citric acid cycle activate LDH-5 but exert no influence on LDH-1. By increasing LDH-5, the metabolites limit the production of pyruvate, and also their own concentration. At the same time they intensify glycolysis by increasing the amount of available nicotinamide adenine dinucleotide (NAD).

Product inhibition of LDH has been reported as having a possible significance in relation to the metabolic role of LDH isoenzymes. Stambaugh and Post (1966) suggested that as the concentrations of pyruvate and lactate in contracting muscle were sufficient for differential product inhibition by lactate, the metabolic role of the isoenzymes could be more dependent on the latter. Those tissues containing a preponderance of H sub-units will be adapted to aerobic metabolism, since product inhibition by lactate would direct the pathway to oxidation by the citric acid cycle. Tissues with high levels of M sub-units would allow lactate to accumulate.

Salthe (1965), studying heart muscle LDH of terrestrial and aquatic amphibia, obtained data demonstrating a positive correlation with oxygen availability and the isoenzyme pattern. Further examples of regulation of sub-unit synthesis by oxygen tension have been provided by Goodfriend et al. (1960) studying LDH isoenzyme patterns in tissue cultured cells. They investigated the effect of various oxygen concentrations on the synthesis of M submits, before and after the addition of chelating agents or inhibition of protein synthesis. The results indicated that oxygen tension was not the sole regu lator of LDH synthesis, but that oxygen exerts a repressive effect on M sub-unit synthesis in particular, and is mediated by heavy metals.

The effects of oestradiol on LDH synthesis in rat and rabbit uteri, and testosterone in rat seminal vesicles (Goodfriend and Kaplan, 1964), and thyroxine for inducing tadpole metamorphosis (Kim et al., 1966), have implicated a hormonal control of sub-unit synthesis in tissues dependent on endocrine stimulation.

Tissue isoenzyme patterns of LDH have shown significant alterations during ontogeny, in studies with mice (Markert and Ursprung, 1962), rats and human tissue (Latner and Skillen, 1964), in guinea pigs (Prochazka and Wachsmuth, 1972) and many other species. The differences observed in the isoenzyme patterns probably reflect the changing physiological roles of the developing tissues.

The LDH isoenzyme pattern represents a suitable criterion for the identification of the type of energy production in a particular type of cell or tissue, and any changes could probably indicate an alteration of the cellular metabolism. Such

changes have been observed during the onset of intestinal metaplasia in human gastric mucosa, where preneoplastic intestinalisation and carcinoma are characterised by a reorientation of the LDH isoenzyme pattern, with a predominance of the M4 and M3H1 isoenzymes (Leese, 1965; Prochazka et al., 1968b).

An increase in the LDH-1 isoenzyme in normal fundic mucosa of human stomach was observed by Leese (1965), and attempts have been made to correlate this with the parietal cells. LDH isoenzyme patterns were investigated in samples of human normal and diseased gastric mucosa, and abrasions from the surface of the mucosa (Prochazka et al., 1968b), and histochemical studies were made on gastric mucosa using different substrates and inhibitors (Lojda and Fric, 1970), in an attempt to ascertain the contribution of individual cell types to the overall pattern of the gross tissue sample.

The energy used in acid secretion is derived from adenosine triphosphate (ATP), which is generated by both oxidative metabolism and glycolysis (Davenport, 1971). How the energy contained in ATP is made available for secretion is unknown, but the process must involve hydrolysis of ATP. Work by Durbin and Michelangeli (1972) on high energy phosphate compounds in frog gastric mucosa, support the view that ATP is the immediate energy donor in acid secretion. This has been confirmed in studies by Sachs et al. (1972) where ATP-ase activity was demonstrated in a purified oxyntic cell fraction, isolated from amphibian gastric mucosa.

Since creatine phosphokinase (CPK) is involved in the conversion of adenosine diphosphate (ADP) to ATP, and has been implicated as being involved in the transfer of high energy phosphate between intra- and extra-mitochondrial compartments (Jacobs et al., 1964), it would be pertinent to study this enzyme in gastric mucosa.

Experimental evidence for a dimeric structure for CPK has been presented by Dawson et al. (1965). By dissociating isoenzymes from chick brain, heart and muscle with guanidine, or by freezing and thawing, a hybrid isoenzyme has

been produced. The enzyme associated with skeletal muscle is probably composed of two identical sub-units (MM), and the brain enzyme of two identical but different sub-units (BB). The hybrid enzyme found in heart muscle would then have the structure MB (Dawson et al., 1965).

CPK is thought to be restricted to muscular and nervous tissue but the enzyme has been demonstrated in extracts of liver, kidney, spleen, lung and erythrocytes from rats and humans (Van Der Veen and Willebrands, 1965). It is unlikely that the small amounts of muscle and nerves extracted along with the tissue cells would have made a significant contribution to the observed isoenzyme patterns.

CPK is similar to LDH in that the isoenzyme pattern changes during development from foetus to adult. The isoenzymes in this respect have been studied in rat (Eppenberger et al., 1964), and guinea pig tissues (Prochazka and Wachsmuth, 1972). Eppenberger et al. (1964) correlated the CPK isoenzyme patterns during ontogeny with physiological, morphological and biochemical events.

For each hydrogen ion secreted, one hydroxyl ion (OH^-) is left behind within the cell. Neutralisation of the OH^- ion is accomplished by carbonic acid. The hydration of carbon dioxide to give carbonic acid is relatively slow unless catalysed by the enzyme carbonic anhydrase (CA). CA has been demonstrated in the canaliculi of the parietal cell in mammals, by histochemical techniques (Hausler, 1958; Cross, 1970), and biochemically, in subcellular fractions of isolated, amphibian oxyntic cells (Sachs et al., 1972).

Current evidence does not favour a controlling function for CA in acid secretion, since the potent CA inhibitor, acetazolamide, when administered in high levels, will inhibit the formation of acid secretion substantially, but not completely (Hogben, 1960).

Multiple forms of CA have been demonstrated in erythrocytes, brain, kidney and lens homogenates by Korhonen and Korhonen (1965) using cellulose acetate electrophoresis. Tappan et al. (1964) compared the isoenzymes from

bovine, guinea pig and human erythrocytes by acrylamide disc electrophoresis and DEAE-cellulose column chromatography, and found that the isoenzyme patterns differed from species to species. Isoenzymes of CA in erythrocytes have also been determined using an immunochemical technique (DeSimone et al., 1971).

The significance of CA isoenzymes is not yet clear, and no reports appear to have been published on their presence in gastric mucosa.

The availability of a simple, reliable technique for isolating and separating gastric mucosal cells, would prove an invaluable asset in studying the aspects of cell metabolism involved in the various cell types. The exact mechanism of hydrochloric acid production for example, still evades explanation, even though Prout recognised the existence of hydrochloric acid in gastric juice, as early as 1824.

2.3. Objective of the present study.

Examination of individual cells or an homogenous preparation of cells is a prerequisite for understanding the biochemistry of cells and tissues in normal and pathological conditions. In complex cellular structures such as the glandular mucosa of the mammalian stomach, the physiological role of individual cell types is difficult to elucidate from tissue samples because of the heterogeneous cell population.

Previous indirect approaches to study aspects of gastric physiology, have involved techniques of histochemistry (Bradford and Davies, 1950; Kommich, 1963), and electron microscopy (Helander, 1962; Lillibridge, 1964; Rohrer et al., 1965), to suggest that parietal cells are responsible for acid secretion. Pepsinogen granules in chief cells have been demonstrated with a special staining procedure (Bowie, 1940) and mucous secreting cells are characterised by their mucopolysaccharide content after the PAS reaction.

The majority of methods previously described for isolating cells have utilised tissues that yield relatively homogenous cell suspensions, such as liver and intestine. Suspensions of isolated cells from stomach however, would contain a melange of the different cell types found in this tissue. A requirement of any study with isolated cells is to obtain preparations of one cell type. Subsequent observations of metabolic or enzyme activity may then be related to that cell.

A survey of the relevant literature has revealed that investigations on isolated gastric mucosal cells, when successful, have concentrated on the isolation of parietal cells and an investigation into the metabolic events involved in the process of hydrochloric acid production.

The inherent difficulties encountered in earlier studies, including contamination of the preparations by other cell types, mucus, cellular debris and clumping of cells, have discouraged previous workers in this field. Some of the difficulties were eliminated in later investigations, but most methods still incur disadvantages and none present evidence of the technique being equally adaptable to human tissue. This fact served as a stimulus for the present

investigation to develop a method to prepare homogenous suspensions of one or more of the cells found in the gastric mucosa. It would provide a unique opportunity to study isoenzyme patterns in the cells in an attempt to contribute to the understanding of the processes underlying their different physiological functions.

Pig stomach was chosen for the study because of its similarity to the human stomach, although in some of the preliminary experiments, rat gastric tissue was also examined.

The selection of a method for isolating cells appears to depend largely on the structural properties of the tissue concerned and the type of studies for which the cells are required. The suitability of a procedure for a particular tissue can only be adequately determined by experiment. Consequently, preliminary experiments to assess the potentiality of the effects of E DTA, collagenase and pronase on the release of cells from gastric mucosa, are described (Section 3.3), along with the development of a suitable density gradient for the separation of the different cell types, with the objective of studying isoenzymes of LDH, CPK and CA in the cell suspensions.

3. MATERIALS AND METHODS. PART I.

3. MATERIALS AND METHODS. PART I.

3.1. Animals and Tissue.

Adult Sprague-Dawley rats of either sex, weighing between 150g and 450g were used. The animals were sacrificed during ether anaesthesia by exsanguination, and the stomach removed.

Entire stomachs were obtained at the abattoir from domestic pigs aged between 16 and 20 weeks. The animals were stunned with an electric shock and sacrificed by exsanguination. The stomachs were transported to the laboratory at ambient temperature, within half an hour of the animal's death.

3.2. Histology.

Samples of rat and pig fundic mucosa were fixed in 10% formol saline, dehydrated in graded alcohols and embedded in paraffin wax in an automatic tissue processor (Histokinette). Sections were stained with either aqueous haematoxylin and eosin or a modified Zimmerman technique (Marks and Drysdale, 1957). In the latter method, sections were stained with Mayer's haematoxylin and PAS reagent, then counterstained with alcoholic aurantia. Sections were examined in a Zeiss photomicroscope and observations recorded with an automatic camera attachment on Agfa CT18 colour film or Ilford Pan F black and white film.

3.3. Preliminary assessment of cell isolation from gastric mucosa.

Plastic apparatus was used throughout the cell isolation procedures used in this study since it has been shown that cells can adhere to glass surfaces (Rodbell, 1965).

Analytical grade reagents were used and obtained from British Drug Houses, Ltd., Boehringer and Koch Light Laboratories, unless stated otherwise.

3.3.1. Cell dispersion using EDTA.

Portions of fundic mucosa from rat and pig stomach were washed with physiological saline to remove mucus and food debris. The tissue was then minced with scissors in phosphate-buffered saline (PBS), containing EDTA (Evans et al., 1971), and having the following composition :

NaCl ..... 96 mM	pH 6.8 ± 0.05	pH 6.3 ± 0.05
KCl ..... 1.5 mM		
Na_2HPO_4 ..... 5.6 mM		
KH_2PO_4 ..... 8.0 mM		
Na_2-EDTA ..... 10 mM	.....or 20 mM	

The tissue was incubated at 37°C for 1 hour and subjected to mild mechanical treatment using a magnetic stirrer at approximately 150 rpm. The suspensions obtained were examined by light microscopy. Smears were made from the cell plugs after centrifugation at 300 xg. The cytological preparations were fixed with 0.3% acetic acid in absolute alcohol, and stained using one of the methods previously described.

3.3.2. Cell dispersion using collagenase.

Fundic mucosa was prepared as before and treated with a solution of 0.375% collagenase (Type 1, from clostridium histoliticum; Sigma, London), in phosphate-buffered saline/glucose (PBSG; Croft and Ingelfinger, 1969).

Composition of PBSG :		
NaCl ....137 mM		
KCl ..... 2.7 mM		
Na_2HPO_4 ..... 8.0 mM	pH 7.4 ± 0.05	
KH_2PO_4 ..... 1.5 mM		
Glucose ..... 1 mg/1 ml		

The tissue was incubated at 37°C with magnetic stirring as before and cytological preparations were made from the cell suspensions.

3.3.3. Cell dispersion using Pronase.

The concentration of pronase initially used was the same as that described by Blum et al. (1971).

Minced fundic mucosa from rat and pig was suspended in a solution of pronase (0.175%; Merck Biochemicals; 70,000 PUK/g), prepared in PBSG. After incubation for 1 hour at 37°C, and magnetic stirring, smears were made of the cell suspension.

On the basis of qualitative assessment by the light microscopical examination, pronase solutions of different concentrations (0.1%; 0.15% 0.2%) were added to further samples of tissue, to examine the effect of concentration and time on cell release. Suspensions of released cells were collected after 30 minutes. Fresh pronase solution was added to the tissue fragments and the process repeated. Cell suspensions were collected in this way every half hour for 2 hours. Cell counts were performed on each cell suspension.

3.3.4. Cell counts.

Cell suspensions from the pronase treated tissue were centrifuged at 300xg and washed with PBSG. Cells were resuspended and diluted so that at least 100 were counted in the 25 squares of an improved Neubauer counting chamber (Hawksley, London). The results were expressed as the total number of cells present in the suspension.

3.3.5. Cell viability.

Cell viability was assessed at the time of cell counting by the dye-exclusion technique (Hanks and Wallace, 1958; Blum et al., 1971; Lentz and Di Luzio, 1971). Equal volumes of cell suspension and PBSG (containing 50 mg % eosin) were incubated at room temperature for 1 minute. The percentage of unstained cells was noted.

3.3.6. Results.

Light microscopical examination of the cell suspensions, obtained after the fundic mucosal tissue was treated with EDTA in physiological buffer, revealed a paucity of isolated cells. Increasing the amount of EDTA caused increased contamination by sub cellular debris and did not improve cell yield.

The individual cells present could be distinguished by their columnar shape as being predominantly surface mucous cells. This was confirmed in the stained cytological preparations (Fig. 6). Other material present consisted of damaged cells, free nuclei and cell debris. Also present in small numbers were sheets of cells, sometimes tubular in shape, displaying blebbing of the cell membrane (Fig. 7). Mechanical treatment after incubation only resulted in an increase in the number of

Fig. 6. Cell suspension obtained from pig fundic mucosa after treatment with 10 mM EDTA. The majority of cells present are surface mucous cells (SM), with occasional parietal (P) and mucous neck cell (M). Modified Zimmerman stain x 200.

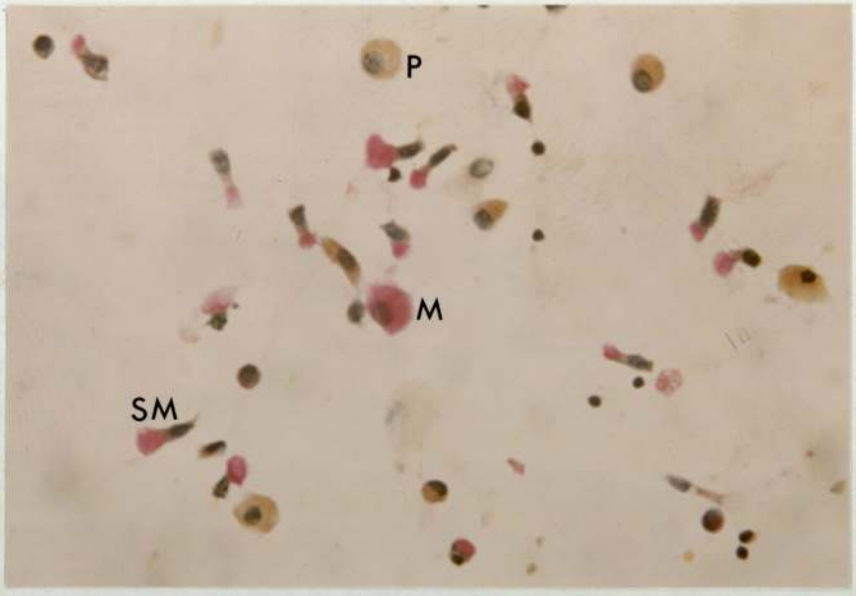
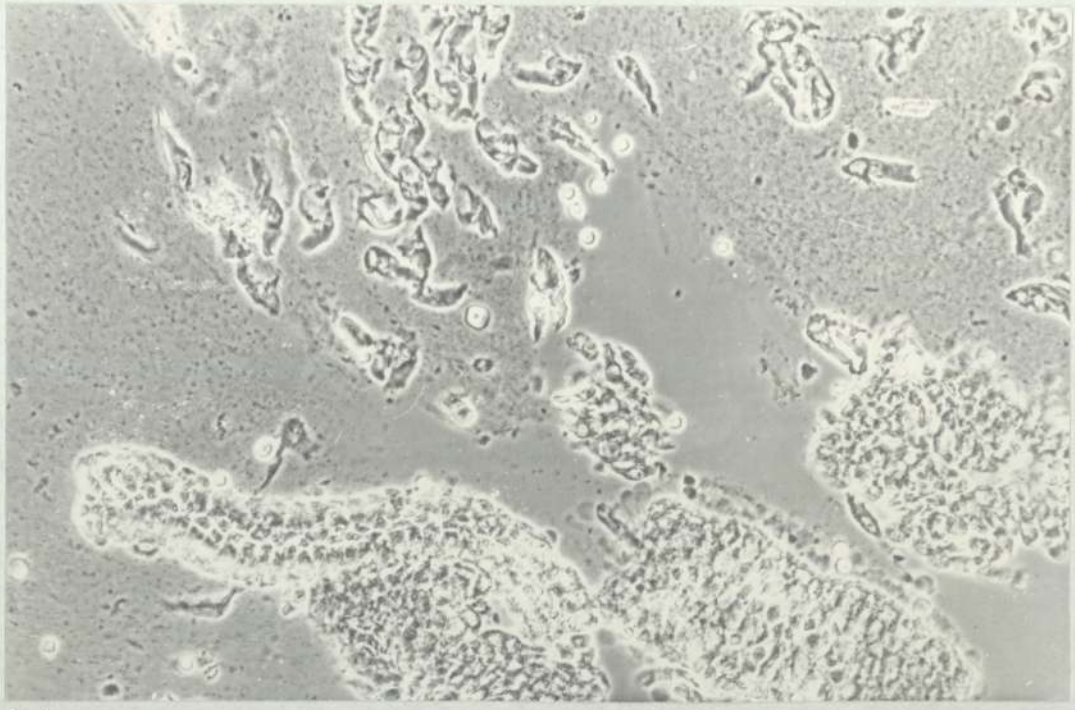


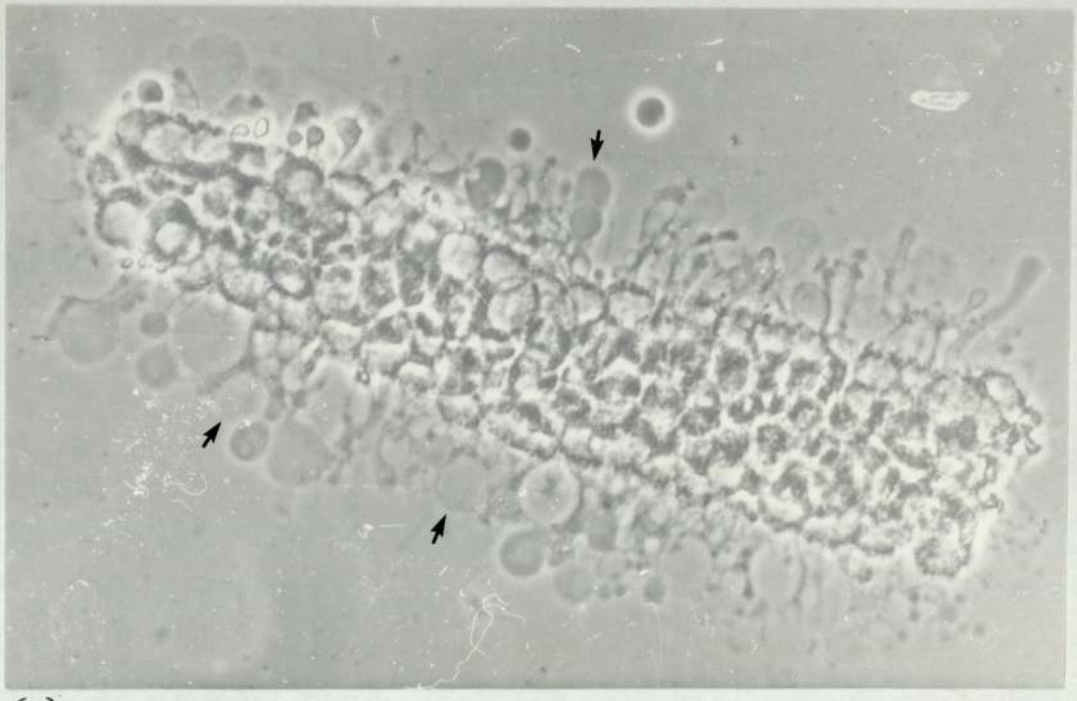
Fig. 6.

Fig. 7. (1) Cells isolated from pig fundic mucosa by treatment with EDTA (10 mM). Both individual and large sheets of surface mucous cells are present. Unstained preparation x 180.

(2) A tubular aggregate of cells displaying considerable blebbing of the cell surfaces (arrowed) following EDTA treatment. Unstained preparation x 450.



(1)



(2)

Fig. 7.

the large aggregates of cells and an accompanying augmentation of the cellular debris.

The presence of mucus in all samples examined created technical difficulties with respect to the centrifugation and resuspension of cells.

Tissue samples treated with buffered collagenase solution resulted in a viscous, gelatinous mass and presented extreme difficulty in examination of the turbid material. Those preparations that afforced study revealed the presence of substantial cellular debris. Occasional aggregates of cells were seen.

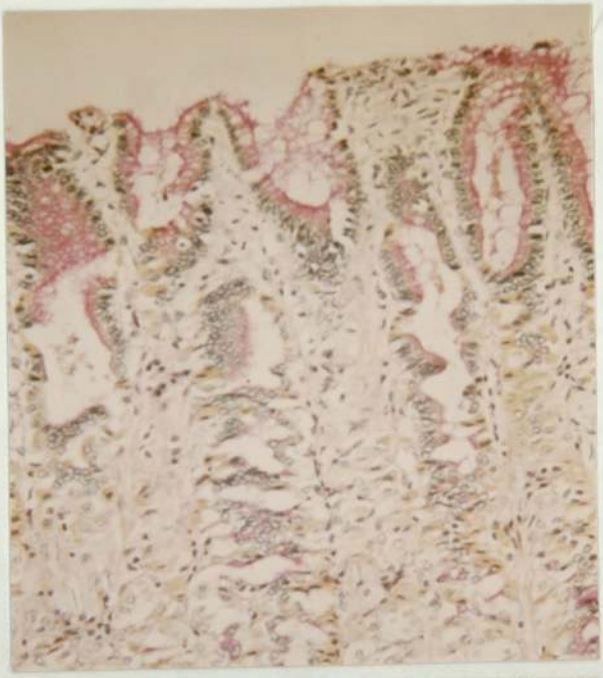
Treatment of fundic mucosa with pronase resulted in the release of large numbers of intact cells, reasonably free from contamination by cell debris and mucus. The centrifuged pellet of cells was easily resuspended in buffer for preparing smears. Stained sections of mucosa (Fig. 8) were compared with the cytological preparations. It was evident that the predominant cell types found in gastric mucosa (i.e. parietal, chief, mucous neck cells) were also present in the cell suspensions (Fig. 9).

Cell counts performed on the cell suspensions obtained at 30 minute intervals, with different concentrations of pronase, displayed wide variation (Table 1). The cell yield for each period was expressed as a percentage of the total number of cells released in 2 hours (Fig. 10). An observed increase in the percentage release of cells after prolonged incubation with pronase was found to be statistically significant for both 0.1% and 0.15% pronase, when comparing the initial 30 minute treatment period with the 90 minute period ($P < 0.0025$).

Unstained preparations of the cell suspensions were examined by light microscopy. The characteristic columnar-shaped surface mucous cells were prevalent in the 30 minute cell suspension taken from both the 0.1% and 0.15% pronase treated samples. Other cell types appeared as spherical cells of varying size. Clumps of attached cells were evident in these samples, occurring much more frequently in the 0.1% pronase treated sample.

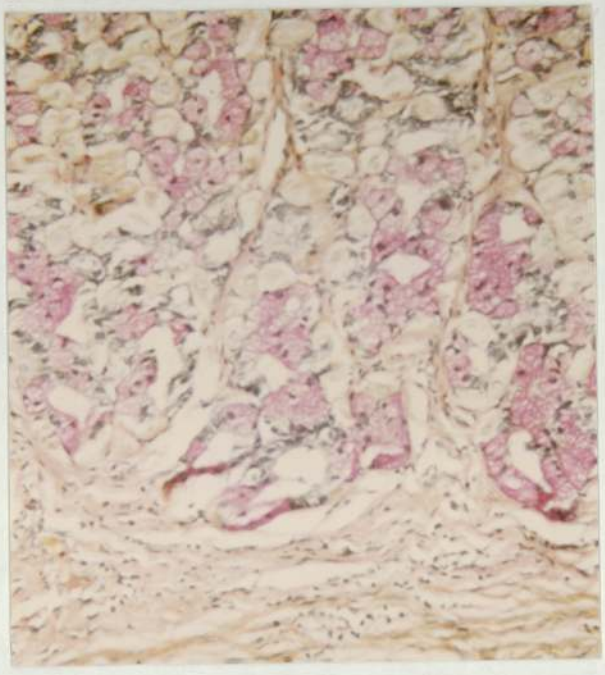
All cells in the 30 minute sample of the 0.2% pronase treatment appeared spherical.

Fig. 8. Sections of pig fundic mucosa showing the surface epithelium and foveolae (1), the isthmus and neck region (2) and the base of the glands with the underlying lamina propria and muscularis mucosa (3). Modified Zimmerman stain. x 180.



(1)

(2)



(3)

Fig. 8.

Fig. 9. A mixed cell suspension from pig fundic mucosa after pronase treatment. The modified Zimmerman staining technique readily demonstrates the different cell types. SM - surface mucous cell; M - mucous neck cell; P - parietal cell; C - chief cell. x 200.

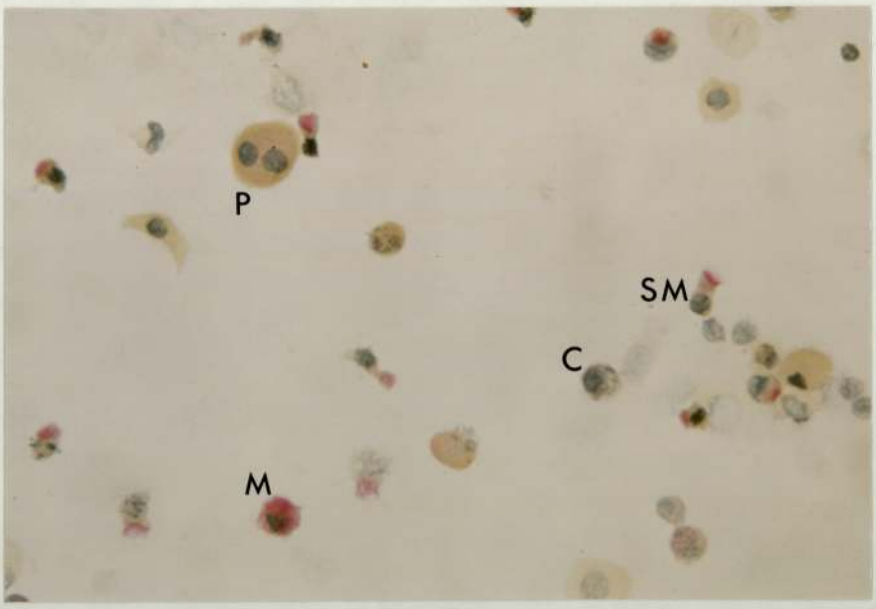


Fig. 9.

Table 1. Total number of cells released in each 30 minute period using different concentrations of Pronase in PBSG.

Concentration of Pronase	30 minutes	60 minutes	90 minutes	120 minutes
0.1 %	$10.0 \pm 7.9 \times 10^3$	$12.7 \pm 7.3 \times 10^3$	$26.9 \pm 14 \times 10^3$	$19.3 \pm 6 \times 10^3$
0.15 %	$5.5 \pm 1.4 \times 10^3$	$13.3 \pm 5.5 \times 10^3$	$19.8 \pm 4.4 \times 10^3$	$28.7 \pm 7 \times 10^3$
0.2 %	$42.2 \pm 23.4 \times 10^3$	$60.3 \pm 38.2 \times 10^3$	$42.3 \pm 19.2 \times 10^3$	$38.9 \pm 23.7 \times 10^3$

Results expressed as mean $\pm$ SEM (5 experiments)

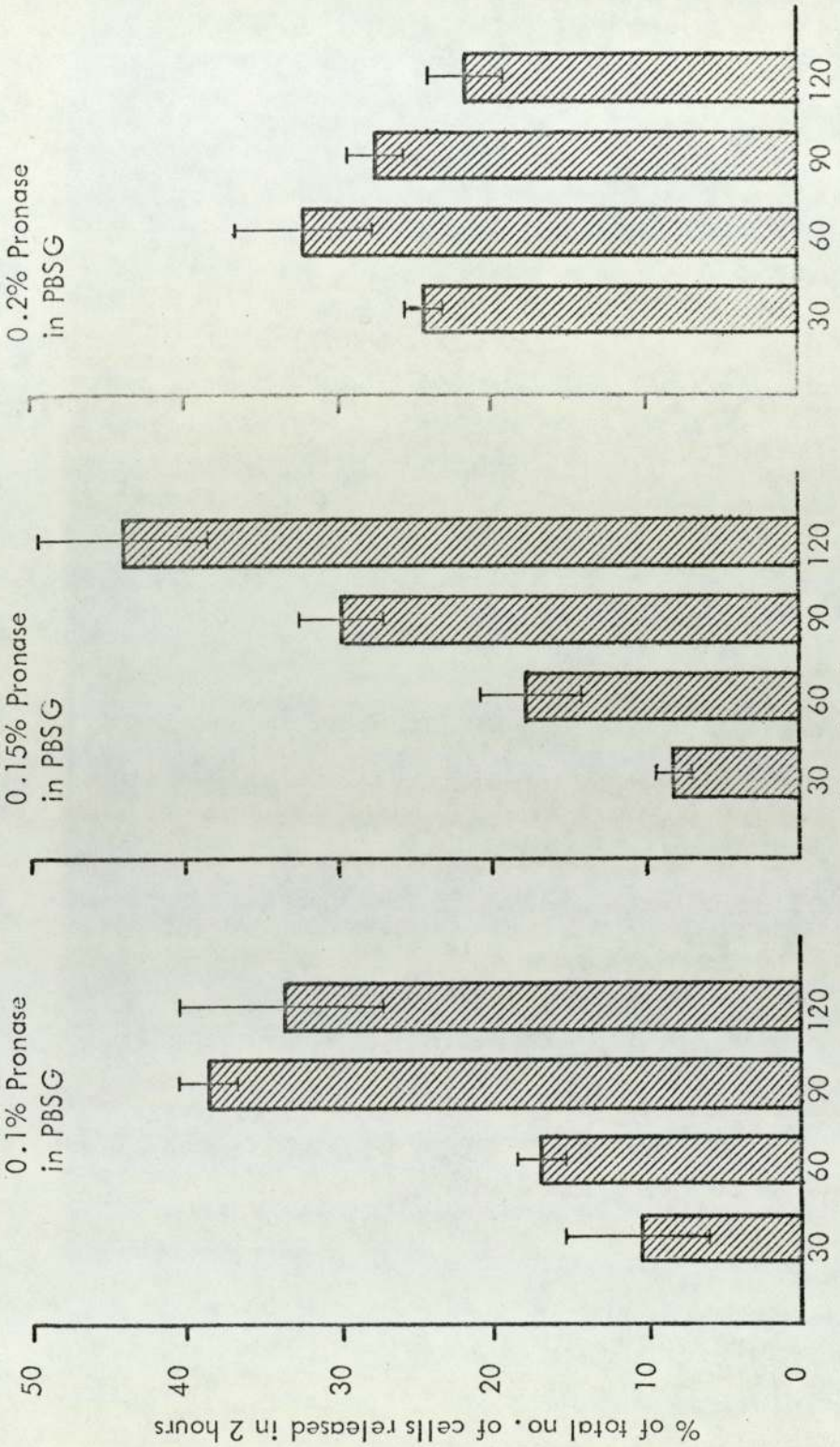


Fig. 10. Effect of pronase concentration and incubation time on cell release from pig fundic mucosa.

Results expressed as mean ± SEM (5 experiments)

There was a progressive increase in the presence of cells from the mid region of the fundic glands (parietal, chief and mucous neck cells), as incubation with pronase was extended (Fig. 11).

The highest concentration of pronase was effective in dispersing the mucus content of the samples, but simultaneously released mucus producing cells (mainly mucous neck cells).

3.3.7. Discussion.

The objective of the previously described experiments was to assess the potentiality of three different tissue dissociating agents for isolating intact, viable cells from the fundic mucosa of rat and pig stomach.

It was clearly evident from a qualitative aspect that EDTA and collagenase were not effective in this respect with the stomach tissue of these two species, and consequently no further detailed experiments were performed beyond the preliminary examination.

Evans et al. (1971), incubated guinea pig small intestine with EDTA and found that epithelial cells were released in sheets. It is therefore interesting to note that after incubation of rat and pig stomach with EDTA some similar sheets of cells were released from the mucosa. Some of the sheets appeared to be derived from the upper part of the foveola and retained the tubular form associated with the opening of the gastric pits.

The effect of EDTA on the junctional complexes between cells in the gastric glands of the frog were studied ultrastructurally by Sedar and Forte (1964). They noted that calcium depletion resulted in a loss of intercellular material from the desmosomes and intermediate junctions. It would appear that mechanical forces after EDTA treatment would therefore be effective in dispersing such 'loosely - attached' cells. With rat intestine, this appears to be a feasible application, but was not in accordance with the results in this study, on rat and pig gastric mucosa.

Similarly, collagenase has been used as a suitable dissociating agent for the isolation of cells from rabbit gastric mucosa (Walder and Lunseth, 1963). The apparent contradiction to the observations on rat and pig stomach may be due to

Fig. 11. A cell suspension obtained from pig fundic mucosa after 90 minutes treatment with pronase solution, showing an increase in the presence of cells from the mid region of the fundic glands (parietal, chief and mucous neck cells).
Modified Zimmerman stain x 100.

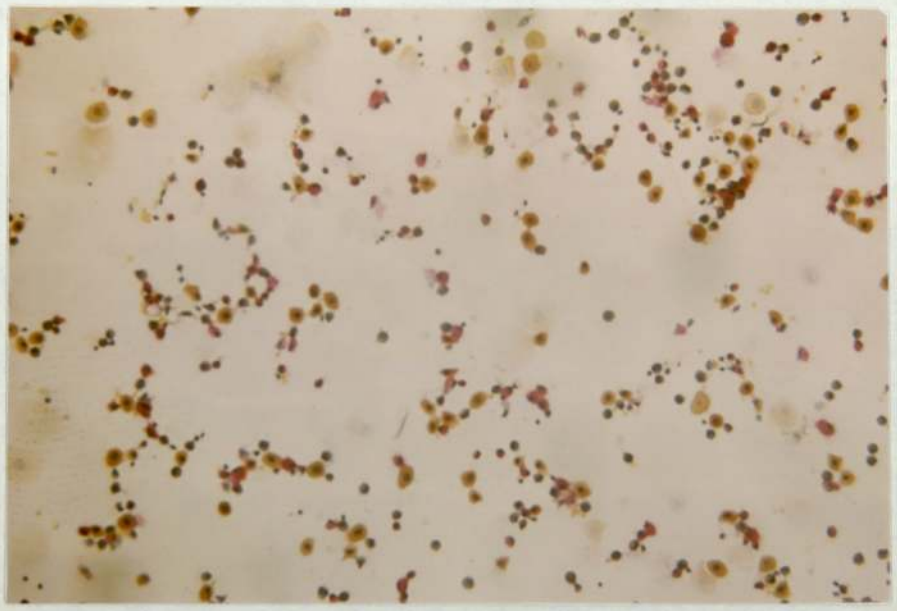


Fig. 11.

differences in the purity of the collagenase. Walder and Lunseth may have used crude forms of collagenase, possessing some non-specific proteases to complement the action of collagenase.

Pronase has been found to be suitable for the isolation of viable cells from necturus gastric mucosa, when used on its own (Blum et al., 1971), or together with collagenase in isolating cells from canine gastric mucosa (Croft and Ingelfinger, 1969).

From the results of the experiments with rat and pig fundic mucosa, it was evident that pronase was singularly capable of releasing viable cells in sufficiently large numbers for further study. The proportionately higher numbers of surface mucous cells present in the initial cell suspensions was probably due to both release of cells in the initial mincing of the tissue, and the earlier release of these cells from the mucosa. Subsequent treatment with the enzyme loosens the matrix of the lamina propria (Fig. 12), and the cells contained in the neck and isthmus region of the gastric pits are released. Providing that sufficient enzyme is available to disperse the mucus present, the cells do not form large aggregates, and are easily resuspended. When the concentration of pronase is increased, the higher yield of cells is invalidated by the presence of large numbers of mucous neck cells. Preparations such as these almost invariably proved unsatisfactory for further investigations because of extensive cell clumping.

Individual samples of rat and pig stomach tissue differed in their susceptibility to the action of pronase and only by comparing the relative increases in cell release with incubation time was it possible to draw conclusions from the results.

A minimum of 90 minutes incubation was found to give satisfactory results and coincided with the finding that cell viability was optimum during this period (Fig. 13).

On the basis of the foregoing investigations, a standard method was developed for the isolation of gastric mucosal cells. It is described in detail in the following section.

Fig. 12. Histological preparation of pig fundic mucosa after pronase treatment. This transverse section through the upper region of the gland illustrates the loosened connective tissue (CT) and a few released cells still present within the glands (arrowed).
Modified Zimmerman stain x 200.

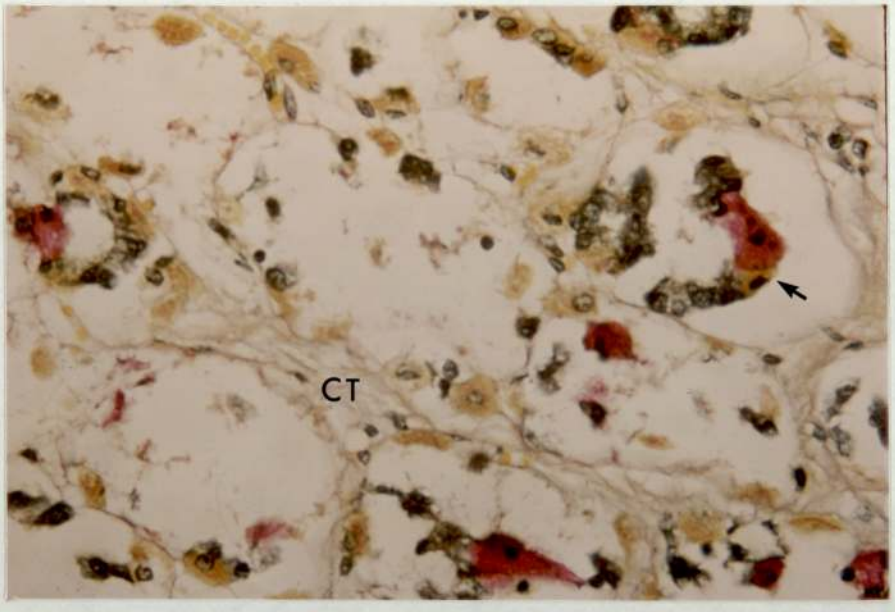


Fig. 12.

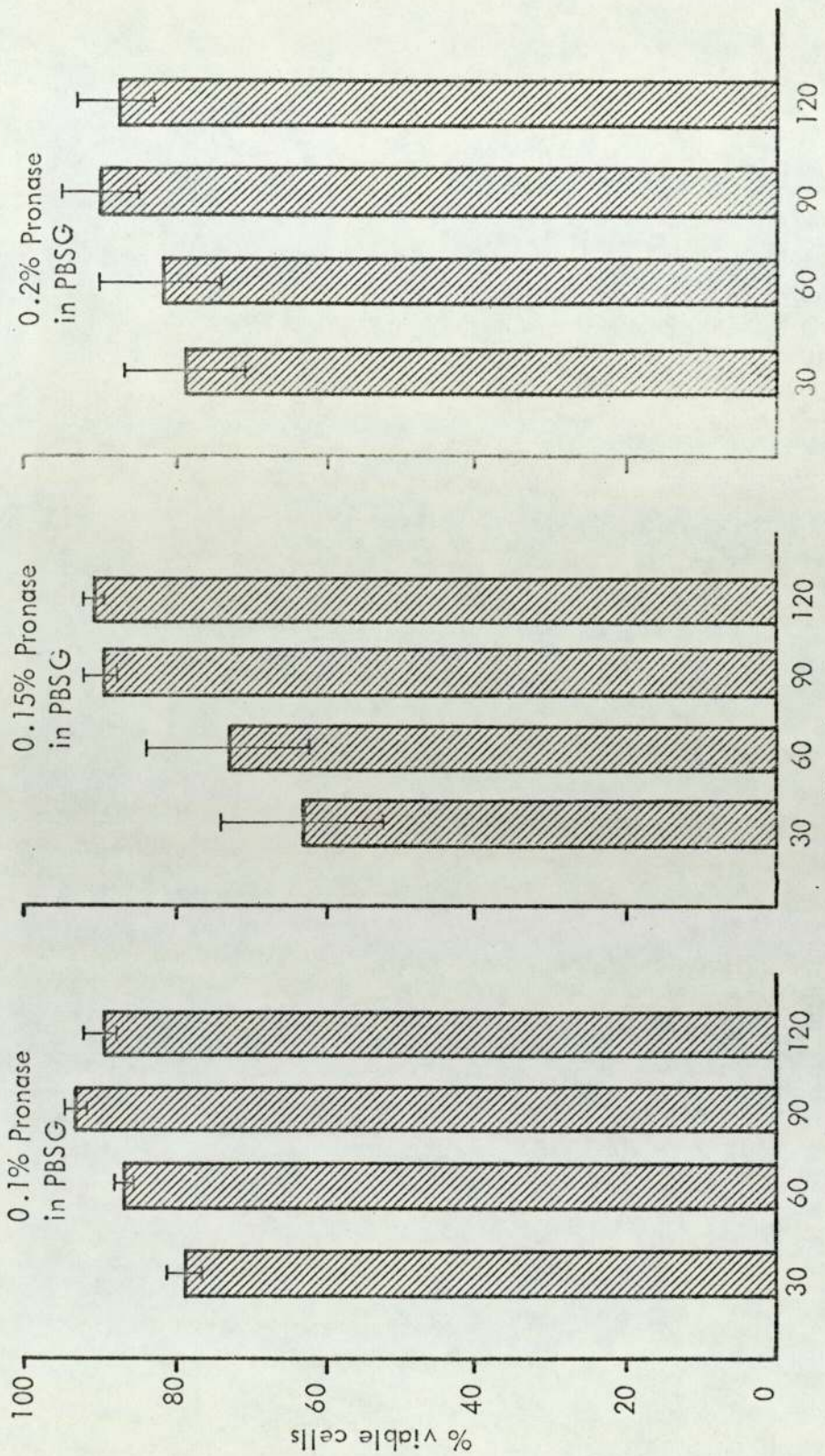


Fig. 13. Effect of pronase concentration and incubation time on cell viability in cell suspensions prepared from pig fundic mucosa.
Results expressed as mean $\pm$ SEM (5 experiments)

4. MATERIALS AND METHODS. PART 2.

4. MATERIALS AND METHODS. PART 2.

4.1. Introductory remarks.

In order to fulfil the objective of this study it was necessary to attempt a separation of the different cell types found in gastric mucosa.

The use of discontinuous density gradient centrifugation employing a synthetic sucrose polymer called Ficoll (Pharmacia, Gt. Britain) for the separation of cells isolated from gastric mucosa, was described by Walder and Lunseth (1963). This method was also employed by McDogual and DeCosse (1970) for the same purpose.

Ficoll is superior to sucrose because it provides high density solutions of low viscosity and osmotic pressure. It has also been used for the successful separation of cells from brain tissue suspensions (Hamberger et al. 1971).

The gradients described by these authors were prepared in different ways and employed slightly different principles for the separation of cell types. Walder and Lunseth (1963) prepared their gradients by layering upon one another solutions of Ficoll of different specific gravities. The cell suspension was layered onto the gradient and centrifuged (Fig. 14a). *Cell fractions were collected from the interphases of the Ficoll solutions. Hamberger et al (1971) achieved cell separation by applying the mixed cell suspension in Ficoll to the central part of the gradient (Fig. 14b). After centrifugation, enriched fractions of brain cell types were obtained from the upper and lower part of the gradient.

*Where the term "cell fraction" is subsequently used, it will refer to suspensions of cells removed from the interphases of the Ficoll solutions in the density gradient.

The latter technique was modified in order to provide the necessary requirements for separating the mixed cell suspensions obtained from gastric mucosa, into fractions enriched with a homogenous population of cells. Before a satisfactory gradient (Fig. 14c) and experimental conditions were found, a large number of variations were tested with respect to the volumes and concentrations of Ficoll solutions and the time and speed of centrifugation.

The cumulation of these results is expressed in the ensuing description of the adopted method. Individual findings are not described in full in order to avoid unnecessary repetition.

4.2. Tissue.

Fresh, unopened pig stomachs were obtained from the abattoir (see Section 3.1.). The prepared fundic mucosa was ready for pronase treatment approximately 30 minutes after the animal's death.

During the course of the study, samples of human fundic mucosa became available from two patients undergoing partial gastrectomy for duodenal ulcer. Normality of the tissue was assessed by histological examination.

4.3. Thermostatically controlled water bath with magnetic stirrer.

A thermostatically controlled water bath was specially constructed for use with the cell isolation technique to combine incubation at 37°C with mild magnetic stirring. A Gallenkamp magnetic stirrer/hotplate was modified by Aptek 70 Ltd. (Swansea, South Wales), to allow control of temperature to within $\pm$ 1°C. Fig. 15 illustrates the arrangement used.

4.4. Isolation of gastric mucosal cells.

The technique used was a modification of the method described by Walder and Lunseth (1963). Plastic apparatus was used throughout the procedure.

A portion of pig stomach, approximately 5 cm. square was removed from the greater curvature of the fundus, the external muscle cut away, and the tissue rinsed several times in PBSG (Section 3.3.2). The mucosa was pinned surface upwards on a

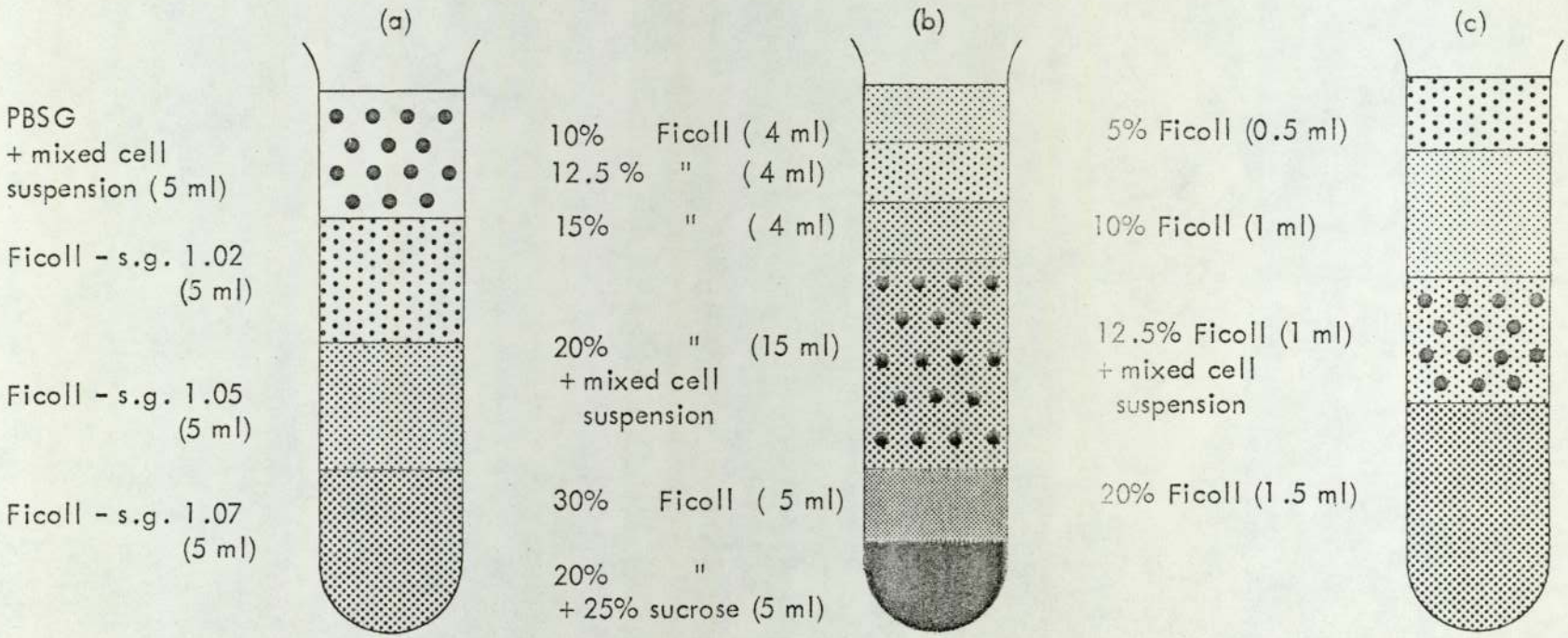


Fig. 14. A comparison of Ficoll density gradients used by (a) Walder and Lunseth (1963), (b) Hamberger et al., (1971) and the gradient used in this study (c).

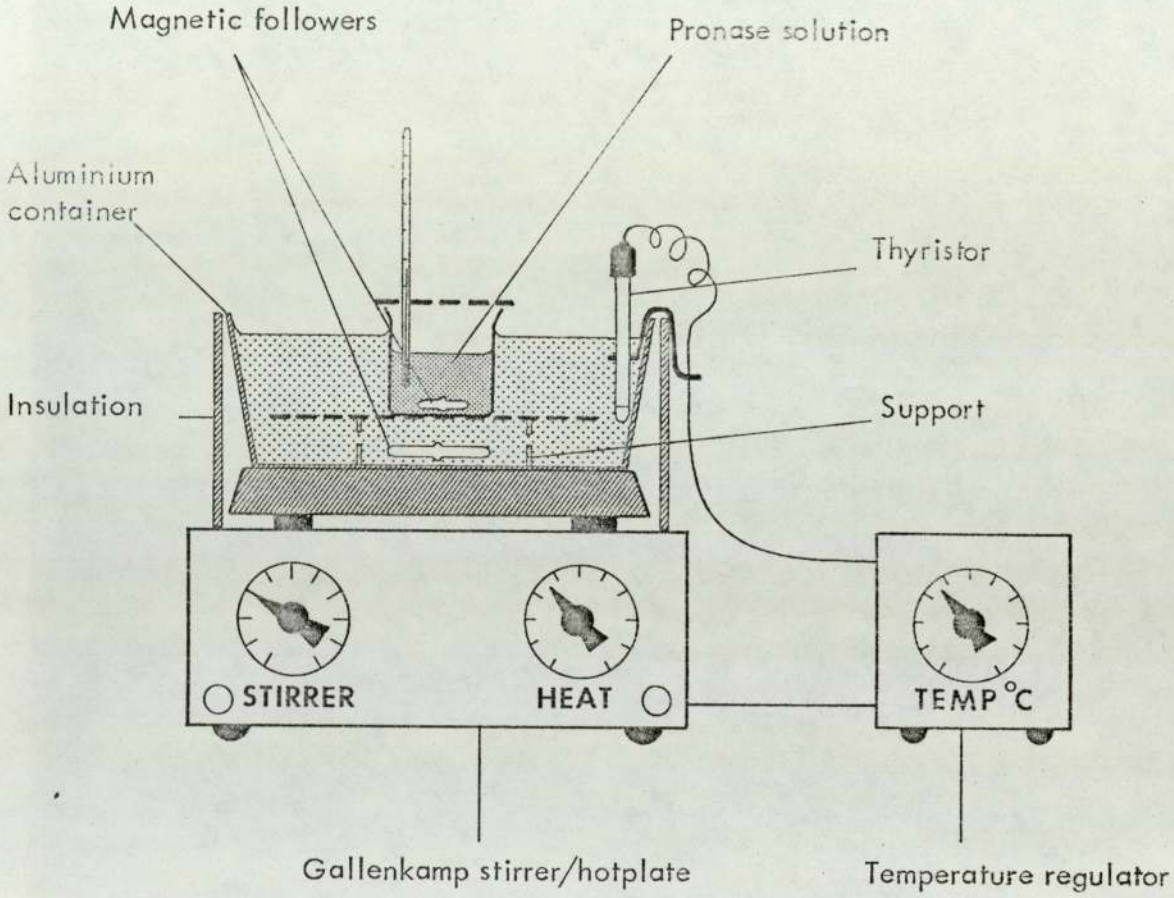


Fig. 15. Thermostatically controlled water bath assembly.

cork mat and blotted with a piece of filter paper to remove superficial mucus and food debris. Using the back edge of a scalpel blade, the surface of the mucosa was abraded several times to remove the mucous epithelium (Fig. 16). The underlying muscularis mucosae and submucosa were separated from the mucosal epithelium by careful dissection (Fig. 17).

Approximately 2g. of the dissected and abraded mucosa was minced with scissors in 2- 3 ml. of PBSG. The suspension of tissue fragments was transferred to 20 ml. of pre-warmed (37°C) pronase solution (0.15% in PBSG) in a 100 ml. beaker, placed in the incubating bath at 37°C and stirred for 20 minutes at approximately 150 rpm.

The beaker was removed to allow the tissue.to settle, and the pronase solution poured off and discarded. Cytological examination of this suspension revealed that this initial treatment removed a great deal of mucus, food debris and subcellular particles, and helped to reduce this contamination in the subsequent cell suspension. A further 50 ml. of fresh, pre-warmed pronase solution was added to the tissue and stirred as before at 37°C for another 40 minutes.

The tissue and pronase were then filtered through a blood administration set filter (Baxter Division, Travenol Laboratories), the pronase and released cells being collected in a clean 100 ml. beaker which was returned to the incubating bath. The tissue fragments remained in the nylon mesh compartment of the filter set. 2-3 ml. of fresh pronase solution was added to the filter set and retained by a pressure clamp on the outlet tube (Fig. 18). By gentle squeezing of the pliable filter set between thumb and index finger, isolated cells still retained within the gastric pits and enveloped by the framework of the lamina propria were released, and the resultant cloudy cell suspension was pooled with the incubating filtered cell suspension. After repeating this treatment twice, the pooled suspension was stirred for a final 30 minutes to disperse the mucus and small aggregates of cells released by squeezing the tissue.

The suspension was strained through a clean filter set into five 15 ml. conical centrifuge tubes, followed by centrifugation at 300 xg. for 2 minutes. The cells were resuspended in 0.5 ml. of PBSG with the aid of a siliconised pasteur

Fig. 16. Upper region of pig fundic mucosa following abrasion, showing absence of the surface epithelium (arrowed).
Modified Zimmerman stain x 180.

Fig. 17. The muscularis mucosa (MM) and the lower region of the fundic glands was separated from the upper mucosa by dissection.
Modified Zimmerman stain x 180.

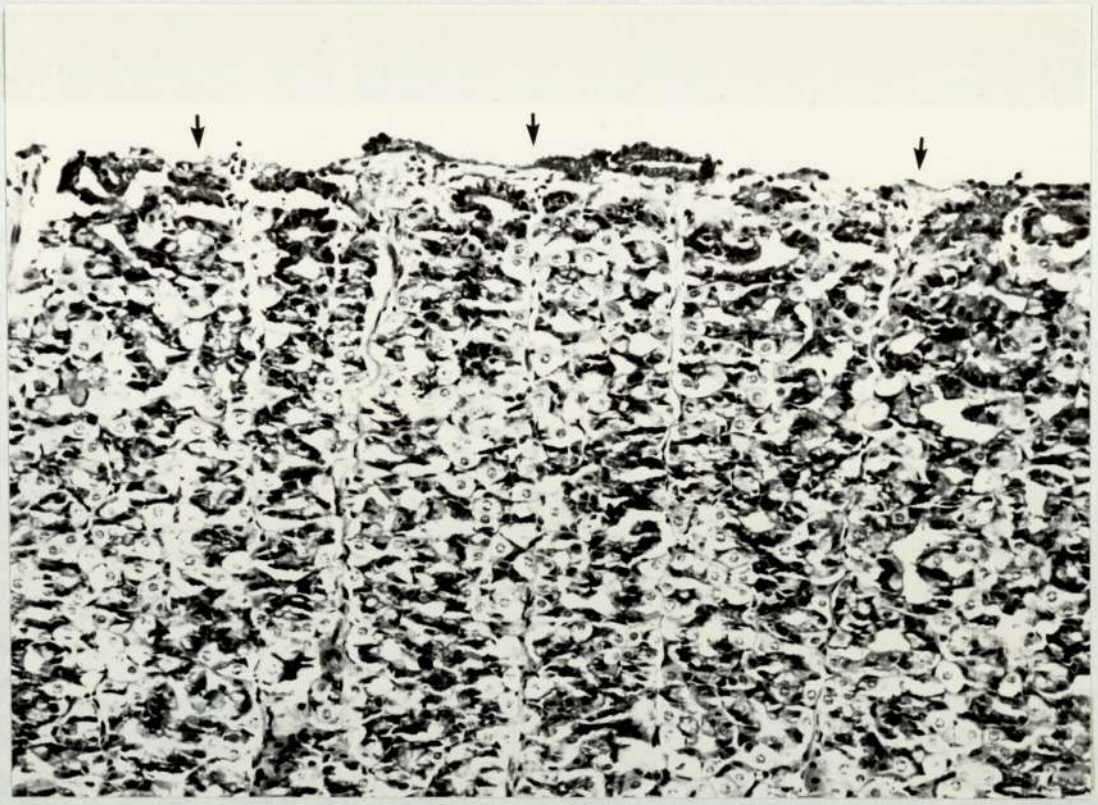


Fig. 16.

Fig. 17.

pipette and a further 4.5 ml. of PBSG added before centrifuging at 300 xg. for 1 minute. The PBSG was carefully drained from four of the cell plugs before resuspending the cells in 1 ml. of 12.5% of Ficoll (vide infra for preparation).

The remaining cell plug was drained and used as a sample of the mixed cell suspension for cytological examination and isoenzyme studies.

4.4.1. Preparation of cell fractions by density gradient centrifugation.

Ficoll was dissolved slowly in PBSG by constant stirring. Solutions with concentrations of 20%; 12.5%; 10% and 5% were prepared, and kept at 4°C when not in use.

Each mixed cell suspension (4 x 1 ml.) was subjected to centrifugation in a discontinuous Ficoll gradient. The gradients were prepared in 5 ml. tubes (7.5 x 1.0 cm.), calibrated in 0.5 ml. increments, in the following manner : 1.5 ml. of 20% Ficoll was added, followed by 1 ml. of cell suspension in 12.5% Ficoll, 1 ml. of 10% Ficoll and finally 0.5 ml. of 5% Ficoll. Pasteur pipettes were used to add the solutions to the tubes, taking care not to disturb the interphases between the Ficoll layers. The tubes were capped and centrifuged at 1000 xg. for 30 minutes.

4.4.2. Collection of cell fractions.

Cells were collected from the interphases of the Ficoll solutions and the bottom of the gradient using the device illustrated in Fig. 19, and transferred to pre-weighed Eppendorf micro reaction tubes, which contained a small amount of PBSG. If necessary, more PBSG was added to fill the tube, after which the cell suspensions were mixed and centrifuged at 300 xg. for 5 minutes. The supernatant was removed as completely as possible and smears were made of the various fractions, before the tubes plus cells were re-weighed.

For simplification, the cell fractions were designated : fraction A, mixed cell suspension obtained 90 minutes after pronase treatment: fraction B, cells collected from the 5-10% Ficoll interphase; fraction C, cells collected from the

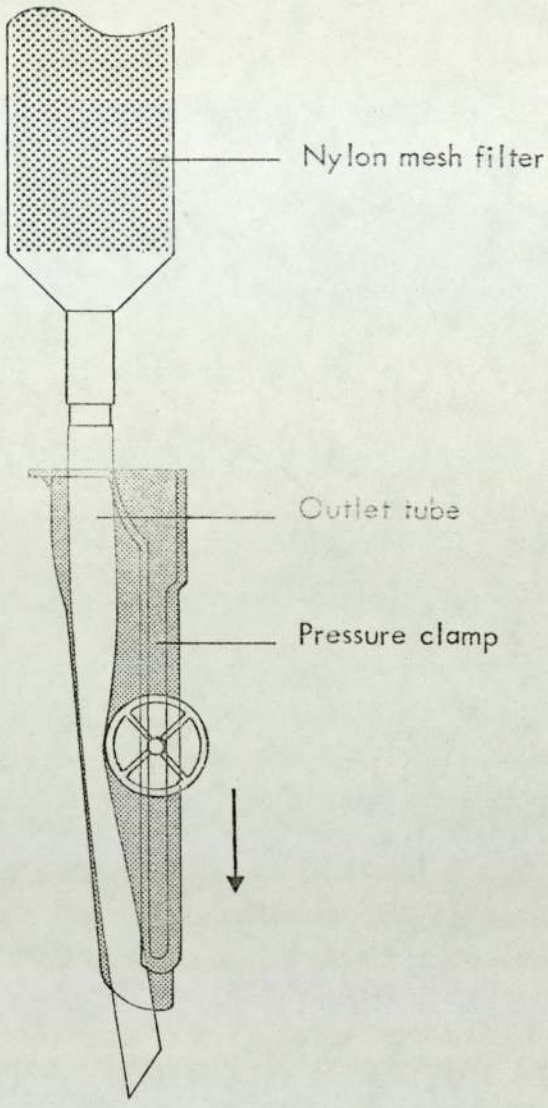


Fig. 18. Filter set

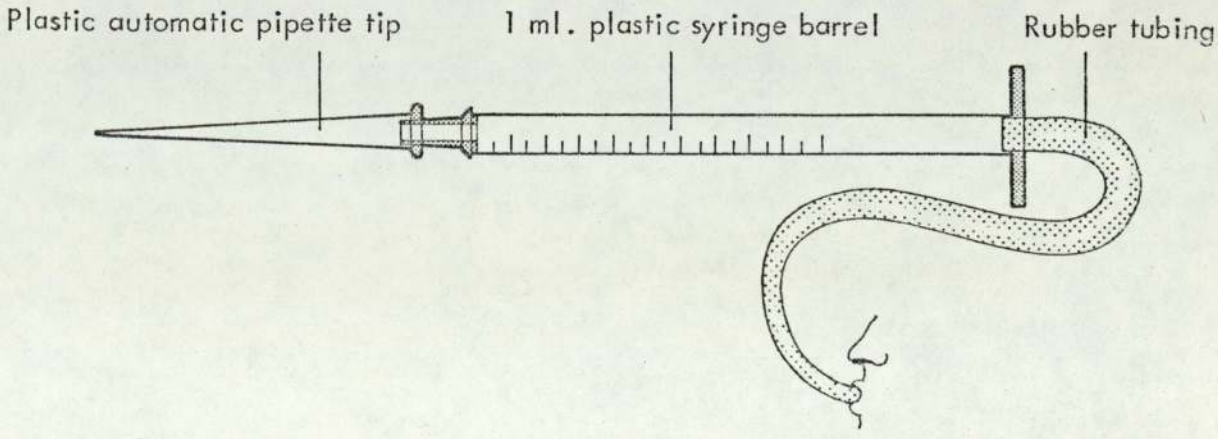


Fig. 19. Pipette used for collecting cell fractions from the density gradients.

10-12.5% Ficoll interphase; fraction D, cells collected from the 12.5 - 20% Ficoll interphase; fraction E, cells collected from the bottom of the gradient.

A typical gradient after centrifugation is illustrated in Fig. 20.

4.4.3. Cell size estimations.

Cell fractions obtained from the gradient were washed and resuspended in PBSG. The diameter of 100 cells was measured using a calibrated eye-piece micrometer (1 division ≡ 2.27 μm.)

4.4.4. Differential cell counts.

Cytological preparations of the cell fractions were examined by light microscopy after staining by the modified Zimmerman technique. The mucous secreting cells were characterised by the red PAS - positive cytoplasm, the chief cells had coarse blue granules, and the parietal cells contained fine, closely packed yellow granules. All these cells were readily distinguished from one another in the isolated cell preparations. Differential cell counts were performed by counting 100 cells in each fraction, and noting the contributions made by the various cell populations.

4.5. Homogenisation and extraction of tissue and cell samples.

Weighed samples of fundic and pyloric mucosa (including muscularis mucosae and submucosa), prepared fundic mucosa and abrasion in addition to fractions A - E from the same stomach were homogenised* in barbitone buffer, pH 8.4, ionic strength 0.05 (see Section 4.6.1).

The ratio of the wet tissue weight (in milligrams) to the volume of buffer(in microlitres) was 1:3, and for the cell fractions 1:1. The samples were centrifuged at 1000 xg. for 30 minutes, and duplicate aliquots of the supernatant used immediately for isoenzyme studies.

*A micro tissue homogeniser was specially prepared for use with the small amount of cellular material. It was constructed from epoxy resin (Araldite) using an Eppendorf micro reaction tube as a mould. A tube was half filled with resin and centrifuged briefly to remove air bubbles before inserting a glass rod for the handle. When the resin had set, the tube was cut away, and the surface of the resin was roughened with fine emery paper. After thorough washing with distilled water, the pestle was used to homogenise the cell samples contained in the micro tubes.

Fig. 20. Ficoll density gradient used for the separation of isolated gastric mucosal cells. The mixed cell suspension was applied to the central part of the gradient in the 12.5% Ficoll. After centrifugation, the cell fractions appeared at the interphases of the Ficoll solutions.

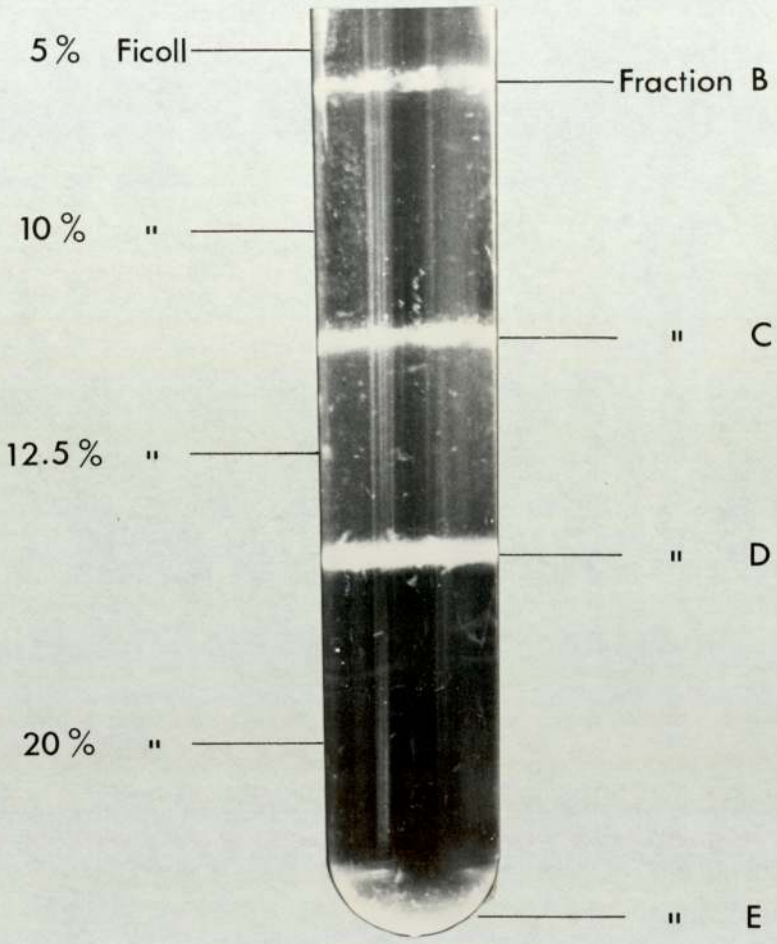


Fig. 20.

4.6. Agar gel electrophoresis.

Samples of supernatant from the extracted cell fractions and tissue were subjected to agar gel electrophoresis, according to a method developed by Wieme (1959).

4.6.1. Preparation of agar gel.

One per cent special Agar-Noble (Difco Laboratories), was prepared in barbitone buffer, pH 8.4, ionic strength 0.05. The stock buffer was prepared by dissolving 17 g. of sodium barbitone in distilled water, to which had been added 23.5 ml. of 1M HCl. The solution was made up to 1 litre with distilled water. pH of the buffer was 8.4, and ionic strengh 0.1. This buffer was diluted with an equal volume of distilled water before use.

The agar was added to the buffer in an Erlenmeyer flask and placed in a boiling water bath. A small filter funnel was placed in the mouth of the flask to reduce evaporation. The solution was boiled for 20 minutes and stirred occasionally, after which time it was removed from the heat and cooled to about 50°C.

4.6.2. Preparation of agar gel layers.

Using a 10 ml. graduated pipette, 3 ml. of warm agar was added to each of several microscope slides (76 x 26 mm.) which were rested on a levelled plate glass surface. When cool and gelified the slides were placed in petri dishes containing moistened filter paper. These were kept in a cool place for 24 hours before use, giving greater rigidity to the gel and minimising the effects of electro-osmotic flow during electrophoresis.

4.6.3. Preparation of electrophoresis tank.

An electrophoresis tank was constructed from 4 mm. perspex similar to the one described by Wieme (1959), except that the dimensions were slightly larger to allow accommodation for 6 microscope slides during electrophoresis.

The constructional details are illustrated in Fig. 21. To prepare the tank, warm agar of the same composition as that used for the agar gel layers was poured into the two outside electrode vessels, until the adjacent chambers, which freely communicate, were completely filled. When solidified, the agar was removed from the electrode vessels. The tank was left for 24 hours before use. Usually, 18 - 20 runs were obtained before the agar was replaced, providing the polarity of the electrodes were reversed after each run. The tank was kept covered in a cool place when not in use. The platinum electrodues were supported on perspex carriers to facilitate removal when filling the tank with agar.

4.6.4. Sample application.

Small strips of Whatman filter paper (4 x 12 mm.) were inserted (narrow end down) into the agar gel on the microscope slides, 30 mm. from one end. When the strip of paper had been completely soaked it was carefully removed, leaving a small slit into which 5 ul of supernatant fluid from the samples was placed with a micropipette. After the sample had penetrated the gel, the groove was filled with warm agar (ca. 40°C). This prevented distortion of the electrophoresed protein bands. Each sample was applied in duplicate to one slide and immediately electrophoresed.

4.6.5. Electrophoresis.

The microscope slides were placed in the tank with the agar layer face down on the agar supports. Petroleum ether (low boiling point) was then added to fill the central chamber and covered. The buffer vessels were filled with the diluted barbitone buffer. Electrophoresis was carried out for 30-35 minutes with approximately 150V between electrodes and a current of 130 - 140 mA (6 slides). After the electrophoresis was completed the slides were incubated with solutions for the detection of enzyme activity.

4.7. Location of Enzyme Activity.

A small perspex box was used to support the microscope slides with the agar gel layer face down. The incubating medium was added to the space between the gel layers and the bottom of the container (Fig. 22).

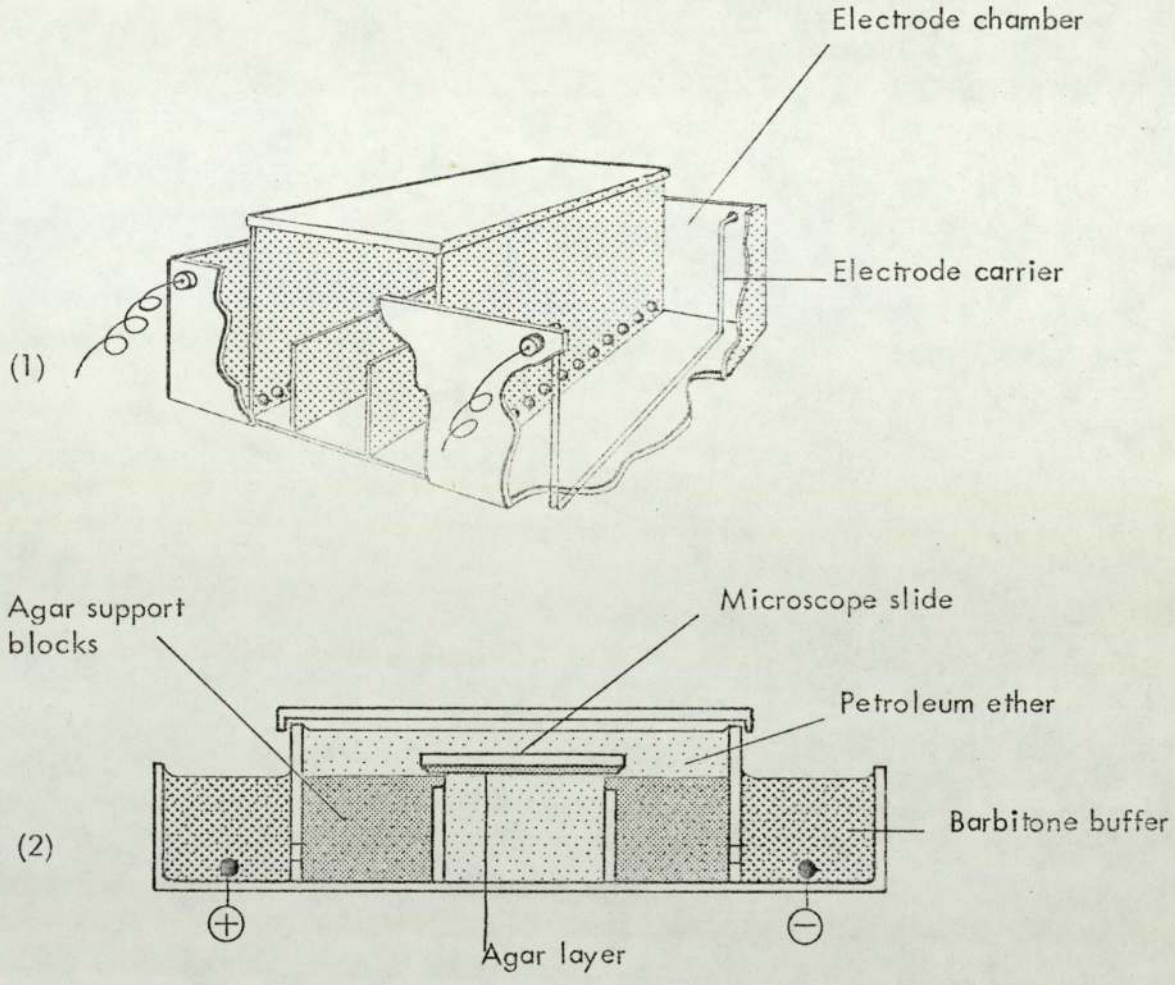


Fig. 21. (1) Apparatus used for agar gel electrophoresis according to Wieme (1959).
(2) Schematic view illustrating tank prepared for electrophoresis.

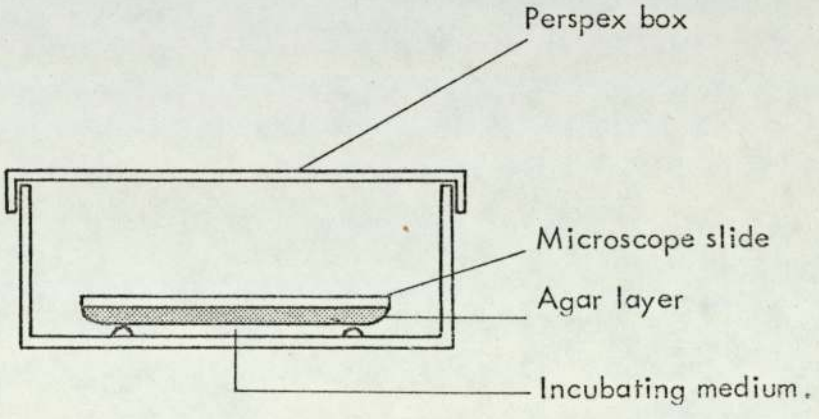


Fig. 22. Container for incubating the agar gel layers with substrate medium after electrophoresis.

4.7.1. Lactate dehydrogenase (LDH; l-lactate nicotinamide-adenine oxidoreductase, E.C.1.1.1.27).

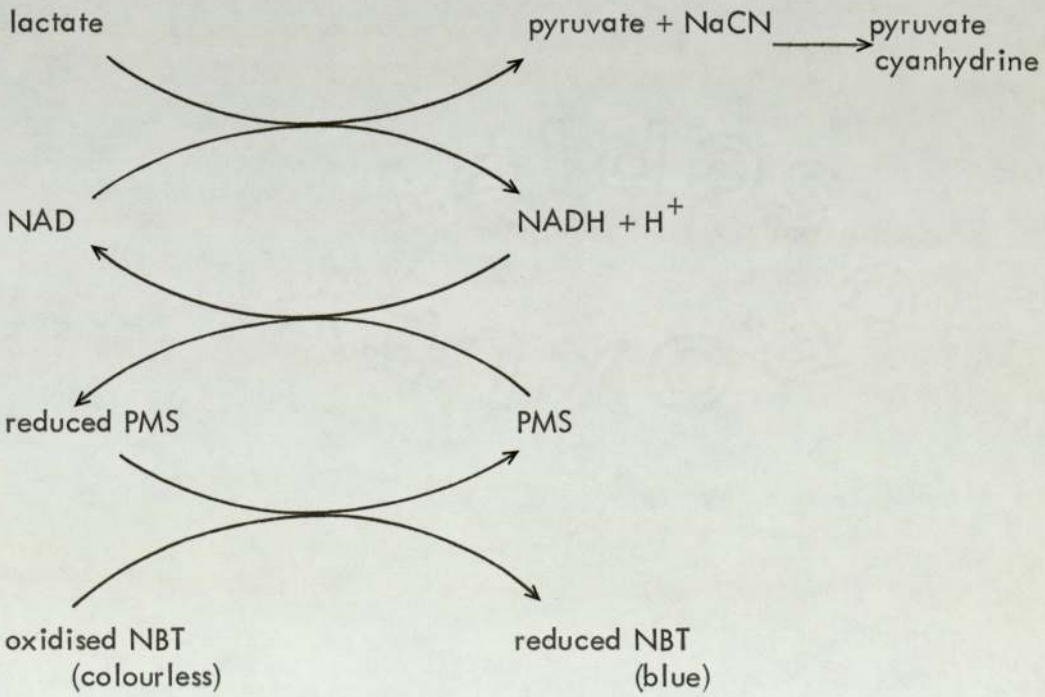
Location of LDH activity was made using the reagents described by Prochazka and Wachsmuth (1972).

LDH catalyses the reaction :

$$\text{Lactate} + \text{NAD} \rightleftharpoons \text{pyruvate} + \text{NADH} + \text{H}^+$$

The equilibrium is normally on the side of the lactate, but adding cyanide to the incubating mixture causes production of pyruvate cyanhydrine. Removal of pyruvate in the form of this extremely stable compound, keeps the reaction moving towards the right. Visualisation of enzyme activity is then effected by coupling this reaction to the reduction of a colourless dye (nitro blue tetrazolium (NBT), to a blue insoluble formazan in the presence of an intermediate electron carrier (phenazine methosulphate (PMS)).

The sequence of events is summarised :



After electrophoresis the slides were incubated with a freshly prepared medium, at 37°C for 1 hour in the dark.

Incubation medium 0.1M Tris (hydroxymethyl methylamine)-HCl buffer, pH 7.6, was prepared from stock solutions in the following proportions :

0.4M Tris ..... 25 ml.
0.2M HCl ..... 40 ml.
Distilled water.... 35 ml.

Reagents marked with an asterisk were freshly prepared for each analysis, the others were stable at 4°C.

0.1M Tris-HCl buffer	10 ml.
0.1M NaCN (pH adjusted to 7.6)	1 ml.
0.2M $MgCl_2.6H_20$ (pH adjusted to 7.6)	1 ml.
2M sodium lactate (pH adjusted to 7.6)	1 ml.
*0.02M NAD	1 ml.
*0.6% NBT	1 ml.
*0.06% PMS	1 ml.
Distilled water	4 ml.

4.7.2. Creatine phosphokinase (CPK; ATP : creatine phosphotransferase, E.C. 2.7.3.2.)

CPK catalyses the transfer of phosphate from creatine phosphate to ADP to form the high energy phosphate compound ATP. In order to utilise the PMS/NBT technique for the visualisation of CPK activity, the components of two further enzyme systems were included in the incubation mixture. The sequence of events is expressed by the following reaction chain :

$$\text{ADP + creatine phosphate} \xrightleftharpoons{\text{CPK}} \text{ATP + creatine}$$

$$\text{ATP + glucose} \xrightleftharpoons{\text{hexokinase}} \text{glucose-6-phosphate (G-6-PO}_4\text{)} + \text{ADP}$$

$$\text{G-6-PO}_4 + \text{NADP} \xrightleftharpoons{\text{G-6-PO}_4 \text{ dehydrogenase}} \text{6-phosphogluconic acid} + \text{NADPH} + \text{H}^+$$

$$\text{NADPH} + \text{H}^+ + \text{PMS} \longrightarrow \text{NADP + reduced PMS}$$

$$\text{reduced PMS + NBT} \longrightarrow \text{PMS + reduced NBT.}$$

Another enzyme, adenylate kinase is visualised by this technique, and can be partially inhibited by the addition of adenosine monophosphate (AMP) to the incubation medium (Dawson et al., 1965).

AMP shifts the equilibrium of the following reaction to the left :

$$\text{ADP} \xrightleftharpoons{\text{adenylate kinase}} \text{ATP + AMP}$$

The barbitone buffer used for homogenising tissue and cell samples for CPK studies, contained 1% mercaptoethanol to activate the enzyme.

Incubation was performed at 37°C for 1 hour in the dark.

Incubation medium.

0.1M tris-HCl buffer, pH 7.6	10 ml.
0.2M $MgCl_2.6H_2O$ (pH adjusted to 7.6)	1 ml.
*0.02M NADP	1 ml.
*0.04M ADP	1 ml.
*0.2M AMP	1 ml.
0.074M D (+) glucose	1 ml.
*0.2M creatine phosphate	1 ml.
Hexokinase (140U/mg/0.5 ml.)	0.05 ml.
Glucose-6-phosphate dehydrogenase (140U/mg/1 ml.)	0.1 ml.

*freshly prepared.

*0.6% NBT	1 ml.
*0.06% PMS	1 ml.
Distilled water	1.85 ml.

*freshly prepared.

4.7.3. Carbonic anhydrase (CA; carbonate hydro-lyase, E.C. 4.2.1.1.)

The reversible combination of carbon dioxide and water to form carbonic acid, is catalysed by the enzyme carbonic anhydrase.

A histochemical technique described by Pearse (1972) was adapted for use with agar gel electrophoresis. The technique is based on a specific staining method described by Hausler (1958) and Hansson (1967). The principle is derived from the following reactions :

$$2HCO_3^- \rightleftharpoons CO_3^{--} + H_2CO_3$$

$$H_2CO_3 \xrightleftharpoons{CA} H_2O + CO_2$$

The carbon dioxide from the dehydration of carbonic acid is removed with the result that more carbonate is formed and the equilibrium of the first spontaneous reaction shifts to the right. Large numbers of CO_3^{--} ions are produced and these are precipitated by the cobalt ions present in the incubation medium. The cobalt carbonate is then visualised as a black precipitate of cobalt sulphide.

After electrophoresis, the agar gels were floated off the microscope slides into the incubation medium contained in a petri dish. The gels were incubated for 1 hour at room temperature.

Incubation medium.

Solution A : (0.1M $COSO_4$ ..... 10 ml.
(0.05M H_2SO_4 ..... 60 ml.

Solution B : 1 g. $NaHCO_3$ dissolved in 50 ml. 0.1M Na_2SO_4 (freshly prepared).

7 ml. of solution A was added to 50 ml. of Solution B just prior to use.

In order to control any non-specific staining, control samples were incubated in medium containing 1 mM acetazolamide (Diamox, Sodium Salt, Lederle), a specific inhibitor of CA.

After incubation the gels were washed with distilled water before being treated with 1% ammonium sulphide for 3 minutes.

Destaining. The gels were thoroughly washed with distilled water, then submerged in 7% acetic acid for a minimum destaining period of 48 hours (Hodgen and Gomes, 1969).

The gels were resupported on microscope slides for drying and qualitative evaluation.

4.8. Densitometry.

When the isoenzymes of LDH and CPK had been visualised, gels were fixed in 5% acetic acid for 3 hours and washed distilled water. A strip of blotting paper was placed on the gel surface and left at room temperature for 24 hours to dry.

The agar dries, leaving a transparent film firmly adhering to the slide.

Quantitation was made using a Vitatron scanning densitometer, with transmitted light at 510 μm. The appartus was attached to a Smith's Servoscribe potentiometric recorder. As the slides were scanned, the areas of the curves corresponding to the individual isoenzymes were automatically integrated, and expressed as a percentage of the total area of the curves.

5. RESULTS.

5. RESULTS.

5.1. Examination of cell fractions obtained from pig gastric mucosa.

Unstained suspensions of cells collected from the interphases of the Ficoll solutions in the discontinuous density gradients and stained preparation of these suspensions were examined by light microscopy (Figs. 27a, 28a, 29a, 30a).

Total cell counts were not performed, but an indication of the cell yield in each fraction was given by the weights of packed cell samples obtained after centrifugation and removal of supernatant (Table 2). Each fraction was pooled from four gradients.

Although fraction C existed in most gradients, it was only possible in a few experiments to collect a sufficient quantity of cells to permit further analysis.

Occasionally results of isolation procedures were unsatisfactory because of mucus production which led to clumping of isolated cells in mucus plugs. Repeated washing of the cells did not facilitate dispersion. One particular batch of pronase produced an extremely low yield from treated tissue, and mucus contamination was very high, presumably due to some loss of proteolytic and mucolytic activity.

5.1.1. Cell size.

Unstained suspensions of cell fractions revealed that intact isolated cells exhibited a variety of sizes and shapes. The majority of cells were round or oval. A few cells were cuboidal or polygonal in shape.

The fractions were compared to one another with respect to cell diameter and morphological appearance using phase contrast microscopy. Cell diameters were measured in increments of approximately 2 μm.

Cells in fractions B and C were large, with diameters ranging from 12 to 45 μm., and exhibiting a uniformly dense, fine granular cytoplasm. The nuclei could be clearly seen and some cells were binucleate.

Table 2. Cell yield (expressed in milligrams) obtained from pig stomach after pronase treatment (fraction A), and from the density gradients (fractions B, C, D and E).

Fraction A	Fraction B	Fraction C	Fraction D	Fraction E
29.1 ± 4.4	11.3 ± 1.2	1.9 ± 1.0	24.5 ± 5.0	27.1 ± 4.5

Results expressed as mean $\pm$ (12 experiments).

Fraction D contained cells with diameter of 9 - 30 μm. The cells in this fraction exhibited heterogeneity in their morphological appearance, and had either (a) similar characteristics to the cells of fraction B and C; (b) fairly dense cytoplasm with large and small granules and a centrally located nucleus, or (c) translucent cytoplasm, large granules and an eccentric nucleus occupying a large proportion of the cytoplasm.

The cell diameter of Fraction E were much smaller (9 - 20 μm.) with the majority of cells having a diameter between and 11 and 14 μm. These cells in general had uniformly dense cytoplasm with scattered large and small granules and a concentric nucleus. A few cells with different characteristics were present. Erythrocytes, and subcellular material were also present in this fraction in larger proportions.

A summary of the cell diameter estimations on these fractions is represented in Fig. 23.

5.1.2. Cytological preparations.

Cell fractions stained by the modified Zimmerman technique were examined by light microscopy. Differential cell counts were performed to ascertain the contributions made by the various cell populations found in the fundic mucosa. Cells were classified into five groups : parietal, chief, mucous secreting cells, erythrocytes and others (to include lymphocytes, leucocytes and connective tissue cells). Both mucous epithelial and mucous neck cells were included in the same group because the surface epithelium of the mucosa used for the experiments was abraded, and in most cases the number of epithelial cells present was very small. The results are summarised in Table 3. The abrasion sample was not included in this Table, as it was difficult to make an objective assessment of the relative amounts of cell types present due to the high mucus content. By studying histological preparations it was possible to conclude that the abraded surface layer consisted mainly of the surface epithelial cells and mucus with a few lymphocytes and erythrocytes. Parietal or chief cells were rarely seen.

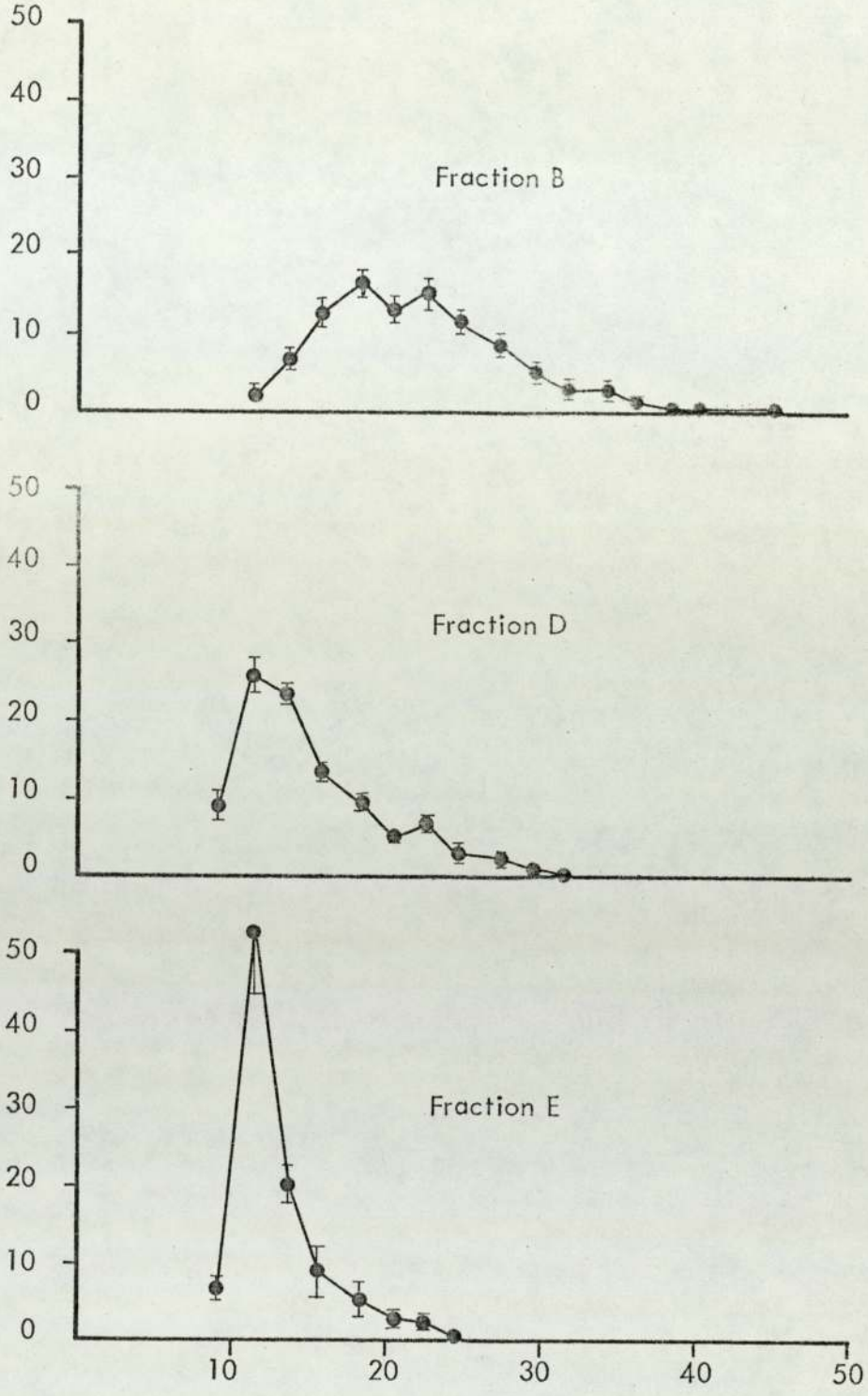


Fig. 23. Cell size distribution in fractions B, D and E obtained from the Ficoll density gradient.
Abscissa : cell diameter (μm); ordinate : percentage.

Table 3. Differential cell counts on fractions obtained from pig fundic mucosa.

Fraction	No. of Experiments	Percentages of cell types				
		Parietal	Chief	Mucous	Erythrocytes	Others*
A	12	11.6 ± 1.6**	14.3 ± 3.1	38.8 ± 6.3	22.3 ± 6.7	13.0 ± 4.5
B	10	68.3 ± 4.4	4.3 ± 1.6	8.5 ± 2.2	5.0 ± 2.6	13.9 ± 3.5
C	3	67.6 ± 13.3	6.7 ± 2.6	7.7 ± 4.3	1.0 ± 0.7	17.0 ± 9.7
D	9	19.2 ± 3.6	17.0 ± 4.9	31.9 ± 6.4	12.6 ± 4.6	19.3 ± 5.9
E	9	5.7 ± 1.9	11.1 ± 3.3	49.4 ± 8.2	20.5 ± 7.0	13.3 ± 4.7

* Includes lymphocytes, leucocytes and connective tissue cells

** Results expressed as mean ± SEM

The cells obtained from the interphase of the 5% and 10% Ficoll layers (fraction B) were predominantly parietal cells of varying size (Fig. 27a), representing a mean of 68.3% (SD 13.3) of the cells in this fraction. Although a similar high proportion of parietal cells were found in fraction C (Fig. 28a), their morphological appearance differed from those in fraction B in that the former exhibited greater intensity of cytoplasmic staining, and the nuclei in some cells were pyknotic and sometimes fragmented.

The yield of chief cells was relatively low in all fractions, the highest percentage being found in fraction D. In preliminary experiments, using the dissected lower part of the gastric gland, it has been possible to concentrate the amount of chief cells to between 30% and 60% (Fig. 32). This was achieved by resuspending the cell suspension obtained from fraction D in 1 ml. of 20% Ficoll, and subjecting this to a second density gradient made up from 30%, 20% (+ cells), 15% and 10% Ficoll in the same proportions as before.

Mucous secreting cells were present in the highest concentrations at the bottom of the gradient. Cellular conglomerates occurred to a certain degree in this fraction and were comprised mainly of mucous cells together with a small proportion of the other cell types.

The remaining cell types in the suspensions were present in relatively constant amounts, and consisted mainly of lymphocytes with occasional leucocytes and connective tissue cells. Erythrocytes contamined fraction D and E, but were present in negligible amounts in fraction B and C.

In unstained preparations of fraction B, 65.5% (SD 18.5) of the cells had diameters greater than 16 μm. When these fractions were stained, a positive correlation existed between the percentage of cells classified as parietal cells and the percentage of large cells observed in the unstained preparations ($r = 0.943$, Fig. 24). Cells from this fraction, larger than 16 μm. in diameter, could therefore be designated parietal cells without the need to perform specialised staining techniques. Simple light microscopical examination of this fraction, using a calibrated eyepiece micrometer would be sufficient for confirmation of purity. Owing to the more mixed populations in the remaining gradients it was more difficult to make similar comparisons.

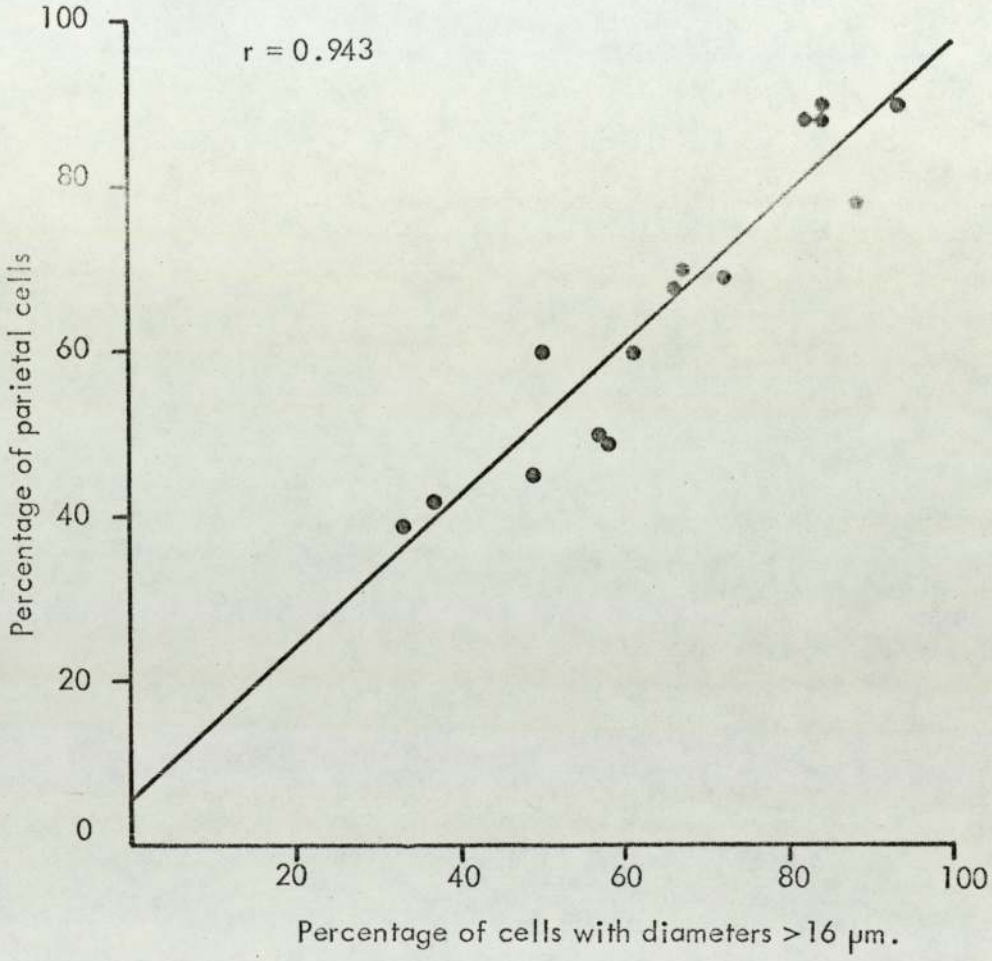


Fig. 24. Comparison between cells classified as parietals using the modified Zimmerman stain, and cells with diameter greater than 16 μm in the unstained preparation (Fraction B).

In fractions D and E, cells with diameters of more than 16 μm., only represented 28% (SD 3.6) and 12% (SD 9) respectively. When these figures were compared with the cell population percentages in Table 3, it was apparent that the presence of these large cells was due to contamination of these fractions by parietal cells.

There was some overlapping in the ranges of cell diameters, since a few chief and mucous neck cells could be larger than 16 μm., a few parietal cells were probably less than 16 μm. in diameter.

Erythrocytes formed part of the contamination in fractions D and E, but since their diameter is normally less than 10 μm. (Geigy, 1962), then cells in these fractions having diamters between 10 μm. and 16 μm., were assumed to be predominantly chief and mucous neck cells together with small numbers of lymphocytes and leucocytes. In Table 3, approximately 70% of the cells fall into this category, and in the cell diameter estimations 63% (SD 3.8) and 81 % (SD 6.9) of the cells in fraction D and E respectively, had diameters between 10 μm. and 16 μm.

5.2. Examination of cell fractions obtained from human gastric mucosa.

Using the techniques described for pig stomach, cell fractions were prepared from normal human fundic mucosa. Results of the differential cell counts are presented in Table 4. Insufficient material was available for studying unstained preparations.

Stained cytological preparations were examined and found to be comparable to those obtained from pig stomach. A suspension of cells from fraction B is illustrated in Fig. 33.

5.3. LDH isoenzyme patterns (Pig Stomach).

Four major bands of LDH enzyme activity were separated by electrophoresis in the cell fractions and tissue samples. A fifth minor band was sometimes present.

Table 4. Differential cell counts on fractions A, B, D, and E, obtained from two samples of human fundic mucosa, using the techniques and nomenclature described as for pig stomach.

Fraction	Percentages of cell types									
	Case 1					Case 2				
	Parietal	Chief	Mucous	Erythrocytes	Others	Parietal	Chief	Mucous	Erythrocytes	Others
A	4	2	30	60	4	6	1	68	15	10
B	90	1	6	0	3	66	0	21	5	8
D	4	0	40	54	2	2	5	85	4	2
E	1	1	43	53	2	1	1	80	12	6

Results are mean of duplicate determinations.

From preliminary visual examination of the electrophoretograms it was apparent that an outstanding difference existed between the pattern of the fraction containing a high proportion of parietal cells (fraction B), to those of the remaining cell fractions (Fig. 25a). After quantitation by densitometry this difference proved statistically to be highly significant (Table 5).

Photomicrographs of the different cell fractions together with a representative sample of their corresponding LDH isoenzyme patterns are presented in Figs. 26-31. In Table 6, the isoenzyme patterns are expressed as mean values of the percentages of individual isoenzymes.

A constant feature of the patterns derived from fraction B was (a) the predominance of LDH-1 with a progressive decrease in LDH-2 and LDH-3; (b) a virtual absence of LDH-4 and a complete absence of LDH-5, even in samples exhibiting high enzyme activity, and (c) a greater ratio of monomer H subunits to M subunits, which was calculated from the mean percentages of isoenzymes to be 13 : 1 in contrast to the H : M ratio of 2 : 1 for entire fundic mucosa (Fig. 34). An example of the H : M ratio calculation is shown in Table 7.

Although limited in number, fraction C which contained a similar high proportion of parietal cells, revealed the same characteristic isoenzyme pattern as fraction B and no statistical difference existed between them. The isoenzyme patterns of fractions D and E were very similar and differed only with respect to their LDH-1 and LDH-3 percentages.

The majority of electrophoretograms did not reveal the presence of LDH-5, but small amounts were detected in fraction D and in samples of the abraded surface mucous epithelium.

The abrasion pattern was characterised by the prevalence of LDH-4 and a concomitant decrease in LDH-1, in comparison with the other fractions.

LDH-1 and LDH-3 prevailed in all fractions except B and C. In samples of fundic and pyloric mucosa this was still evident (Table 8). The greatest differences between fundic and pyloric mucosa isoenzyme patterns were in the relative amounts of LDH-1 and LDH-4. This difference was enhanced by dissecting away the muscularis mucosae.

Table 5. Statistical significance of the difference between the LDH isoenzymes percentages in cell fractions A, B, D, E, and abrasion.

Significance of difference between cell fractions	LDH isoenzymes				
	LDH 1	LDH 2	LDH 3	LDH 4	LDH 5
Abrasion and A	0.05[a]	0[b]	0	0.01	.[c]
Abrasion and B	0.001	0	0.005	0.001	.
Abrasion and D	0.01	0	0	0.005	.
Abrasion and E	0	0	0	0.05	.
B and A	0.02	0	0.001	0.01	.
B and D	0.001	0.005	0.001	0.005	.
B and E	0.001	0.005	0.001	0.02	.
A and D	0.02	0.05	0.05	0.02	.
A and E	0.005	0.01	0.005	0.05	.
D and E	0.05	0	0.01	0	.

[a]The level of significance was calculated using a paired-sample t-test

[b]$p > 0.05 = 0$

[c]The significance was not calculated.

Table 6. Percentages of LDH isoenzymes in fractions A - E, and abraded surface epithelium of pig fundic mucosa.

Fraction	No. of Experiments	LDH isoenzymes				
		LDH 1	LDH 2	LDH 3	LDH 4	LDH 5
A	12	60.1 ± 4.6	16.2 ± 2.2	19.4 ± 1.8	4.3 ± 1.1	-
B	10	79.7 ± 1.6	12.5 ± 1.5	7.0 ± 1.4	0.8 ± 1.2	-
C	3	77.2 ± 4.9	10.9 ± 0.7	9.6 ± 2.6	2.3 ± 1.7	-
D	9	44.6 ± 3.9	21.2 ± 1.9	25.2 ± 1.9	8.7 ± 2.0	0.3 ± 0.3
E	9	37.0 ± 4.0	21.8 ± 2.0	31.2 ± 2.6	10.0 ± 3.2	-
Abrasion	5	30.4 ± 4.9	17.7 ± 2.2	27.5 ± 2.0	23.9 ± 4.1	0.5 ± 0.6

Results expressed as mean ± SEM.

Table 7. An example of the calculation of the H : M ratio from the percentage of LDH isoenzymes.

Isoenzyme	Percentage	Monomer Composition	H %	M %
LDH-1	20	H4	20	0
LDH-2	18	H3M1	13.5	4.5
LDH-3	22	H2M2	11	11
LDH-4	20	H1M3	5	15
LDH-5	20	M4	0	20
	100		49.5	50.5

Table 8. LDH isoenzyme percentages in the fundic and pyloric regions of pig stomach.

Tissue	No. of Experiments	LDH isoenzymes				
		LDH 1	LDH 2	LDH 3	LDH 4	LDH 5
Fundic mucosa	4	35.7 ± 3.9	17.8 ± 1.6	30.9 ± 1.3	14.6 ± 2.4	1.0 ± 0.4
Fundic mucosa (free from muscularis mucosae and submucosa)	8	38.7 ± 4.0	20.3 ± 1.6	27.6 ± 1.0	12.8 ± 2.4	0.6 ± 0.3
Pyloric mucosa	4	24.5 ± 4.4	21.8 ± 1.4	31.9 ± 0.9	20.5 ± 3.4	1.3 ± 0.6

Results expressed as mean ± SEM.

Further differences between the two regions of the stomach were observed when the H : M ratios were calculated (Fig. 34).

5.4. LDH isoenzyme patterns (Human Stomach).

Five isoenzymes of LDH were separated by electrophoresis in the cell fractions obtained from human fundic mucosa (Fig. 25b). Of particular interest was the LDH isoenzyme pattern of fraction B in Case 1 (Table 9), which clearly exemplified the characteristic elevated LDH-1 and progressive decrease of LDH-2, LDH-3 and LDH-4 that was found in fraction B of pig gastric mucosa.

This decrease was not so evident in Case 2 (Table 9). The differences however, between the isoenzyme pattern of fraction B, and the patterns of fractions D and E were most striking. The latter two fractions contained relatively pure suspensions of mucous secreting cells and their patterns reflected this in the preponderance of LDH-4 and LDH-5, and marked decreases in the levels of LDH-1 and LDH-2.

In Case 1, LDH-3 was the predominant isoenzyme in fractions D and E. There was a similar prevalence of LDH-3 in the abrasion sample, a further distinction of which was the elevation of the LDH-4 and LDH-5 fraction with a parallel decrease in LDH-1 and LDH-2.

5.5. CPK isoenzymes.

Only samples of cell fractions and tissue from pig stomachs were examined for CPK isoenzymes. There was insufficient material available from human fractions to make a comparison.

The separation of CPK isoenzymes was performed on the same samples used for LDH studies.

In samples of external muscle from the stomach, three bands of CPK activity were detected. The intermediate isoenzyme (MB) exhibited the highest activity, with a much lower activity of the fast-moving anodic band (BB). Only trace levels of the cathodic isoenzyme (MM) were detected.

Table 9. LDH isoenzyme percentages in fractions A, B, D, and E, from two samples of human fundic mucosa.

Fraction	LDH isoenzymes									
	Case 1					Case 2				
	LDH 1	LDH 2	LDH 3	LDH 4	LDH 5	LDH 1	LDH 2	LDH 3	LDH 4	LDH 5
A	34[+]	24	25	13	4	20	18	22	20	20
B	71	18	8	2	1	45	9	13	20	13
D	24	24	33	15	4	1	3	23	39	34
E	18	27	39	14	2	2	7	24	34	33
Abrasion	19	16	31	19	15	-	-	-	-	-
Fundic mucosa (b)	32	31	20	12	5	-	-	-	-	-

[+] results are mean of duplicate determinations.

(b) abraded mucosa free from muscularis mucosae and submucosa.

The only enzyme activity detected in the cell fractions and tissue apart from the muscle, was found in the fundic mucosa sample. Small amounts of the BB isoenzyme were found in extracts of this tissue (Fig. 35).

Increasing the amount of sample did not reveal the presence of any additional regions of enzyme activity.

5.6. CA isoenzymes.

In a separate study, the extracts of cell fractions and tissue samples from pig fundic mucosa were electrophoresed in an attempt to separate isoenzymes of CA.

Using the method described, no CA activity was detected in any of the samples analysed (Fig. 36). Modifying the reagents according to the technique described by Hodgen and Gomes (1967), did not produce any better results.

Fig. 25 (a) LDH isoenzyme patterns of fractions A - E, and abrasion. Fraction B, the cell fraction containing the highest percentage of parietal cells, exhibits an almost exclusive presence of the pure H4 tetramer, LDH-1. (Pig stomach).

Fig. 25 (b) LDH isoenzyme patterns of fractions A, B, D, E, abrasion and fundic mucosa. (Human stomach).

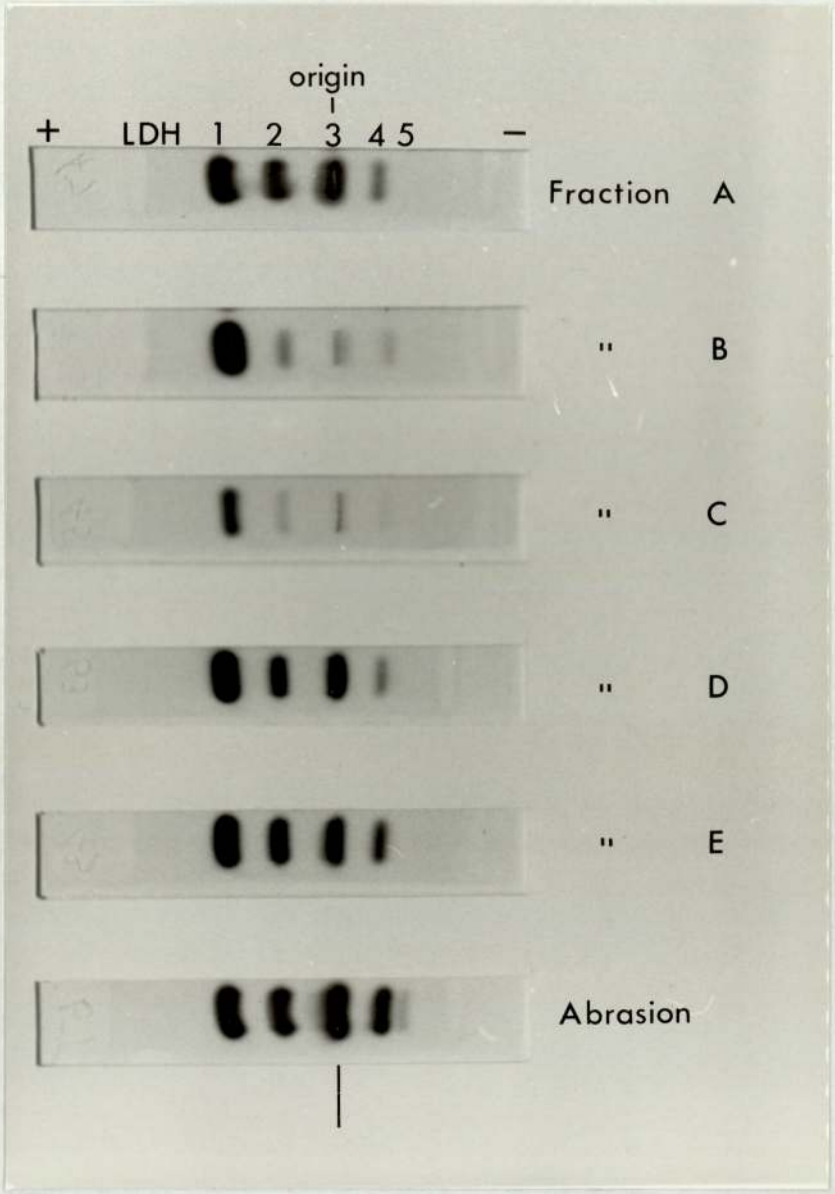


Fig.25.(a)

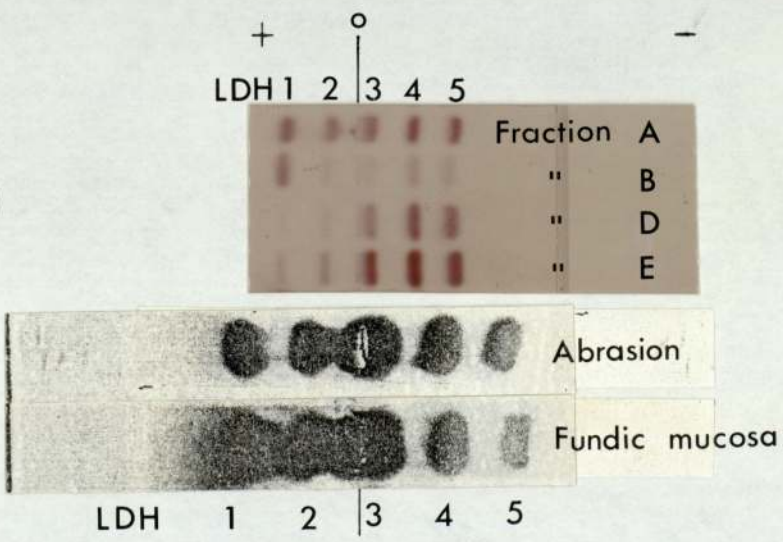
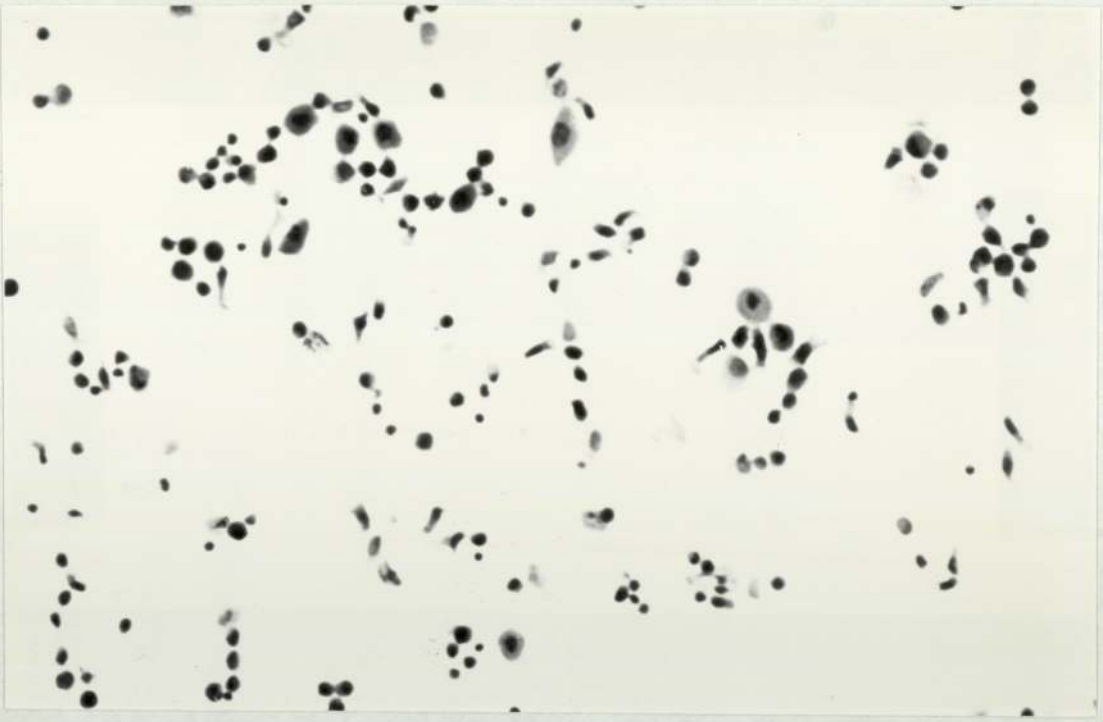


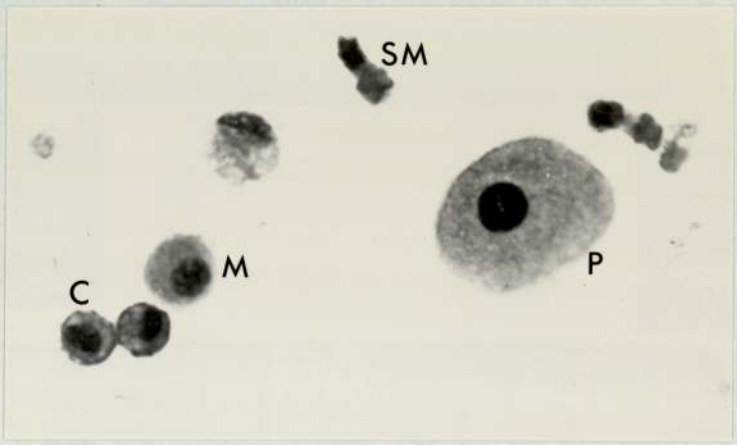
Fig.25.(b)

Fig. 26 (a) Fraction A : Mixed cell suspension obtained from pig fundic mucosa by incubation with pronase for 90 minutes x 200.
The different cell types found in this suspension are illustrated at higher magnification in the lower photograph. SM - surface mucous cell; M - mucous neck cell; C - chief cell; P - parietal cell.
Modified Zimmerman stain x 900.

(b) Xerox photocopy of the LDH isoenzyme pattern of fraction A.



(a)



(b)

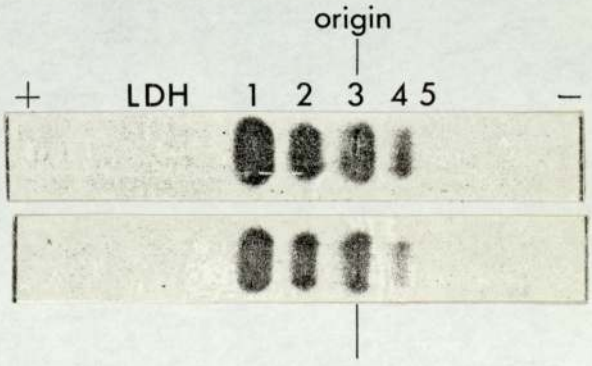
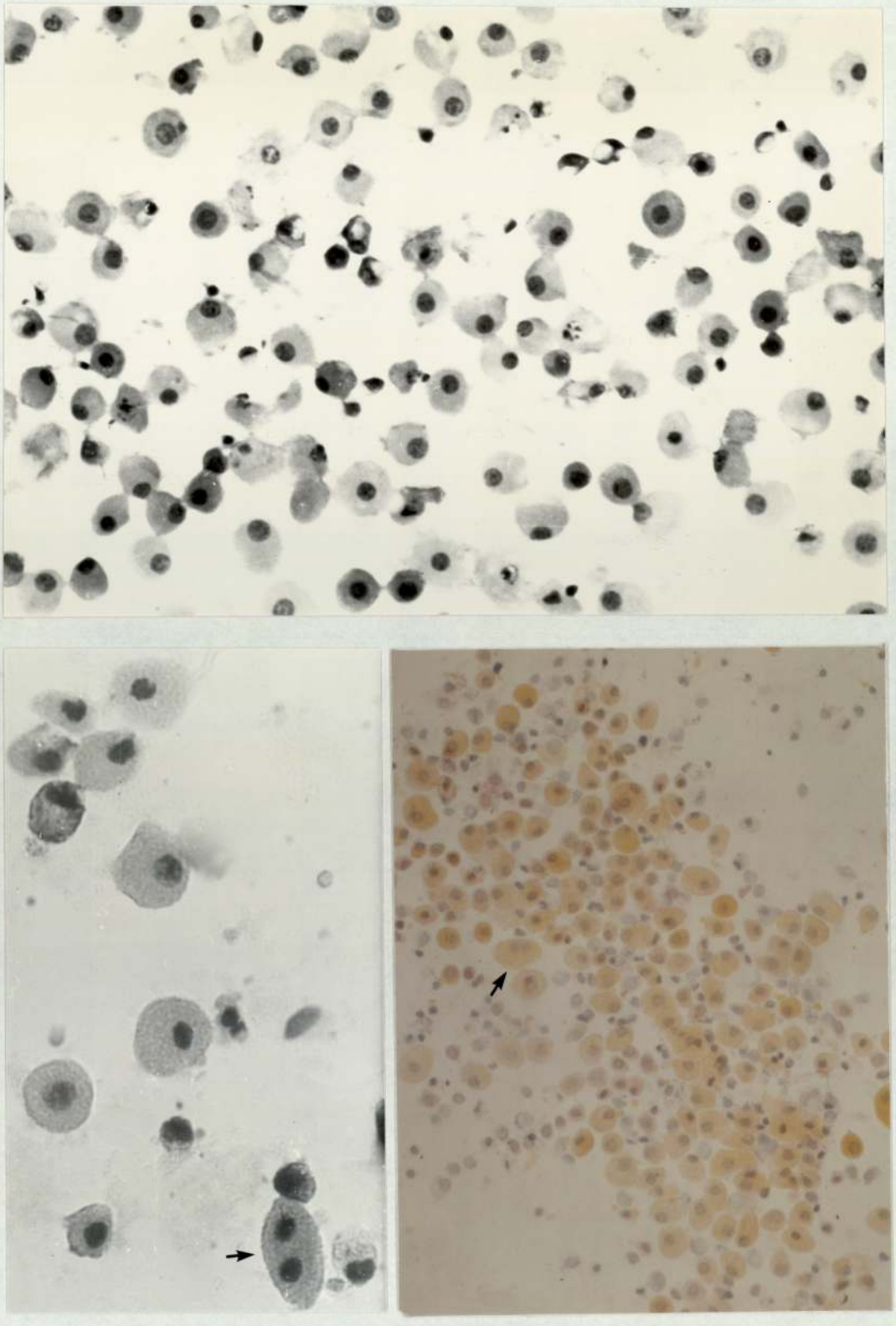


Fig. 26.

Fig. 27 (a) Fraction B : Photomicrographs at different magnifications showing the relatively pure suspensions of parietal cells collected from the interphase of the 5% and 10% Ficoll. Binucleate cells are often seen (arrowed). Modified Zimmerman stain.

(b) Xerox photocopy of the LDH isoenzyme pattern of fraction B, which exhibits an almost exclusive presence of the pure H-4 tetramer, LDH-1.



(a)

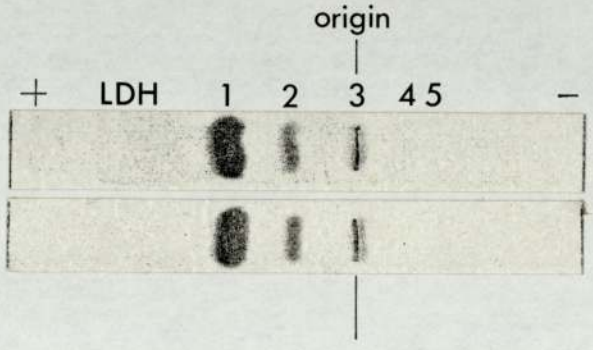
(b)

Fig. 27.

Fig. 28 (a) Fraction C : A suspension of cells obtained from the 10 - 12.5% Ficoll interphase. The cells are predominantly parietal cells exhibiting various intensities of cytoplasmic staining. The nuclei are sometimes fragmented or pyknotic.
Modified Zimmerman stain x 200.

(b) Xerox photocopy of the LDH isoenzyme pattern of fraction C.

(a)

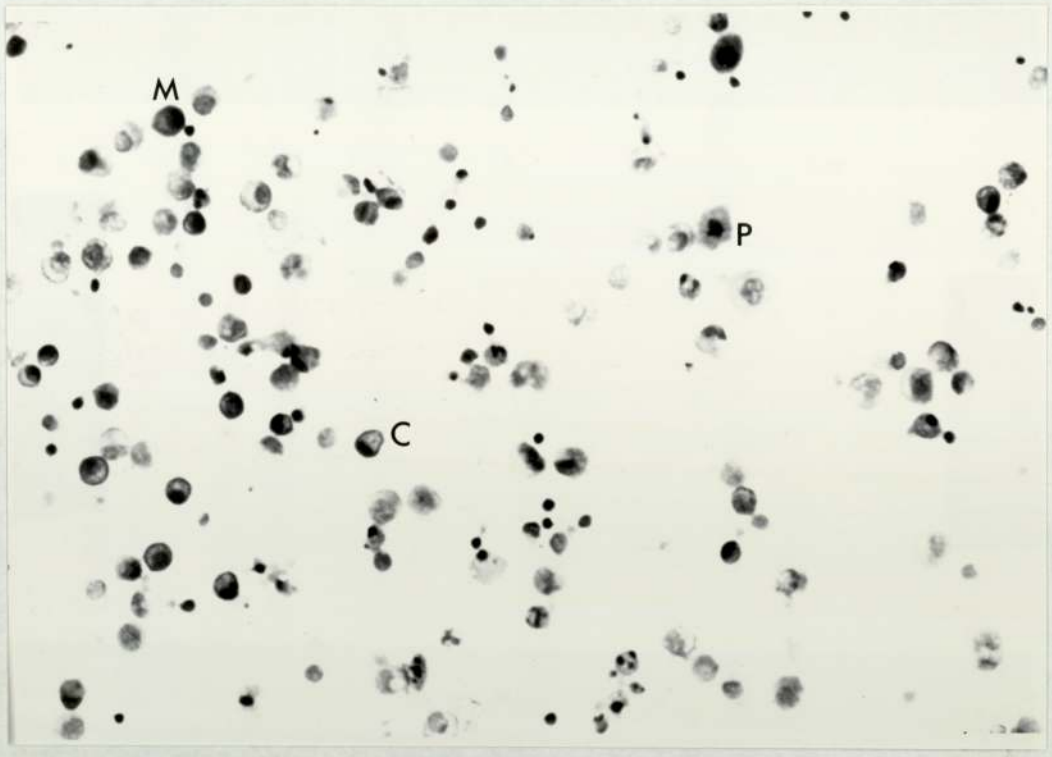


(b)

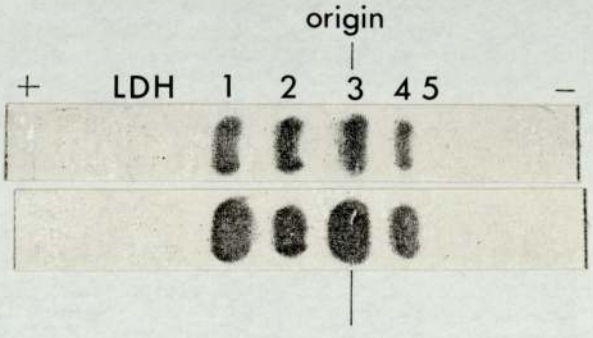
Fig. 28.

Fig. 29 (a) Fraction D. Cell suspension obtained from the 12.5 - 20% Ficoll interphase. This fraction contained mainly mucous neck cells (M) with a small percentage of chief (C) and parietal cells (P). Modified Zimmerman stain x 200.

(b) Xerox photocopy of the LDH isoenzyme pattern of fraction D.



(a)

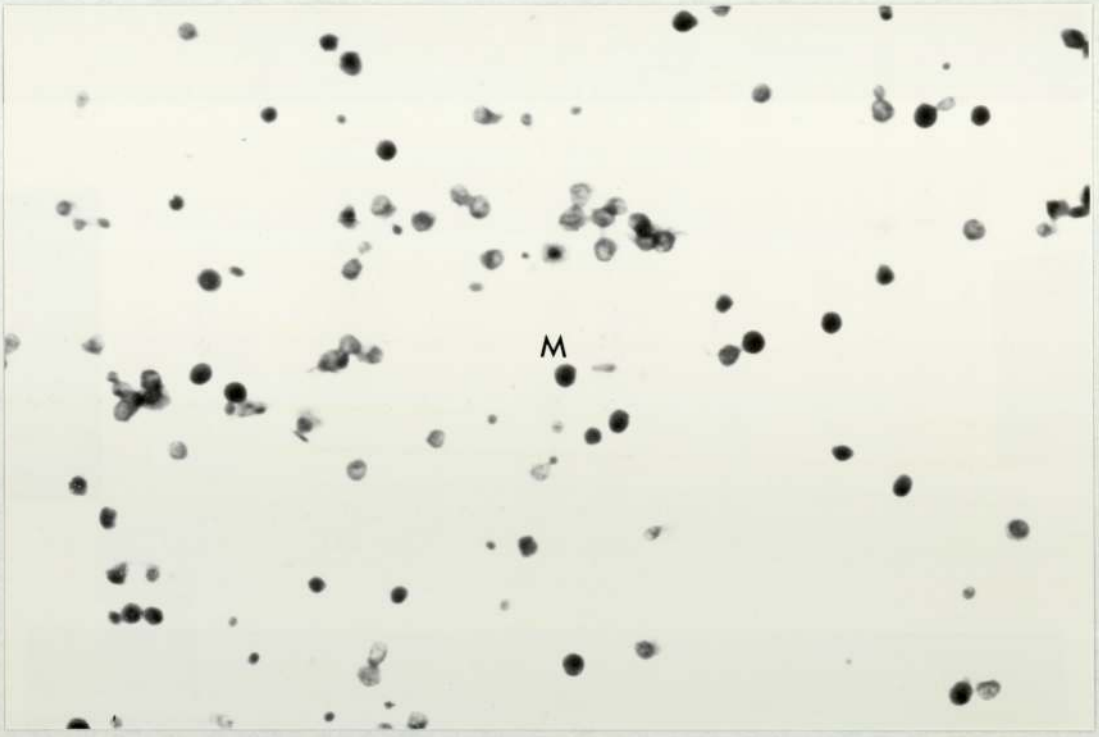


(b)

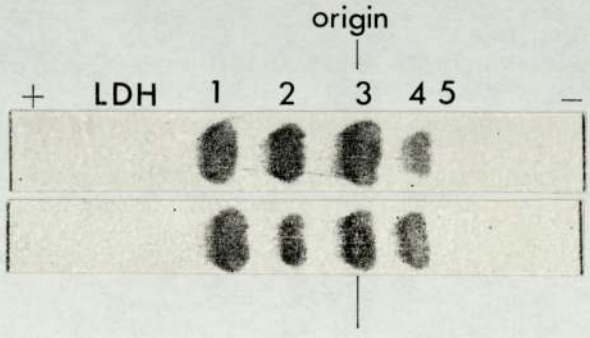
Fig. 29.

Fig. 30 (a) Fraction E : cell suspension obtained from the bottom of the Ficoll gradient. This fraction contained the highest proportion of mucous neck cells (M). Modified Zimmerman stain. x 200.

(b) Xerox photocopy of the LDH isoenzyme pattern of fraction E.



(a)

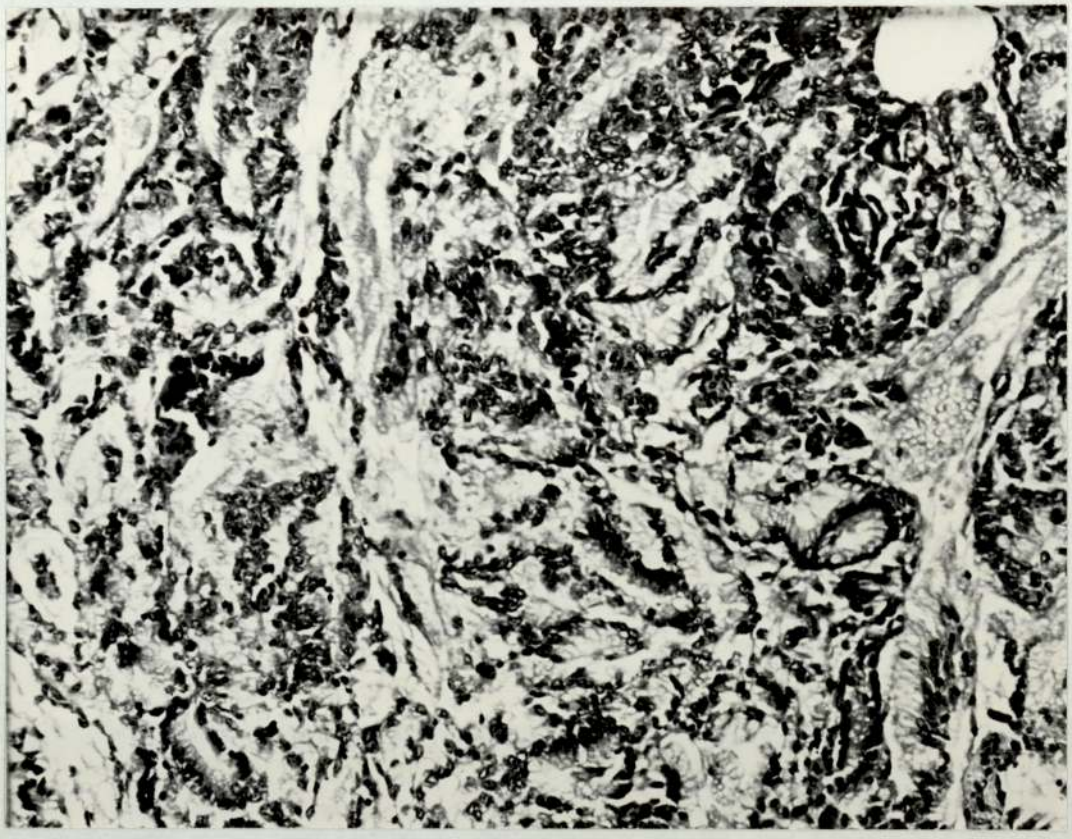


(b)

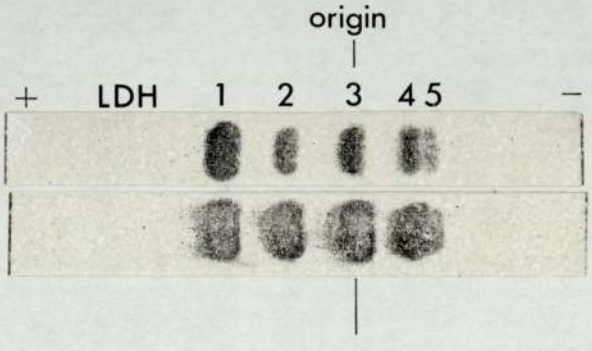
Fig. 30.

Fig. 31 (a) Histological preparation of the abraded surface epithelium of pig fundic mucosa.
Modified Zimmerman stain x 200

(b) Xerox photocopy of the LDH isoenzyme pattern of the abrasion sample.



(a)



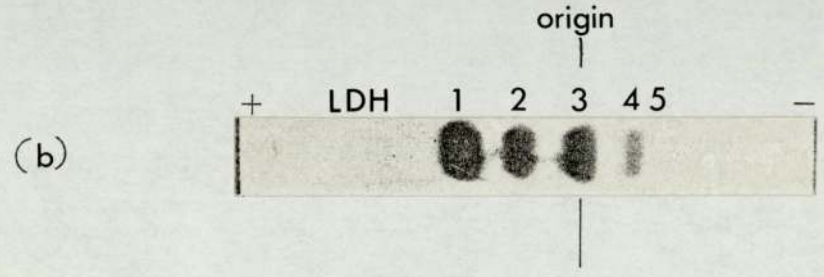
(b)

Fig. 31.

Fig. 32 (a) A suspension of cells containing a high proportion of chief cells (C) obtained by subjecting fraction D to a second Ficoll gradient of higher density. A few parietal (P) and mucous neck cells (M) are present. Modified Zimmerman stain x 400.

(b) Xerox photocopy of the LDH isoenzyme pattern of the above cell suspension.

(a)

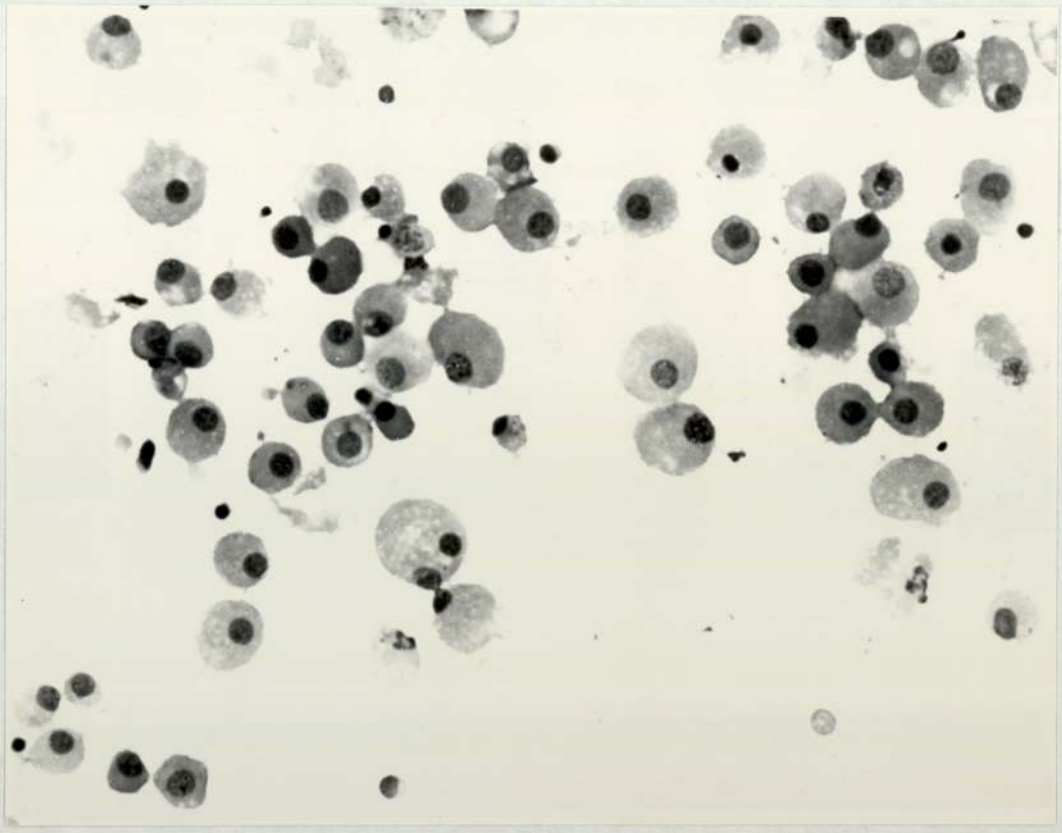


(b)

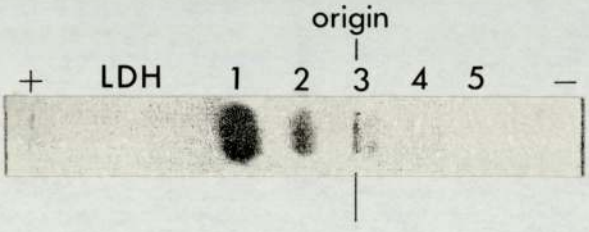
Fig. 32.

Fig. 33 (a) A suspension of parietal cells (fraction B) obtained from human fundic mucosa, using the techniques described for pig stomach. Modified Zimmerman stain x 500.

(b) Xerox photocopy of the LDH isoenzyme pattern of the above suspension.



(a)



(b)

Fig. 33.

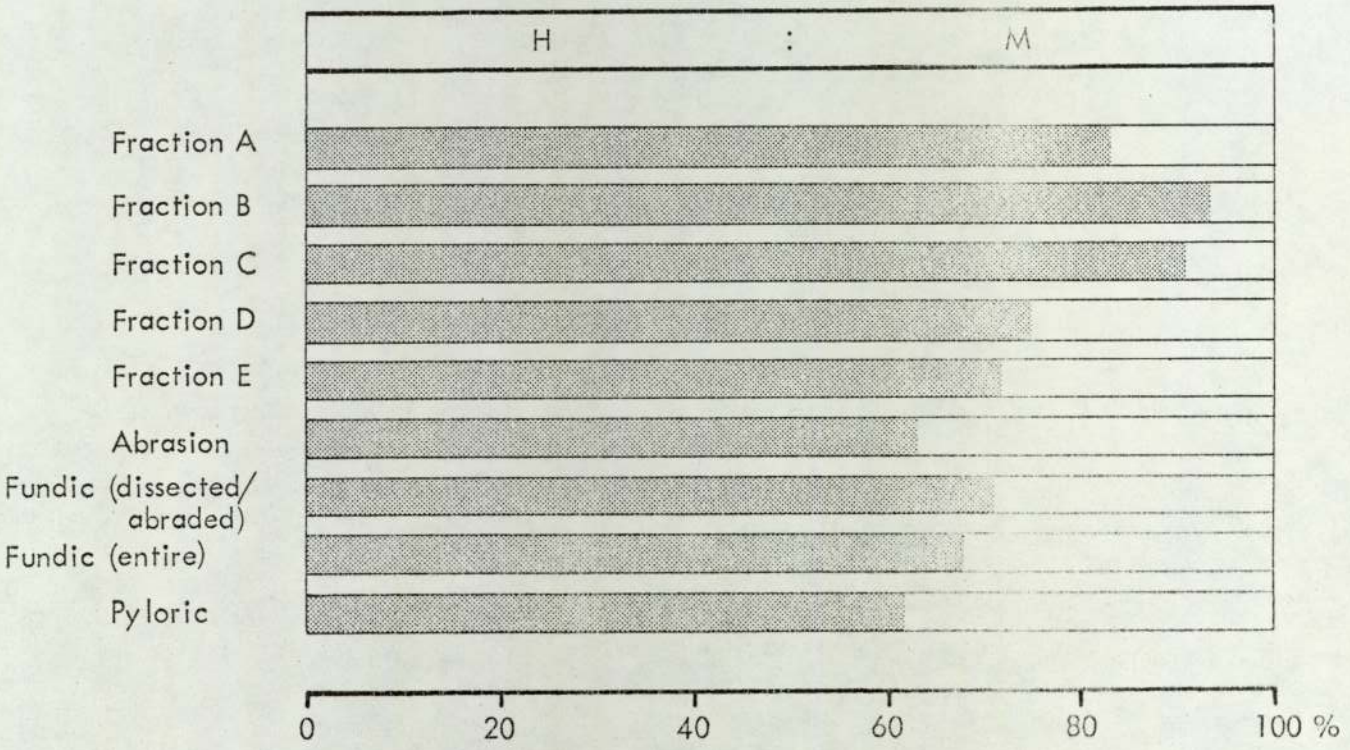


Fig. 34. The proportions of the monomer H and M (in percentage) in fractions A - E, abrasion and in fundic and pyloric mucosa of pig stomach.

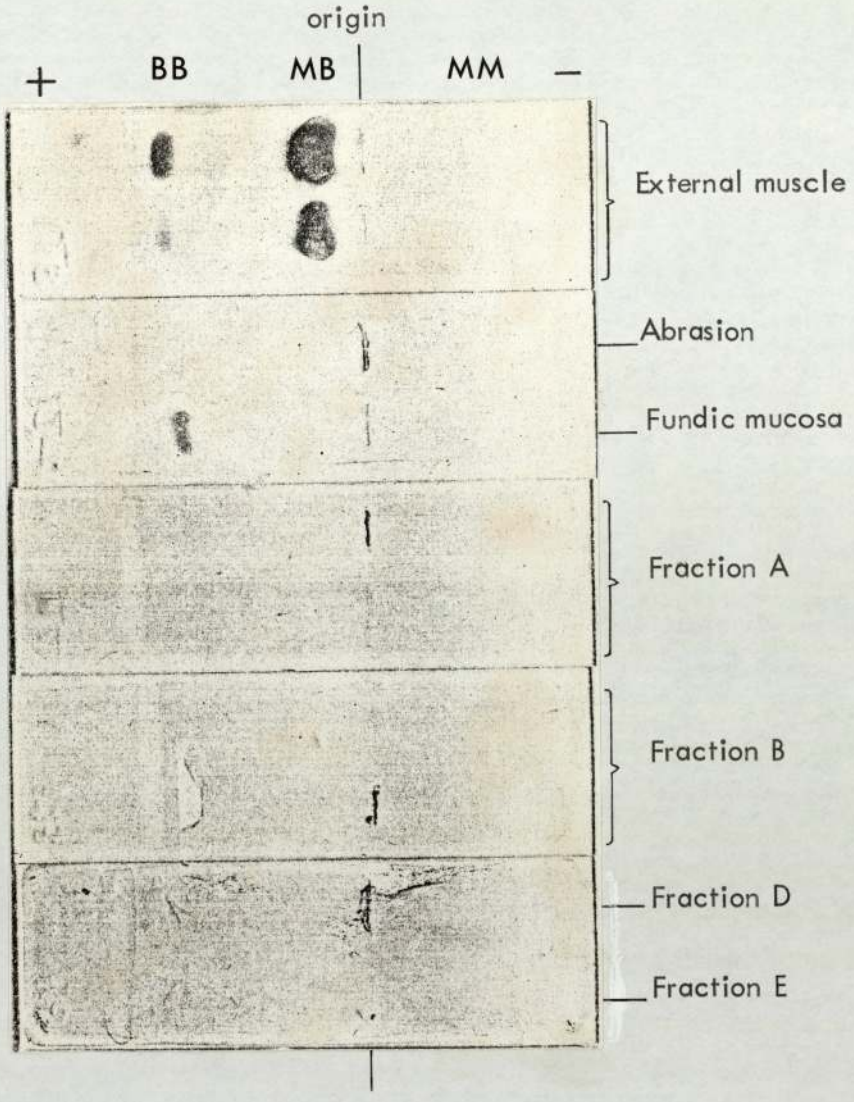


Fig. 35. Xerox photocopy of CPK isoenzymes. No activity could be detected in fractions A, B, D, E and abrasion.
A small amount of the BB isoenzyme was present in the fundic mucosa sample.

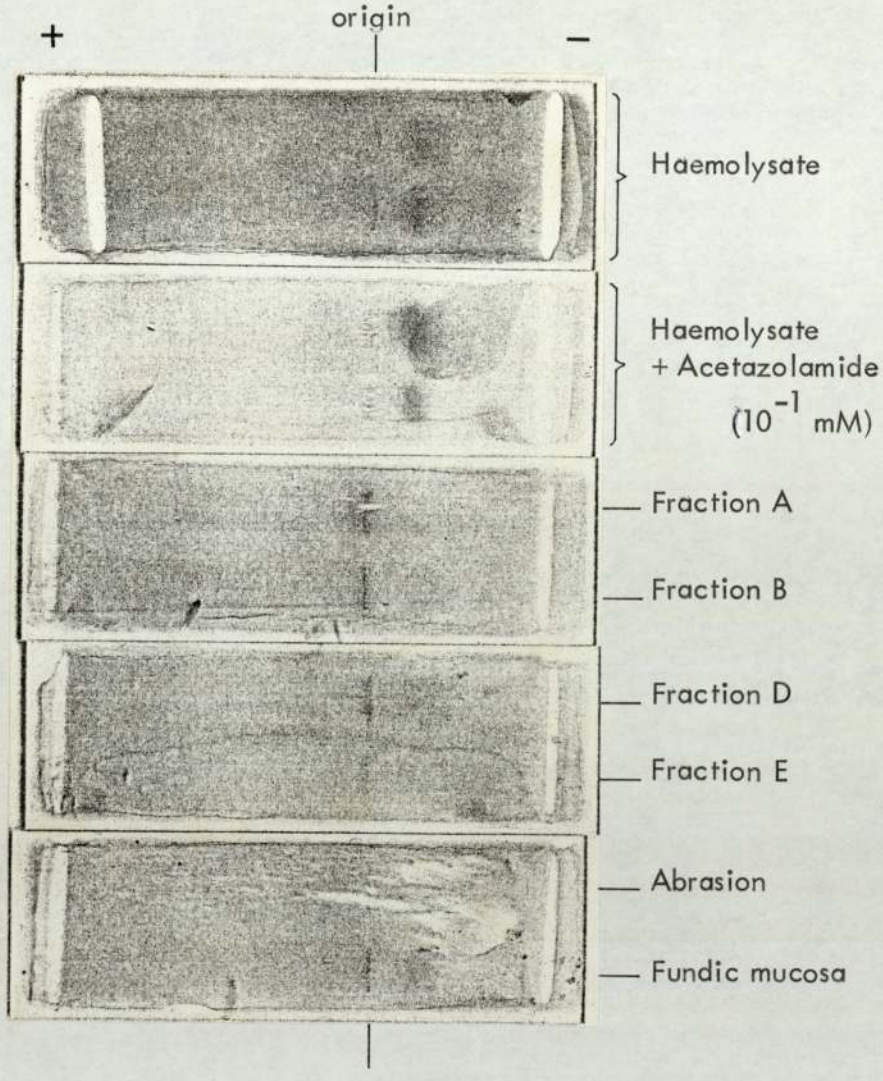


Fig. 36. Xerox photocopy of dried agar gel layers after staining for carbonic anhydrase activity. The dark bands in the haemolysate are haemoglobin.
No carbonic anhydrase activity could be detected in any of the cell fractions or tissue samples in pig fundic mucosa.

6. DISCUSSION.

6. DISCUSSION.

Cell isolation. The aim of developing a technique for separating the different cell types of fundic mucosa into homogenous populations was not completely fulfilled, nevertheless the method described proved to be a reliable procedure for isolating relatively pure suspensions of parietal cells. As a result, considerable attention was directed towards their study.

An homogenous preparation of surface mucous cells was prepared by simply abrading the surface of the mucosa.

The mucous neck cells prevailed in fraction E, and provided a means to compare this fraction with other samples containing fewer cells of this type.

Isolation of chief cells proved difficult within the framework of the relatively straightforward technique. Preliminary work has shown that it is possible to purify cell suspensions containing these cells by centrifugation in a second gradient of higher Ficoll concentrations. Unfortunately, for practical reasons it was not possible to divert the procedure towards that objective in more than a few experiments. In addition, the lower part of the fundic glands, where the chief cells predominate, was dissected away with the muscularis mucosae.

Abrading the surface of the mucosa, and dissecting the mucosa from the underlying tissue served two purposes. Firstly, it eliminated a large majority of the surface mucous cells and mucus, thereby decreasing the number of cell types to be separated and lessening the effects of mucus contamination. Secondly, by using a relatively pure 'mucosal' tissue it increased the cell yield and diminished the contamination by other cell types found in the region of the muscularis mucosae (lymphocytes, connective tissue cells, leucocytes and erythrocytes).

In the preliminary experiments when the pronase method was being evaluated, the mucosa was left intact and not dissected from underlying tissue. The effect of the pronase was to loosen this tissue and give it the appearance and consistency of thick mucus. This impeded the release of cells, as they became trapped within this mass. Also, during these experiments histological examination of the tissue after pronase treatment revealed that although large proportions of cells had been

separated they were still trapped within the connective tissue surrounding the gastric pits. The innovation of squeezing the tissue after pronase treatment provided the necessary mild mechanical action needed to release these cells and provided excellent improvement in cell yield.

Croft and Ingelfinger (1969) described a method of obtaining canine gastric mucosa free of muscularis mucosae and submucosa. Using a blunted needle attached to a syringe, they injected phosphate buffer into the tissue in such a way that the tissue stripped from the muscularis mucosae. This was not found to be a simple or satisfactory method of obtaining 'mucosal' tissue when applied to pig or human stomach.

Discontinuous density gradients prepared from Ficoll in the manner described were found to be effective in separating the cells in the suspensions. By applying the mixed cell suspensions to the centre of the gradient and using minimal gravitational forces, cells migrated to the upper and lower fractions of the gradient. Since only the parietal cells travelled to the upper Ficoll interphase they were less likely to be contaminated with the other cell types that migrated to the lower fractions.

If mixed cell suspensions in phosphate buffer were layered onto the gradient and centrifuged, separation was only partly achieved. This may be why Walder and Lunseth (1963) using similar Ficoll concentrations, obtained suspensions containing only 30% - 50% parietal cells, using this technique. They, however, collected this parietal cell fraction from the lower layers of the gradient, using rabbit, dog and human gastric tissue.

This contradiction to the finding of parietal cells in the upper fraction in this study is difficult to explain. Differences in technique and methods of cell identification are possible reasons. McDogual and DeCosse (1970) using the same technique as Walder and Lunseth, used haematoxylin and eosin stain for cell identification. This method of staining was found to be unsatisfactory for the purpose of identifying and distinguishing the gastric mucosal cell types in isolated suspensions in this study.

Isoenzyme patterns. Although the LDH isoenzyme patterns observed in fundic and pyloric regions of pig gastric mucosa (Fig. 37) do not exhibit the characteristic differences found in similar samples from rat stomach (Prochazka et al., 1968a) and human stomach (Baume et al., 1966; Prochazka et al., 1968b), the pattern of the cell fractions used in this study revealed marked differences in individual isoenzyme levels when comparing one fraction with another (Fig. 25). These differences appeared to be related to the proportion of individual cell populations present in the suspensions.

Characteristic of the fraction enriched with parietal cells was the almost exclusive presence of the pure H4 tetramer, LDH-1. These results indicate that the parietal cells have a high rate of synthesis of monomer H subunits, and the prevalence of LDH-1 found in the fundic mucosa can therefore be unequivocally attributed to the unique LDH isoenzyme pattern of these cells.

LDH-1 is associated with aerobic oxidation and the complete oxidation of pyruvate and lactate in mitochondria (Dawson et al., 1964) providing a constant supply of energy. The demonstration of a high level of LDH-1 in the parietal cell fraction is in accordance with the energy demands required for the secretion of hydrochloric acid.

In fraction C, a similar high proportion of parietal cells were present, and even though the amount of material was considerably less than in fraction B, the LDH isoenzyme pattern was almost identical.

In comparison, the remaining cell fractions, which contained a high proportion of mucous secreting cells, and few parietal cells, revealed a decrease in the H monomer production relative to the M monomer (Fig. 34).

The abrasion sample could be regarded as an homogenous preparation of surface mucous cells, and in this respect the pattern of LDH isoenzymes indicated the presence in these cells of a different type of metabolism to the parietal cells, in that the production of M monomer was greatly increased by comparison. Where M monomer production prevails it indicates a cellular metabolism utilising energy from the anaerobic breakdown of carbohydrate.

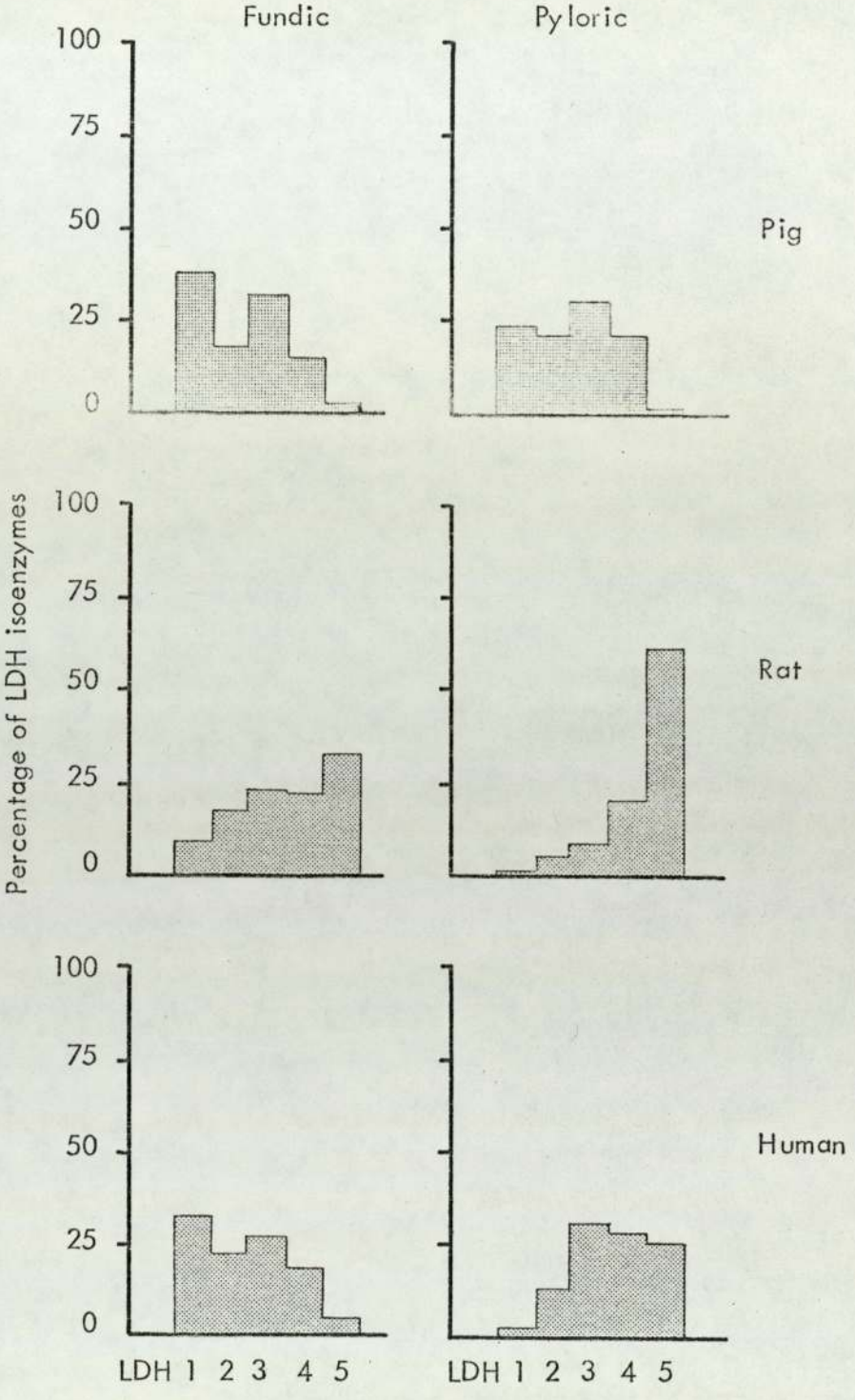


Fig. 37. LDH isoenzyme patterns in fundic and pyloric regions of pig, rat (Prochazka et al., 1968a) and human stomach (Prochazka et al., 1968b)

The level of LDH-1 was however, still higher than that found in samples of pyloric tissue, where there is a preponderance of mucous secreting cells. Since the epithelium of the gastric mucosa has been shown to rapidly proliferate (Lipkin et al., 1963), it is possible that the intracellular contents of extruded cells from the gastric pits could be present in the mucus secretion covering the epithelium, and influence the H : M ratio of the abrasion sample.

Fractions D and E were not homogenous samples of cells but each contained different proportions of cell types. This was reflected in their respective isoenzyme patterns. Fraction E, which contained the highest percentage of mucous secreting cells, exhibited a corresponding increase in LDH-3 and LDH-4. It is assumed that in these two fractions, the LDH-1 isoenzyme is derived partly from contaminating parietal cells, erythrocytes and possibly chief cells.

The presence of LDH-1 and LDH-2 in tissue samples indicates that the isoenzymes may originate from erythrocytes (Neremberg and Pogojeff, 1969). Samples of pig erythrocytes were electrophoresed and found to contain mainly LDH-1 with a trace of LDH-2. Cell fractions heavily contaminated with erythrocytes revealed correspondingly higher levels of LDH-1.

The pattern of the mixed cell suspension (fraction A), demonstrated higher levels of LDH-1 than would be expected from the differential cell count studies, and does not appear to be satisfactorily explained by the percentage of erythrocytes and parietal cells present. One possible explanation is that since this fraction was not subjected to the density gradient centrifugation and subsequent additional washing procedures, a small amount of pronase may have been present and interfered with the LDH activity. It has been shown that proteolytic enzymes can be adsorbed to cell surfaces and are not removed without stringent procedures (Poste, 1971).

In cell fractions where it had been possible to concentrate the number of chief cells, there was a tendency for a prevalence of the LDH-1 isoenzyme fraction (Fig. 38), and suggests the possibility of these cells also synthesising larger amounts of H monomer subunits when compared with the mucous secreting cells.

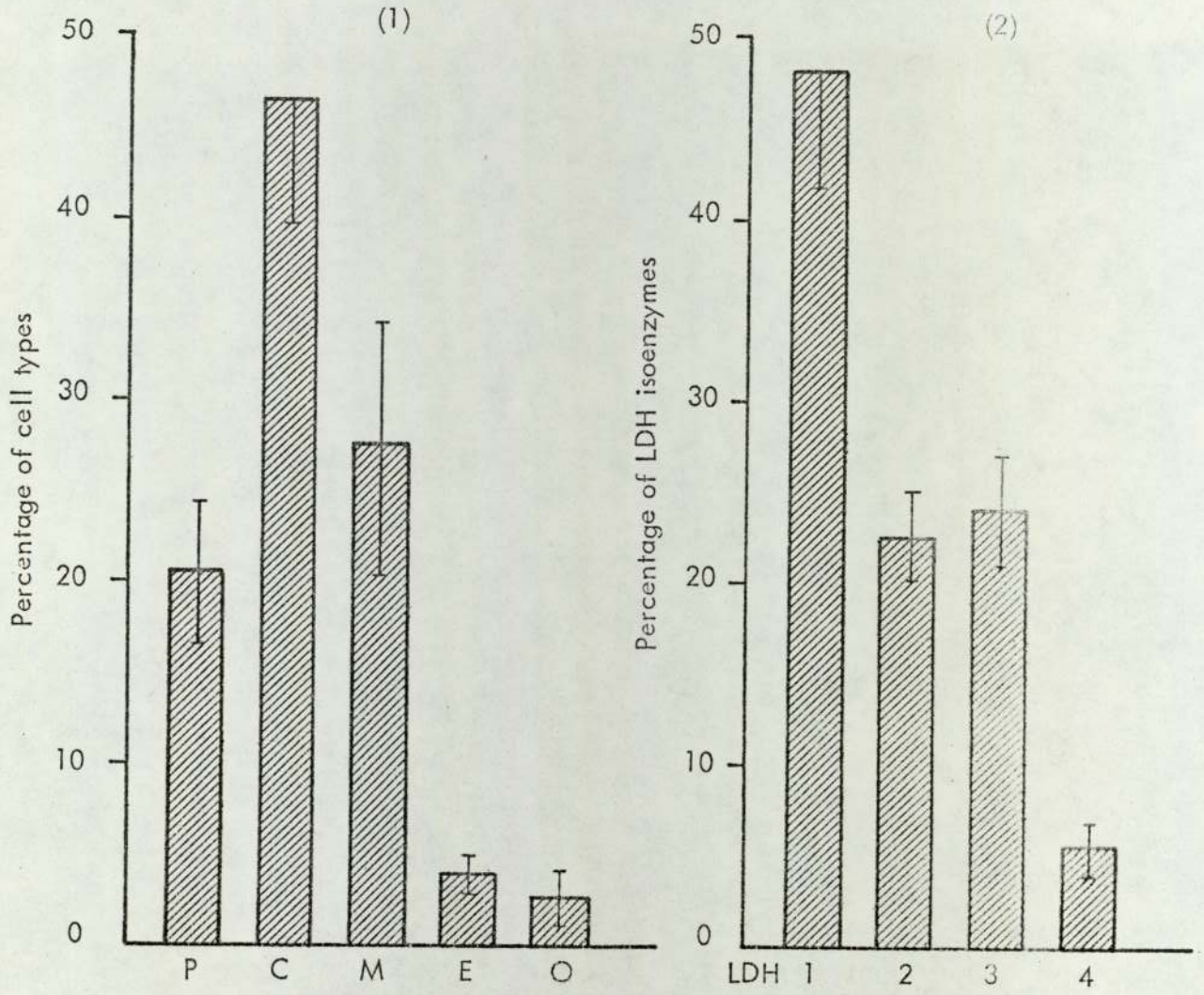


Fig. 38. (1) Differential cell count of cell fractions containing a high percentage of chief cells obtained by subjecting Fraction D to a second gradient of higher Ficoll concentrations (see section 5.1.2). P - parietal cell, C- chief cell, M- mucous cell, E - erythrocytes and O - other cell types.

(2) LDH isoenzyme percentages of the same fractions.
Results are mean ± SEM (4 experiments)

A low level and sometimes the complete absence of the LDH-5 isoenzyme in the cell fractions exhibiting an elevated LDH-4, was thought to be due to selective leakage of this isoenzyme from the isolated cells. Evans et al. (1971) have shown that LDH leakage from isolated intestinal epithelial cells, is influenced by the osmolarity of the suspending medium used. Samples of the PBSG supernatants obtained after washing the cell fractions from the pig stomachs were electrophoresed and examined for LDH isoenzymes. No activity could be detected in any of the samples.

Substrate inhibition by lactate is more likely to effect the faster moving isoenzymes, LDH-1 and LDH-2 (Brody, 1964) unless a pH below 7.0 is used in the incubation mixture (Vesell, 1966). This was not therefore considered to be a contributing factor to the low LDH-5 levels.

Kreutzer and Eggels (1965) demonstrated that different preparations of Agar affected the mobility of LDH-5. The recommended Difco Agar Noble was used in this study. Since LDH-5 was detected in the samples of human stomach, the decreased levels of this isoenzyme found in the pig samples was assumed to be a result of the prevalence of the faster moving enzymes in pig gastric mucosa.

The cell fractions obtained from samples of human gastric mucosa, though limited in number, displayed outstanding differences in their isoenzyme patterns. The prevalence of LDH-1 in human parietal cells again reflects a metabolism requiring energy supply from oxidative cycles.

The LDH pattern of the almost pure suspension of mucous cells in Case 2, indicates a completely different type of cell metabolism occurring in these cells, similar to that observed in pig stomach.

In a comparative qualitative study on the distribution of LDH isoenzymes in human fundic mucosa made by Leese (1965), using freeze-dried tissue sections, it was found that LDH-1 was elevated in sections taken from the upper gastric gland, and was thought to be associated with parietal cells. This assumption was further justified by subsequent experiments on diseased human gastric mucosa. Prochazka et al. (1968b) demonstrated that progressive decreases in the number of parietal

cells, due to the presence of varying degrees of intestinal metaplasia, resulted in a parallel decrease in the amount of LDH-1. Both studies reported an increase in LDH-4 and LDH-5 in samples where there was a predominance of mucous secreting cells.

It has been reported that a variety of human malignancies are associated with a reorientation of LDH isoenzyme distribution when compared with their tissue of origin (Richterich and Burger, 1963; Goldman et al., 1964; Leese, 1965). Since the precursor of the differentiated cell types in the stomach is thought to be the mucous neck cell, it is interesting to note that the fraction obtained in the human sample in particular, and the pig fractions as a whole, containing a preponderance of mucous secreting cells, exhibited high levels of the M-type isoenzyme.

The pattern obtained in samples of intestinalised gastric mucosa, with LDH-5 predominating (Leese, 1965, Prochazka et al., 1968b), is a reflection of the isoenzyme pattern of the mucous secreting cells. This pattern is enhanced by the loss of parietal cell mass and associated high LDH-1, and its influence on the overall tissue pattern.

It was postulated by Prochazka et al. (1968a) that the isoenzyme pattern of homogenised gastric mucosa was the sum of the different patterns of the individual cell populations. To test this hypothesis, a simple calculation was made using results from this investigation. The LDH isoenzyme percentages in one random sample of pig fundic mucosa were : LDH-1, 39%, LDH-2, 24%, LDH-3, 26% and LDH-4, 12%. The sum of the individual isoenzyme patterns of fractions B, D, and E and the abrasion (representative of a mixed cell population isolated from the fundic mucosa) expressed as percentages were found to be : LDH-1, 44%, LDH-2, 21%, LDH-3, 24% and LDH-4, 11%. The two patterns are almost identical and provide a novel demonstration to confirm the foregoing assumption.

Using tissue biopses, Nerenberg and Pogojeff (1969) found differences in the patterns of LDH isoenzymes in samples taken from restricted anatomical sites of the human kidney. They also assumed that complex organs probably produced isoenzyme

patterns that varied from area to area, and that in whole organ extracts these different patterns would be mixed together, resulting in a composite pattern.

Unfortunately, the inability to detect any CPK activity in the cell fractions other than the fundic mucosa samples, does not permit extrapolation of the results to corroborate the participation of CPK in the energy transfer reactions thought to be involved in hydrochloric acid secretion.

The CPK isoenzyme found in the fundic mucosa sample may be due to the presence of imperceptible amounts of muscle tissue not completely removed during dissection. It is unlikely, however, that the amount present would be responsible for the level of activity detected. The fact that the BB isoenzyme was present may prove to be of significance, but further study is required.

CPK is thought to be restricted to muscle and nerve tissue (Eppenberger et al., 1964; Dawson et al., 1965), with the MM isoenzyme prevailing in skeletal muscle and the BB isoenzyme predominant in brain. Heart muscle, on the other hand is found to contain mainly the intermediate hybrid isoenzyme MB.

Van Der Veen and Willebrands (1965) have demonstrated the presence of the BB isoenzyme in extracts of rat and human liver, kidney, lung, spleen and red blood cells, but have not attempted to explain their findings.

Since a suspension of parietal cells would be the ideal material for confirming the presence of CA activity, an adaption of the histochemical technique was applied to samples of fraction B after electrophoresis. The remaining cell fractions were analysed for comparison.

The method utilised, failed to demonstrate any CA activity, presumably due to inadequate sensitivity, and difficulty in destaining the agar gels after ammonium sulphide treatment.

Unfortunately it was not possible in the scope of this study to pursue the technical innovations required to modify the method or apply techniques utilising the esterase activity of CA (Tashian et al., 1963).

There is a vast potential for further study on isolated gastric mucosal cells, especially the parietal cells. Pertinent studies might include further isoenzyme and enzyme investigations in an attempt to understand the relative contribution of different metabolic pathways to cell metabolism, electrophysiology, cell culture, preparation of parietal cell antibodies and the effects of endocrine manipulation.

One of the advantages of using suspensions of isolated cells is that metabolic and secretory processes dependent on cell compartmentation can be readily studied. Such studies are not possible with tissue slices or homogenates, in which the biochemical compartments have been disrupted.

In view of the limitations imposed upon this study, the method previously described was recently published (Timms and Prochazka-Perthen, 1975) in the hope that it would provide additional resources to those currently interested in gastric physiology, especially since the method is suitable for human gastric mucosa.

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