THE DISTRIBUTION AND CHARACTERISATION OF ASPARTIC PROTEINASES IN HUMAN TISSUES

by

William Alexander Reid

A thesis presented to the University of Glasgow
for the degree of

Doctor of Medicine

from

The Department of Pathology, University of Leeds

November, 1987

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

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ACKNOWLEDGEMENTS

Numerous people have contributed in one way or another to this thesis, both in its production and in the work described by it. Most thanks should go to Dr. John Kay, University College, Cardiff, who collaborated in much of the work, providing not only biochemical expertise in a confusing field, but also thought provoking and critical discussion. Dr. W.D. Thompson, University of Aberdeen gave me practical help and encouragement in starting the work. Encouragement was given in the early stages by Professor R.B. Goudie, University of Glasgow and later by Professor C.C. Bird, University of Leeds. Professor I.M. Samloff allowed me to visit his laboratory in Sepulveda, California. I also wish to acknowledge the practical biochemical help of Dr. M.J. Valler, Cardiff, Dr. J. Shepherd, Glasgow and Mr. F. Lewis, Leeds.

Recent studies would not have been possible without the assistance of Mr. T. Branch, who has carried out biochemical and immuno-histochemical work under a grant from Leeds Health Authority. This includes the localisation of SMP, the IGSS labelling (Chapter 11) and the electron microscopy (Chapter 3). Mr. K. McGechaen and C. Gorman undertook B.Sc. projects under my charge and contributed to the results of Chapters 7 and 11 respectively. Gastric carcinomas were classified by Drs. M.F. Dixon, Leeds and J. Jass, London and various pathologists supplied fetal stomachs. Thanks are also due to Mr. T. Parker, Glasgow and S. Toms, Leeds, for photographic assistance and to Dr. J.N. Bulmer for valuable discussion.

The manuscript has been typed by Mrs. J. Fearnley, whose accuracy in transcribing illegible writing and a harsh accent into typescript has substantially reduced the effort required to correct the drafts.

Finally I should like to thank my adviser, Dr. A.M. McNicol, University of Glasgow who willingly undertook the task and has offered helpful and constructive criticism of the work.

DECLARATION

The work described in this thesis was, except where otherwise stated, initiated and carried out by me, although much of it was subsequently published collaboratively. Enzyme purification and assay and the development of antisera were carried out personally, cathepsin D being purified by me and M.J. Valler under the supervision of Dr. J. Kay. Most of the immunohistochemical labelling was performed personally, but technical assistance in cutting sections was obtained. The acknowledged work by T. Branch, K. McGechaen and C. Gorman was done under my direction.

The work has not been submitted for any other higher degree.

PUBLICATIONS AND PRESENTATIONS RESULTING FROM WORK DESCRIBED IN THESIS

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Reid, W.A., Valler, M.J. & Kay, J. (October 1985) The distribution of cathepsin D in normal and neoplastic human tissues. International Workshop on Aspartic Proteinases. Tokyo, Japan (by invitation).

Forthcoming presentations

Reid, W.A., Branch, T., Gorman, C. & Kay, J. (January 1988) Progastricsin in metastatic adenocarcinoma from stomach and other sites. Pathological Society of Great Britain & Ireland, London, 1988. (Abstract to be published, Journal of Pathology, 1988.)

Reid, W.A. & Kay, J. (July 1988) Tissue localisation of aspartic proteinases. 18th Linderstrøm-Lang Conference: Aspartic Proteinases. Elsinore, Denmark (by invitation).

Papers submitted

Reid, W.A., McGechaen, K., Branch, T., Gray, H.D.A., Thompson, W.D. & Kay, J. (1987) Immunolocalisation of aspartic proteinases in the developing human stomach. Submitted to Developmental Physiology.

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SUMMARY

The aspartic proteinases are members of a family of enzymes, the catalytic activity of which depends on the presence of two aspartic residues in the active site. This thesis concerns four members of this group - pepsin, gastricsin, cathepsin D and slow moving protease (SMP). The defining characteristics, nomenclature and general biochemistry of the aspartic proteinases are reviewed in Chapter 1 and an attempt is made to clarify the various systems which have been used to classify this group of enzymes. Assay and purification methods, the structure and mechanism of action of the enzymes and their immunological properties are outlined. Pure supplies of these enzymes are not commercially available and Chapter 2 describes the method by which these were obtained and how antisera were developed. The antisera are used to show that cathepsin D and SMP, previously thought to be identical, are separate enzymes. In Chapter 3 the immunohistochemical methods are introduced and their use in determining the distribution of the above enzymes in normal gastroduodenal mucosa is described, at both light and electron microscopic level. It is shown that pepsinogen is mainly confined to the gastric body, while progastricsin co-localises with pepsinogen but is also present in the gastric antrum and duodenum. Cathepsin D is demonstrated in parietal cells, in gastric antral glands and cells in the lamina propria, while SMP is demonstrated mainly in the surface epithelium of the stomach. These studies raise the general problem of the effects diffusion may have on the localisation of tissue antigens. This problem appears not to have been widely discussed in the literature and is dealt with in Chapter 4, in which diffusion was studied by immunolocalisation of pepsinogen and progastricsin in autolysing gastric mucosa and confirmed experimentally by immunolabelling of antigens introduced into various normal tissues.

Enzyme activity at low pH has long been recognised in seminal fluid. In Chapter 5 this is shown to be due to the presence of gastricsin, apparently identical to that in normal gastric juice. The zymogen, progastricsin, was localised to the acinar cells of the prostate, which appear to secrete and presumably synthesise it. In Chapter 6, the distribution of aspartic proteinases in normal tissues apart from stomach, duodenum and prostate is discussed. No evidence of pepsinogen or progastricsin was found in other tissues, while cathepsin D appears widely distributed both in macrophages and in epithelial cells. SMP appears to be confined to the surface membrane or cilia of a few tissues, such as chorionic villi and respiratory epithelium, but its presence even in these tissues could be derived from the red blood cells, on the membranes of which it is demonstrated. Studies on the distribution of the aspartic proteinases are extended to the fetal stomach in Chapter 7, where it is shown that SMP is the dominant enzyme from 12 weeks gestation onwards, while progastricsin becomes prominent by about 17 weeks, when pepsinogen and cathepsin D also appear. Some attempt is made to relate the order of their appearance to the molecular evolution of aspartic proteinases.

The rest of the thesis deals with the presence and distribution of the aspartic proteinases in neoplastic tissues. In Chapter 8, it is shown that gastric carcinomas produce aspartic proteinases, namely cathepsin D (100%), SMP (50%), progastricsin (30%) and pepsinogen (6%). There appears to be no relation to prognosis and both intestinal and diffuse types of carcinoma contain aspartic proteinases. In Chapter 9, the studies which showed progastricsin in normal prostatic epithelium, described in Chapter 5, are extended to carcinomas, up to 40% of which produce progastricsin. Progastricsin co-localises with acid phosphatase but is, however, less widely distributed. In Chapter 10 the distribution of aspartic proteinases in non-gastric tumours is

described. While pepsinogen is highly specific to gastric carcinomas, progastricsin occasionally occurs in tumours of some other sites, especially pancreas, while cathepsin D is found in almost all tumours studied. SMP appears to be present in certain tumours, mainly kidney. In Chapter 11, progastricsin is more fully studied in metastatic tumours, firstly in local lymph node secondaries near primary gastric carcinomas and secondly in deposits further afield, both in lymph node and liver, from a range of primaries in different sites. It is shown that in most cases the presence of progastricsin in a lymph node metastasis of adenocarcinoma indicates a primary in the stomach or in pancreas but that occasionally metastases from other sites contain progastricsin. While progastricsin was commonly found in liver metastases, the correlation with a primary in stomach did not reach statistical significance. These results could be useful to the diagnostic histopathologist in determining the primary site from which a given metastasis has originated. The final discussion (Chapter 12) incorporates recent evidence that the pol gene of the HIV I virus codes for an aspartic proteinase and that retroviral proteinase activity is inhibited by the aspartic proteinase inhibitor, pepstatin. Knowledge of the distribution of the aspartic proteinases might therefore be relevant to possible treatment of AIDS with aspartic proteinase inhibitors.

PREFACE

The work described in this thesis began as an attempt to use immunohistochemical methods to identify the primary site of a given metastatic deposit of malignant tumour. Various enzymes, including pepsin, which was thought might be specific to stomach, were injected into rabbits. Pig pepsin yielded antiserum which gave strong labelling of human gastric mucosa by the peroxidase-antiperoxidase (PAP) method and which was used in a study of a series of gastric carcinomas. Although a small number of tumours were positive, the purity of the antiserum was open to some doubt. With the biochemical advice of Dr. J. Kay, Cardiff, with whom much collaborative work was subsequently undertaken, commercial pig pepsinogen was purified to homogeneity and used to block immune labelling by antipepsin, the specificity of which was thereby proven. Continued scepticism about staining of parietal cells led to further work on diffusion of antigen (Chapter 4). It became clear that the gastric mucosa and juice contained other important enzymes, of which gastricsin and slow-moving protease (SMP) might also prove useful as markers of gastric carcinoma. Initial attempts were made to purify progastricsin from seminal fluid but our supply of gastricsin was obtained as a gift. Antisera to gastricsin were raised and used in studies of gastric carcinoma and lymph node metastases. Subsequent studies on gastricsin in the benign prostate were extended to prostatic carcinoma. During this work the staining rack, described in Chapter 3, was devised. Attempts were made to purify SMP from fetal stomach but, as some difficulty was encountered, attention was turned to another member of the aspartic proteinase family, cathepsin D, which was then thought to be possibly identical to SMP. In Cardiff, under the supervision of Dr. J. Kay, M. J. Valler and I purified cathepsin D from human spleen, obtaining the most homogeneous form of the enzyme then

available. Rabbit antiserum to cathepsin D was then raised and the distribution of the enzyme within human tissues was determined. Cathepsin D was also demonstrated in a variety of tumours, including gastric carcinoma.

In April 1984 I spent three weeks in the laboratory of Professor I.M. Samloff in Sepulveda, California, U.S.A. attempting to set up a radioimmunoassay for cathepsin D. This proved unsuccessful, but a supply of anti-SMP was given to us. It was thus possible to prove that cathepsin D and SMP were not identical and it was soon shown by Professor B. Foltmann, Copenhagen, using our anti-cathepsin D, that SMP was the enzyme known as cathepsin E.

It was suggested that the expression of pepsinogen, progastricsin and cathepsin D by neoplastic stomach might reflect the presence of the enzymes in the normal developing gastric mucosa. A study of fetal stomachs was undertaken under my charge in 1983 - 1984 as part of a B.Sc. project. Later this work was completed by inclusion of SMP (Chapter 7).

In another B.Sc. project (1984 - 1985) which I supervised, the presence of progastricsin in gastric carcinomas and distant metastases was studied. This was subsequently expanded in a study of widespread metastases, with the aim of showing whether progastricsin is specific to carcinoma of gastric origin.

Recently, the distribution of SMP in normal and neoplastic tissues has been determined and localisation of the enzymes at electron microscopic level has been attempted.

As much of the work to be described in the thesis presupposes some knowledge of the enzymes involved, it is with a brief review of their biochemistry that this thesis begins (Chapter 1).

CHAPTER 1

INTRODUCTION

INTRODUCTION

NOMENCLATURE AND DEFINITIONS

In current literature, the terms protease, proteinase and proteolytic enzyme are used synonymously. Protease is an older term than the others and has tended to become replaced by proteinase, which was adopted for the Enzyme Nomenclature (1982) List. Enzymes may be acid, alkaline or neutral proteinases according to optimal pH. Any of these types of proteinase may be an exopeptidase, which cleaves peptide bonds one or two residues from the ends of the protein, or an endopeptidase, which mainly cleaves bonds distant from the ends. Cathepsin is a somewhat ill-defined generic name applied to endopeptidases of mammalian cells and tissues; most are acid proteinases, but some are neutral or alkaline proteinases. The terms 'tissue proteinase' and 'intracellular proteinase' are also rather ill-defined but are intended to describe the proteinases which are stored in lysosomes and other organelles and which act intracellularly and pericellularly, unlike those which are produced, usually as inactive precursors, and stored in secretory granules for subsequent export (Barrett, 1977b).

CLASSIFICATION OF PROTEINASES

Proteolytic enzymes are classified into four groups, according to their catalytic mechanism: serine, cysteine, metallo- and aspartic proteinases (Kay, 1982). The aspartic proteinases have been rather less well studied than many enzymes in the first three groups (Kay, 1985) and, in particular, their distribution in normal and abnormal human tissues is poorly documented.

The proteolytic activity of an aspartic proteinase is dependent on the presence of an aspartic residue in positions 215 and 32 in the linear sequence. These react with the site-specific affinity labels diazoacetyl norleucine methyl ester (DAN) and epoxy (p-nitrophenoxy)

propane (EPNP) respectively, the enzyme in either case being inactivated. By definition, an aspartic proteinase must be inhibited by both DAN and EPNP and also by pepstatin, an acylated pentapeptide produced naturally by various species of actinomycetes (Kay, Valler & Dunn, 1983).

CLASSIFICATION OF ASPARTIC PROTEINASES

The aspartic proteinase group includes enzymes derived from the following sources:

1. Mammals - pepsin, gastricsin, slow-moving protease, cathepsin D, cathepsin E, chymosin and renin.
2. Yeast - proteinase A.
3. Fungi - enzymes from the species Endothia parasitica, Penicillium janthinellum, Mucor pusillus and Rhizopus chinensis.
4. Plants - proteinases from squash seeds and cucumber (Polanowski et al, 1985), rice seeds and wheat leaves (Foltmann, 1985a).

It may be noted that no bacterial aspartic proteinases have yet been found.

Most of these enzymes have molecular weights of 35-42,000 Kd. Most are acid proteinases, being maximally active in acid conditions, but renin, which is a neutral proteinase in physiological conditions, is exceptional. The mammalian aspartic proteinases, unlike the microbial enzymes, are synthesized as precursors, termed zymogens, namely pepsinogen, progastricsin, prochymosin, prorenin (Kay, 1980) and possibly, though less well substantiated, cathepsinogen D (Puizdar & Turk, 1981).

In the nomenclature of the International Union of Biochemistry, enzymes are classified according to catalytic activity and further separated by the substrates on which they act. This system is difficult to apply to proteinases, as their substrate specificities overlap.

These enzymes are more conveniently sub-classified according to the mechanism by which their active sites bind to various inhibitors. By these criteria, the aspartic proteinases occur in the group EC 3.4.23 and are further divided thus (Foltmann, 1985a):

3.4.23.1	Pepsin A
3.4.23.2	Pepsin B (pig only)
3.4.23.3	Gastricsin
3.4.23.4	Chymosin
3.4.23.5	Cathepsin D
3.4.23.6	Aspartic proteinases from fungi and yeasts
3.4.23.15	Renin

Cathepsin E and slow-moving protease (SMP) are not yet classified.

Pepsin B (Ryle, 1970) occurs only in the pig and in the present work pepsin A will be referred to simply as pepsin.

Pepsin

Pepsin was the first enzyme ever identified, following its extraction from human gastric juice (Schwann, 1836). The early history of its discovery and study is discussed by Wolf (1965). The enzyme is synthesised as the zymogen pepsinogen (Langley, 1882a), which is secreted from the gastric mucosa and activated by the low pH in the gastric juice, where it is the main acid proteinase, its concentration in humans being 400 mg/l (Fruton, 1971). Although optimally active at pH 2.0, it is active up to around 5.3, at which it clots milk. Above pH7 it is permanently inactivated and no longer functions, even if the pH is lowered once more (Langley, 1882b). Pepsin survives lyophilisation but if stored in solution, even at $4^{o}C$, it digests itself rapidly, although the fragments resulting from its autolysis may

continue to be active for some time.

Gastricsin

The second important acid proteinase of the human stomach, now called gastricsin, was first demonstrated in pig and dog gastric mucosal extracts, which were found to display activity not explicable by the presence of pepsin alone (Takemura, 1909; Hirayama, 1910). The enzyme was found to be only weakly active at the optimal pH of pepsin. The enzyme was named cathepsin and was mistakenly thought to be derived from lymphocytes (Willstätter & Bamann, 1929; Freudenberg, 1940). The proteinase was found to have optimal pH 3-4 and was extracted from human duodenal as well as gastric mucosa (Buchs, 1953; Buchs, 1954). As this enzyme is present in high amounts (150 mg/l, which is 20-30% of the proteolytic activity of the gastric juice), it seems clear that it is a secretion product and does not really belong to the group of enzymes termed cathepsins. It was therefore renamed gastricsin when it was purified in 1958 (Richmond et al, 1958). Gastricsin is synthesised as the zymogen progastricsin and, like pepsinogen, is activated by exposure to gastric acid. The optimum pH of gastricsin is around 3.0, somewhat higher than that of pepsin. Gastricsin cleaves different peptide bonds from pepsin, but is similarly inactivated by neutralisation.

Chymosin

In 1853 an acid proteinase was discovered in the infant stomach of certain mammals: rennin (Heintz, 1853). Rennin was shown, like pepsin, to be secreted from the gastric mucosa as a zymogen (Langley, 1882a). Rennin is now called chymosin to avoid confusion with renin of the kidney. Chymosin is an aspartic proteinase and has great importance in the manufacture of cheese but does not occur in the human stomach.

Cathepsin D

Cathepsin D appears to have been responsible for the acid proteolytic activity detected in autolysing tissues, such as liver, muscle, adrenal glands (Salkowski, 1890; Schweining, 1894; Biondi, 1896; Jacoby, 1900), thymus (Kutscher, 1901; Jacoby, 1902), spleen (Hedin & Roland, 1901a, Hedin, 1904), lymph node, liver, kidney, skeletal and cardiac muscle, blood cells (Hedin & Roland, 1901b), inflammatory exudates, reactive lymph nodes and bone marrow (Opie, 1906; Jobling & Strouse, 1912). The acid proteinase was thought to be of macrophage origin, which would account for its wide distribution in tissue, and was found to have maximal activity at pH 3-3.5 (Dernby, 1918). The term cathepsin was first applied to an enzyme detected in gastric mucosa, but it seems likely that this enzyme was in fact gastricsin rather than cathepsin D. The term cathepsin is now reserved for any of a group of proteolytic enzymes which are stored within cells, probably within lysosomes, and act intracellularly. Cathepsins vary in catalytic mechanism and include not only aspartic proteinases such as cathepsin D, but also cysteine proteinases, such as cathepsins B, L and H and the serine proteinase, cathepsin G (Kay, 1982). Cathepsin D was named in 1960 (Press, Porter & Cebra, 1960).

By the above criteria the evidence for the existence of a cathepsin in gastric mucosa has until recently not been convincing and will be discussed later in this chapter.

Renin

Renin differs from other aspartic proteinases in that it is active physiologically at neutral pH, although its optimum *in vitro* is 5.5-5.6. In addition, renin is highly substrate-specific for the plasma protein angiotensinogen. Renin is synthesised as the zymogen, prorenin, which is stored in the juxtaglomerular apparatus of the kidney and secreted into the blood. The enzyme has also been extracted from mouse

submaxillary gland (Cohen et al, 1972) and from rat and dog brain (Fischer-Ferraro et al, 1971; Inagami et al, 1982).

Cathepsin E

Cathepsin E is an aspartic proteinase which has been identified in rabbit bone marrow (Lapresle & Webb, 1962), in rabbit, rat and bovine spleen (Lapresle, 1971; Yamamoto, Katsuda & Kato, 1978; Yamamoto et al, 1980; Lapresle et al, 1986) and in rabbit skin homografts (Jasani, Jasani & Talbot, 1978). Cathepsin E has a molecular weight of 85-90,000 Kd and consists of two subunits with molecular weights of around 45,000 Kd (Yamamoto et al, 1978; Lapresle, 1971). It has a pH optimum of 2.5 (Jasani et al, 1978) and is inhibited strongly by the inhibitor protein from Ascaris lumbricoides (Keilová & Tomášek, 1972). It is known not to cross-react immunologically with antiserum to cathepsin D (Barrett & Dingle, 1972; Yamamoto et al, 1980; Lapresle et al, 1986). It has been suggested that cathepsin E is a dimer of cathepsin D (Yamamoto et al, 1978) but recent studies have shown that the amino acid composition of cathepsin E is quite different from that of cathepsin D and that they cleave different peptide bonds (Lapresle et al, 1986).

ASSAY METHODS

Assay methods for pepsin, gastricsin and cathepsin D exploit the ability of the enzymes to digest haemoglobin at certain pH optima to yield products which are not precipitated by trichloracetic acid (TCA) (Anson & Mirsky, 1932; Anson, 1938). Spectrometric measurement of the change in optical density allows accurate estimation of enzyme activity, which is expressed in units compared with a standard specific activity.

The gastric and urine zymogens may be more clearly resolved by electrophoresis on polyacrylamide gel and more bands have been demonstrated than on agar. Even these can be shown by column chromatography to consist of subtypes (Samloff, 1985).

Slow moving protease (SMP)

Etherington & Taylor (1970)'s zone 7 corresponds to a small early peak obtained by DEAE cellulose chromatography and appears to correspond to PIV of Kushner et al (1964) and SMP of Samloff (1969) (Table 1.3). The main property which distinguishes SMP from pepsin and gastricsin is its resistance to sequential acid activation and neutralisation. An enzyme with such a property was isolated from normal human gastric mucosa and named a cathepsin (Mangla, Guarasci & Turner, 1974). Its molecular weight, estimated by its mobility on Sephadex G-100, was 40-50,000 Kd and its pH optimum 3.4. This is consistent with the suggestion that it is cathepsin D and not cathepsin E, which has a molecular weight of about 90 Kd and pH optimum 2.5 (Barrett, 1977a). The identity of this enzyme with SMP was not, however, established by zymography.

The non-pepsin proteinase isolated from human gastric mucosa by Roberts & Taylor (1978) behaved like SMP on zymography and had optimal activity at pH 2.5-3.5. It cleaved preferentially peptide bonds Leu-Tyr and Phe-Tyr, like pepsin and cathepsin E but unlike cathepsin D, which preferentially cleaves Phe-Phe (Etherington & Taylor, 1972). These data supported their previous suggestion that normal gastric mucosa contains an enzyme with properties of cathepsin E and that it is identical with SMP (Etherington & Taylor, 1972).

The previous authors found no evidence of cathepsin D in normal gastric mucosa (Roberts & Taylor, 1978), although they had previously demonstrated it in gastric adenocarcinomas. However, the non-pepsin proteinase purified from normal gastric mucosa by Kageyama & Takahashi (1980) had properties similar to, but not identical with, those of

Cathepsin D

Cathepsin D has only comparatively recently been purified (Table 1.1).

Slow moving Protease (SMP)

SMP was partly purified from human fetal gastric mucosa by acetone-ether extraction (Hirsch-Marie & Touboul, 1973). The method was somewhat crude and although the SMP obtained was used in immunological studies, its purity was not convincingly demonstrated. SMP has recently been purified from human gastric mucosa by chromatography (Samloff et al, 1987).

STRUCTURE

Primary structure

The primary structure of various aspartic proteinases has now been determined (Table 1.2 and Fig. 1.1). There is considerable homology among all the aspartic proteinases, about 10% of their amino acid residues being at identical positions (Foltmann, 1985b). In most cases a propart, about 44 amino acids long, is attached to the N-terminal end, from which the active enzyme is numbered. The catalytic aspartic acids 32 and 215 are present in all the aspartic proteinases, while five clusters of residues around them are particularly well conserved (Fig. 1.1 and 1.2).

Thirty to forty of the approximately 375 amino acid residues of the zymogen carry negative charges, 10-20 carry positive charges and the rest are neutral. Differences in the numbers of these presumably account, at least partly, for variations in electrophoretic mobility among the members of the enzyme family. The enzymes and zymogens are often coupled to a carbohydrate moiety to form a glycoprotein. Cathepsin D, for example, contains about 1% hexosamine and 5% hexose (Barrett, 1980a). Variations in the size and charge of the carbohydrate may also affect electrophoretic mobility.

TABLE 1.1 PURIFICATION OF ASPARTIC PROTEINASES

Zymogen/ enzyme	Species	Tissue	Reference
Pepsinogen/ pepsin	pig	gastric mucosa	Northrop, 1930; Liener, 1960
	cow	"	Nevaldine & Kassell, 1971; Chow & Kassell, 1968; Matyash et al,
	sheep	"	Fox, Whitaker & O'Leary, 1977
	chicken	"	Yasugi & Mizuno, 1981
	monkey	"	Kageyama & Takahashi, 1976
	human	"	Zöller, Matzku & Rapp, 1976; Becker & Rapp, 1979; Ward, Neumann & Chiang, 1978
	salmon	"	Norris & Elam, 1940
	dog	"	Kassell, Wright & Ward, 1976
	rat	"	Muto & Tani, 1979
	cat	"	Shaw & Wright, 1976
Progastricsin/ gastricsin	human	"	Richmond et al, 1958; Tang et al, 1959; Tang & Tang, 1963
	pig	"	Ryle & Porter, 1959
	human	seminal fluid	Samloff & Liebman, 1972; Ruenwongsa & Chulavantnatol, 1975
	human	prostate	Chiang et al, 1981
Cathepsin D	human	gastric mucosa	Kageyama & Takahashi, 1980a; Pohl, Bureš & Slavík, 1981
	rat	gastric mucosa	Muto, Arai & Tani, 1983
	pig	myometrium	Afting & Becker, 1981
	calf	thymus	Kregar et al, 1974
Slow-moving protease (SMP)	human	fetal gastric mucosa	Hirsch-Marie & Touboul, 1973
	human	gastric mucosa	Samloff et al, 1987

TABLE 1.2

PRIMARY STRUCTURE OF MAMMALIAN ASPARTIC PROTEINASES

Zymogen/ enzyme	Species	Reference
Pepsin	pig	Sepulveda et al, 1973; Morávek & Kostka, 1974; Chen, Tao & Tang, 1975
	chicken	Baudyš & Kostka, 1983
Pepsinogen	human	Sogawa et al, 1983
Pepsinogen (activation segment)	human	Kageyama & Takahashi, 1980b
Gastricsin	human	Sepulveda, Jackson & Tang, 1975
Progastricsin (propart)	human	Foltmann & Jensen, 1982
Chymosin	calf	Foltmann, 1981; Foltmann et al, 1979
Cathepsin D	pig	Shewale & Tang, 1984
Renin	mouse	Misono, Chang & Inagami, 1982
	human	Hobart et al, 1984

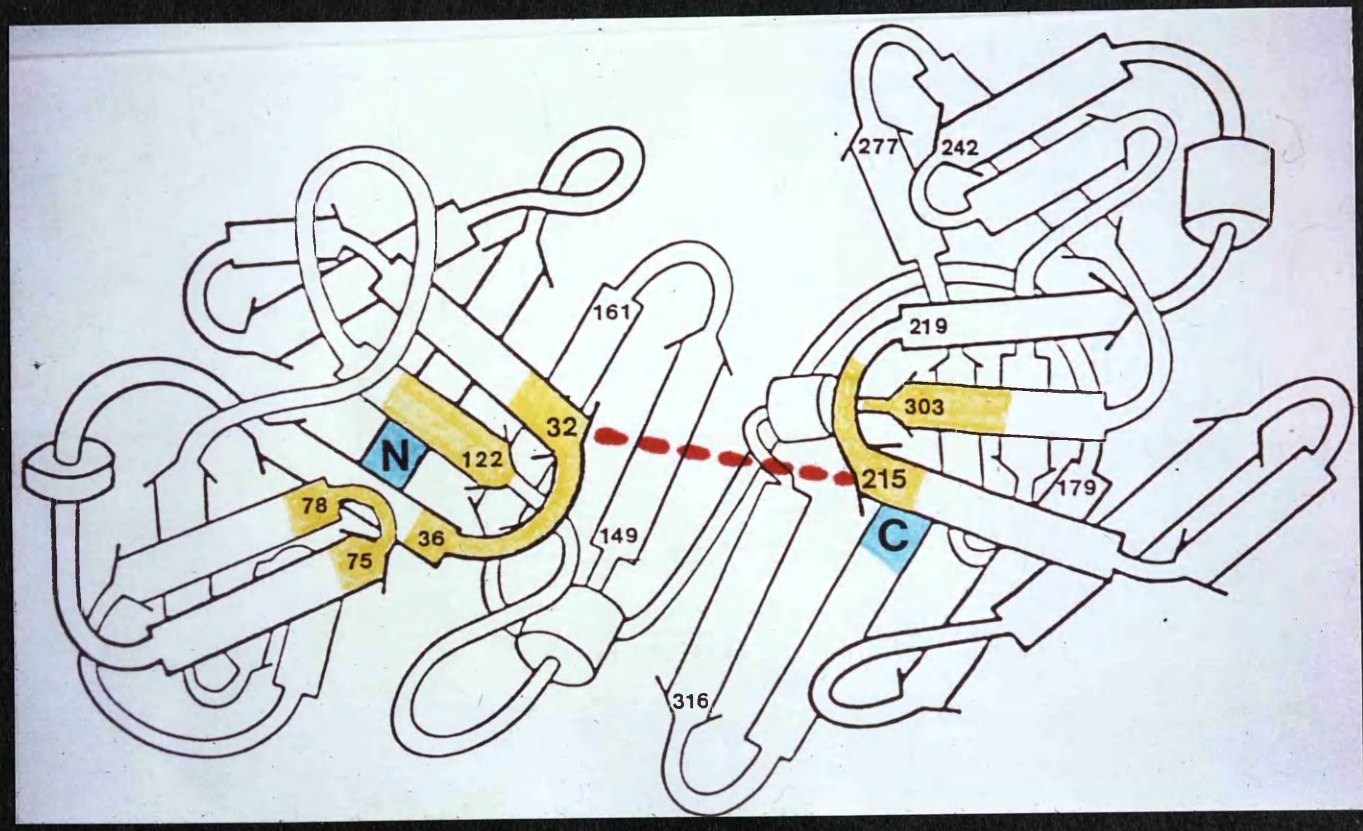
FIG. 1.1

COMPARATIVE AMINO ACID SEQUENCES OF ASPARTIC PROTEINASES

(Valler M.J., personal communication)

	27	32	36	40	46
Human pepsin	-Phe-Thr-Val-Val-Phe-Asp-Thr-Gly-Ser-Ser-Asn-Leu-Trp-Val-Pro-Ser-Val-Tyr-Cys-Ser-				
Pig pepsin	-Phe-Thr-Val-Ile-Phe-Asp-Thr-Gly-Ser-Ser-Asn-Leu-Trp-Val-Pro-Ser-Val-Tyr-Cys-Ser-				
Chicken pepsin	-Phe-Ser-Val-Ile-Phe-Asp-Thr-Gly-Ser-Ser-Asn-Leu-Trp-Val-Pro-Ser-Ile-Tyr-Cys-Lys-				
Bovine chymosin	-Phe-Thr-Val-Leu-Phe-Asp-Thr-Gly-Ser-Ser-Asp-Phe-Trp-Val-Pro-Ser-Ile-Tyr-Cys-Lys-				
Mouse gastricsin	-Phe-Leu-Val-Leu-Phe-Asp-Thr-Gly-Ser-Ser-Asn-Leu-Trp-Val-Pro-Ser-Val-Tyr-Cys-Gln-				
Mouse renin	-Phe-Lys-Val-Ile-Phe-Asp-Thr-Gly-Ser-Ala-Asn-Leu-Trp-Val-Pro-Ser-Thr-Lys-Cys-Ser-				
Human renin	-Phe-Lys-Val-Val-Phe-Asp-Thr-Gly-Ser-Ser-Asn-Val-Trp-Val-Pro-Ser-Ser-Try-Cys-Ser-				
Human cathepsin D	-Phe-Thr-Val-Val-Phe-Asp-Thr-Gly-Ser-Ser-Asn-Leu-Trp-Val-Pro-Ser-Ile-His-Cys-Lys-				

FIG. 1.2 Aspartic proteinases - three dimensional structure. The molecule has been folded open to show the active site cleft, adjacent to which lie the five groups of conserved amino acid residues (Phe 31 to Ser 36, Tyr 75 to Gly 78, before Gly 122, Asp 215 to Gly 217 and around Gly 303), shown in yellow. The catalytic amino acids Asp 32 and Asp 215 normally lie close together and are hydrogen bonded (red dashes). The substrate fits into the cleft, binding site S4 being at the top. The N and C termini are shown in blue. The arrows indicate beta-structures and the cylinders indicate alpha-helices. In the zymogen, the propart is attached to the N-terminal end and occupies the cleft, blocking access by substrate.


277
242
161
219
303
32
N
122
215
179
78
36
149
C
75
316

THREE DIMENSIONAL STRUCTURE

The three dimensional structures of pig pepsin (Andreeva et al, 1985) and of the fungal aspartic proteinases, penicillopepsin (James & Sielecki, 1983), endothiapepsin (Blundell et al, 1985) and Rhizopus chinensis proteinase (Bott, Subramanian & Davies, 1982) have now been determined and are remarkably similar. The pig pepsin molecule consists of two lobes separated by a cleft (Fig. 1.2). The N-terminal half includes amino acids 1-175, while the C-terminal lobe includes residues 176-327. About 35% of the residues in topologically similar positions are identical in the two lobes of the molecule of any one enzyme. Each of the two lobes appears in turn to consist of two symmetrical halves. The active site is in the cleft and is known as an extended site, indicating that tight binding of substrate requires interactions with several side chains of either side of the scissile bond, usually at least seven amino acids in the S_4-S_3' subsites, the bond between the hydrophobic residues in the S_1-S_1' sites being cleaved preferentially (Powers et al, 1977).

Recently the tertiary structure of the zymogen, pig pepsinogen, has been determined (James & Sielecki, 1986). The propart segment occupies the cleft of the pepsin part.

ZYMOGEN ACTIVATION

All the mammalian aspartic proteinases appear to be synthesised as zymogens, although the evidence for a cathepsin D zymogen is less conclusive than for the others. The zymogen is inactive, because the propart occupies the substrate-binding cleft of the enzyme, blocking access to the two catalytic aspartyl residues. On acidification, the propart is detached by sequential removal of short peptides.

In pig pepsinogen the first cleavage at Leu 16 - Ile 17 and the second at Phe 25 - Leu 26 occur intramolecularly. The third cleavage, at Leu 44 - Ile 45 occurs intermolecularly, as this bond is far from the active site of the molecule in which it is cleaved. Removal of the propart thus exposes the active site of the enzyme. The first fragment removed is itself a pepsin inhibitor at or above pH 5 and may help protect the pepsin from inactivation when the pH of the gastric juice rises (Kay, 1980).

At pH 4-4.6 activation of pepsinogen is much slower than at pH 2 and is autocatalytic, i.e. pepsin catalyses its own production (Herriott, 1939).

EVOLUTION OF ASPARTIC PROTEINASES

The homology among the various aspartic proteinases of different species suggests that the enzymes developed long ago, when fungi and mammals had a common ancestor (Foltmann, 1981). The bilobed structure and the symmetry within each of the lobes suggests that the aspartic proteinases evolved from a precursor gene which coded for about 80 amino acids. Gene duplication and fusion led to a gene coding for about 160 amino acids and a second duplication and fusion led to an enzyme group with about 320 amino acids (Tang et al, 1978; Andreeva & Gustchina, 1979; Blundell, Sewell & McLachlan, 1979; Foltmann, 1981). Sequence divergence would explain the non-homology between enzymes within and between different species and is considered more likely than convergence (Burt et al, 1985).

It has been speculated that cathepsin D might be the primitive ancestor of the aspartic proteinases and was the intracellular digestive

enzyme of a unicellular organism. On this basis, pepsin, gastricsin and renin presumably evolved later (Barrett, 1980a). It may be that the critical step in evolution from fungal to mammalian proteinases was the acquisition of the zymogens (Stepanov, 1985). The evidence suggests that the mammalian genes were derived from a common sequence around 160 million years ago and that the mammalian gene precursor and that of the fungus Penicillium janthinellum diverged about 1000 million years ago (Burt et al, 1985).

CATALYTIC MECHANISMS

The cleavage of a peptide bond by an enzyme involves at least three steps:

1 binding of substrate to the active site of the enzyme

2 cleavage of the peptide bond

3 release of the products from the active site

The various aspartic proteinases differ widely in their selectivity for substrates. Naturally occurring proteins are generally too complex for use in catalytic studies and much has been learned from experiments with synthetic substrates, such as

Pro-Thr-Glu-Phe - NO_2Phe-Arg-Leu

P_4 P_3 P_2 P_1 P_1' P_2' P_3'

which is cleaved at the scissile bond between phenylalanine at position P_1 and p-nitrophenylalanine in the P_1' position. The scissile bond is orientated close to the catalytically essential aspartic residues 32 and 215. In most cases cleavage is at or between aromatic amino acids, which have hydrophobic groups, but the rate of cleavage is altered considerably by the amino acid residues at the ancillary positions P_4, P_3, P_2, P_2' and P_3'. Moreover, the corresponding subsites in the enzyme vary between the different aspartic proteinases. The scissile bond and the rate at which it is cleaved depends therefore on the structure of

the substrate and of the particular aspartic proteinase, as well as on the ionic concentration of the solution (Pedersen, 1977). The mechanisms have been discussed in detail elsewhere (Kay, 1985; Dunn et al, 1985). Naturally occurring inhibitors, such as pepstatin, and various synthetic inhibitors have also proved useful in such studies. Renin inhibitors have clinical importance in the treatment of hypertension.

MOLECULAR VARIANTS OF ASPARTIC PROTEINASES

Because of their clinical interest, some of the aspartic proteinases have been further classified by their electrophoretic mobility. In most studies electrophoresis was carried out on 1 to 1.5% agar gel buffered at pH 7.3 to 8.6 in a slab around 1mm thick on a glass plate, kept cool during the run. More recently, polyacrylamide gel has been found to give better separation. Some studies have been carried out on urine, but most work has been performed on gastric mucosal extracts, prepared from the supernatants obtained by centrifuging homogenates of tissue in weak neutral or slightly alkaline buffer. Samples were placed in wells, usually slit-like, and an electric current was passed through the gel. Most aspartic proteinases are negatively charged and moved towards the anode. Enzyme activity was demonstrated by the method of Uriel (Uriel & Scheidegger, 1955; Uriel & Grabar, 1956; Uriel, 1960), in which the gels are impregnated with protein, usually haemoglobin, buffered to suitable pH, usually around 2; the plate is then incubated, dried and stained for protein with amido black or Coomassie blue. Any enzyme activity at the incubation pH is marked by a clear lytic zone, where the protein has been digested. This technique is known as zymography. When electrophoresis is performed as above, only the zymogens may be demonstrated, as any enzymes in the sample are inactivated at the neutral or alkaline pH at

which the electrophoresis is carried out. If, however, electrophoresis is performed at pH 5.3, the active enzymes are not denatured and so may be demonstrated.

The number of isoforms of the zymogens detected depends on the conditions of electrophoresis, but the isoforms have usually been named in order of decreasing anodal mobility. In several studies, four zymogens, PI, II, III and IV, were demonstrated in human gastric mucosa (Rapp et al, 1964) and in gastric juice (Hirsch-Marie, Conte & Burtin, 1965; Hirsch-Marie, 1968). Other workers, studying gastric mucosa from pig, human, rabbit and dog, obtained only three zymogens, named PGI, II and III. Samloff (1969), on the other hand, found that human gastric mucosal extracts yielded no less than eight proteolytic bands. Like Rapp et al. (1964), he found that acidification and neutralisation abolished the activity in the fastest seven bands, which he therefore considered to be pepsinogens and named Pg 1 to 7; activity persisted in the eighth band, which he called slow-moving protease (SMP), as he considered it not to be a true pepsin. Pg 1-5 but not 6 and 7 or SMP were detected in the urine, whereas all were present in the blood (Samloff & Townes, 1970). Seven proteolytic fractions, numbered 1 to 7, were separated from human gastric mucosa by Etherington & Taylor (1967; 1970).

A number of different zymogen fractions have also been separated from gastric mucosa by DEAE cellulose chromatography, but these are often resolved further by electrophoresis (Turner et al, 1970; Samloff, 1971a).

By the early 1970's the nomenclature of the gastric zymogens and enzymes had become complex, as each group of authors accorded different names or numbers to the fractions identified (Etherington & Taylor, 1967). The various terminologies are compared in Table 1.3.

Samloff (1971a) showed the fastest 5 bands (Pg 1 to 5) to be

immunologically identical but different from the next two (Pg 6 and 7) and from SMP. He grouped Pg 1 to 5 together as Group I pepsinogens and Pg 6 and 7 together as Group II pepsinogens. An additional Group I zymogen, band 5a, cathodal to 5, has been found in the urine (Gedde-Dahl et al, 1978). While clinicians tend to use these terms, biochemists usually call the Group I pepsinogens simply pepsinogen and the Group II pepsinogens, progastricsin. In the latter terminology, then, zymography separates gastric mucosal extracts into 1) pepsinogen, of which there are 5 or 6 isozymogens, 2) progastricsin, of which there are 2 isozymogens and 3) SMP. These will be considered in turn.

Pepsinogen and progastricsin

The reasons for the different bands are still not understood. The molecular weights appear to vary from 43,800 to 34,600 Kd (Roberts & Taylor, 1972). Variations in the amount of phosphate or carbohydrate attached to the zymogen molecule could be important (Meitner & Kassell, 1971; Kageyama & Takahashi, 1977; Ryle & Foltmann, 1985). It is also possible that the fractions are breakdown products of the same molecule (Hirsch-Marie, 1968). Differences in substrate specificity have apparently been demonstrated between the isozymogens but the significance of this is not clear (Walker & Taylor, 1978; Roberts & Taylor, 1979).

The concentrations of the pepsinogen isozymogens vary genetically. Indeed, about 14% of Caucasians in the U.S.A. lack pepsinogen band 5 on zymography of both urine and gastric mucosal extracts (Samloff & Townes, 1970) and are said to be of phenotype B. This is inherited as an autosomal recessive trait. By contrast, all individuals of East Asian origin are of phenotype A, none lacking band 5 (Samloff, Taggart & Hengels, 1985). Altogether, nine phenotypes have been identified, explicable by the codominant expression of four alleles at a single genetic locus (Taggart et al, 1979).

The gastric and urine zymogens may be more clearly resolved by electrophoresis on polyacrylamide gel and more bands have been demonstrated than on agar. Even these can be shown by column chromatography to consist of subtypes (Samloff, 1985).

Slow moving protease (SMP)

Etherington & Taylor (1970)'s zone 7 corresponds to a small early peak obtained by DEAE cellulose chromatography and appears to correspond to PIV of Kushner et al (1964) and SMP of Samloff (1969) (Table 1.3). The main property which distinguishes SMP from pepsin and gastricsin is its resistance to sequential acid activation and neutralisation. An enzyme with such a property was isolated from normal human gastric mucosa and named a cathepsin (Mangla, Guarasci & Turner, 1974). Its molecular weight, estimated by its mobility on Sephadex G-100, was 40-50,000 Kd and its pH optimum 3.4. This is consistent with the suggestion that it is cathepsin D and not cathepsin E, which has a molecular weight of about 90 Kd and pH optimum 2.5 (Barrett, 1977a). The identity of this enzyme with SMP was not, however, established by zymography.

The non-pepsin proteinase isolated from human gastric mucosa by Roberts & Taylor (1978) behaved like SMP on zymography and had optimal activity at pH 2.5-3.5. It cleaved preferentially peptide bonds Leu-Tyr and Phe-Tyr, like pepsin and cathepsin E but unlike cathepsin D, which preferentially cleaves Phe-Phe (Etherington & Taylor, 1972). These data supported their previous suggestion that normal gastric mucosa contains an enzyme with properties of cathepsin E and that it is identical with SMP (Etherington & Taylor, 1972).

The previous authors found no evidence of cathepsin D in normal gastric mucosa (Roberts & Taylor, 1978), although they had previously demonstrated it in gastric adenocarcinomas. However, the non-pepsin proteinase purified from normal gastric mucosa by Kageyama & Takahashi (1980) had properties similar to, but not identical with, those of

TABLE 1.3

TERMINOLOGIES OF ZYMOGENS USED BY DIFFERENT AUTHORS

Current	Pepsinogen	Progastricsin	SMP	Cathepsin D
Rapp et al, 1964 Kushner et al, 1964	PI, PII	PIII	PIV	
Hirsch-Marie et al, 1965) Hirsch-Marie et al, 1968)	PgI, II	PgIII	PgIV	
Hanley et al, 1966	PGI	PGII	PGIII	
Seijffers et al, 1963)	(pepsinogen III	pepsinogen I	pre IA	
Turner et al, 1970)	(pepsinogen II			
Etherington & Taylor, 1970	zones 1, 2, 3a, 3, 4	zones 5 and 6	zone 7	
Samloff, 1969	Pg 1 to 5 (Group I pepsinogen: PGI)	Pg 6 and 7 (Group II pepsinogen: PGII)	SMP	
Roberts & Taylor, 1978			proteinase 2	proteinase 1
Pohl et al, 1980			non-pepsin (gastric) proteases	protease 1
Mangla et al, 1974			gastric cathepsin	
Foltmann, 1985	pepsinogen A (PgA)	pepsinogen C (PgC)		

cathepsin D from human liver and pig spleen. The enzyme consisted of two identical monomers, covalently bound to form a molecule of weight 85,000 Kd. However, these workers did not convincingly demonstrate by zymography that this 'cathepsin D-like protease', as they termed it, was SMP, which they had been attempting to purify. Soon afterwards, Pohl, Bureš & Slavík (1981) demonstrated two non-pepsin proteinases in normal gastric mucosa. The first, which they termed 'protease 1', consisted of two polypeptide chains, of molecular weights 33,000 and 18,000 Kd, had optimal pH 3.2-3.6 and cleaved the Phe-Phe bond. On agar gel electrophoresis it moved cathodally, reflecting a higher proportion of basic amino acid residues than are known to exist in pepsinogen or progastricsin. These and other properties supported the view that this enzyme was identical to cathepsin D isolated from other tissues. The second enzyme, termed 'non-pepsin protease', moved electrophoretically like SMP. These results suggest that normal gastric mucosa contains cathepsin D and that SMP is a different enzyme.

Recently a cathepsin D-like proteinase has been demonstrated in the rat gastric mucosa (Muto, Arai & Tani, 1983).

IMMUNOLOGICAL PROPERTIES OF THE ASPARTIC PROTEINASES

It has been recognised that pepsin is immunogenic since Northrop (1930) raised rabbit antisera to his newly purified crystalline pig pepsin and demonstrated a precipitin line between the antisera and both active and denatured enzyme. The immunological non-identity of pepsinogen, progastricsin and SMP has been demonstrated by immunoelectrophoresis of human gastric mucosal extracts against rabbit antisera (Kushner et al, 1964; Hirsch-Marie et al, 1965; Samloff,

1971a). There appears to be partial immunological identity between the zymogens and their corresponding active enzymes (Kushner et al, 1964; Kalinovsky & Okylov, 1978), suggesting that there are shared antigenic determinants. As aspartic proteinases are normally inactivated at pH over 7, they may, when used as immunogens, stimulate production of antisera which react better with the zymogen than with the active enzyme (Foltmann, 1981).

There is immunological identity among the five pepsinogen isozymogens (Pg 1-5) and between the two progastricsin fractions (Pg 6 and 7) (Samloff, 1971a), presumably reflecting the strong correlation known to exist between antigenic similarity and the extent of sequence homology (White, Ibrahimi & Wilson, 1978). Similarly, pepsin, gastricsin and chymosin from the same species are less closely related immunologically than is each enzyme to its counterpart in different species. It has been suggested that this indicates that development of separate genes for these enzymes preceded the divergence of mammals (Foltmann, 1981).

COMMENT

Most work on the aspartic proteinases has been carried out by biochemists. Perhaps this partly explains why the precise location of the enzymes in tissues is poorly documented and why their potential use in tumour diagnosis has not been examined. The immunohistochemical techniques used by pathologists can readily be applied to such problems and the answers may, in turn, be of biochemical as well as pathological interest. In the work described in this thesis, four human aspartic proteinases, pepsin, gastricsin, cathepsin D and SMP were selected. Before their immunolocalisation is discussed, however, certain preliminary studies will be described in Chapter 2.

CHAPTER 2

BIOCHEMICAL AND IMMUNOLOGICAL STUDIES OF ASPARTIC PROTEINASES

INTRODUCTION

A prerequisite of any immunohistological investigation is a supply of antibody and ideally some pure antigen, for use in absorption control experiments. As there are no commercially available antibodies to any of the aspartic proteinases, antisera had to be prepared by immunisation of animals. However, the only enzymes or zymogens which are available for this purpose are pig pepsin and pepsinogen. Initially, therefore, pig pepsin was used as an immunogen and the resultant antiserum was employed in immunohistological studies. Subsequently, due to doubts about the homogeneity of the commercial pepsin, it was considered desirable to obtain a pure supply of this enzyme. In order to avoid problems due to autodigestion, the zymogen, pig pepsinogen rather than the active enzyme was further purified, the aim being to use the purified zymogen, and, if necessary, the derived enzyme, for blocking experiments to ensure specificity of immune staining with the previously prepared antipepsin. Later, a small supply of human pepsin was donated to us.

The purification of the other aspartic proteinases, progastricsin, cathepsin D and slow moving protease presents greater difficulties than that of pepsinogen. As the advantage of a partially pure animal form of these enzymes did not exist, human tissues were used. Because of similarities between the enzymes, they are difficult to separate from one another and for this reason sources other than gastric mucosa, in which several members of this family are present, were chosen whenever possible. Progastricsin, for example, appears to be the only acid proteinase zymogen in seminal fluid, which was therefore used as a source, although during the work we were given a supply of pure progastricsin. A small amount of human cathepsin D was also gifted to us but was thought insufficiently pure for use as an immunogen. It was therefore decided to purify cathepsin D anew from human spleen, which

was readily available and known to be rich in this enzyme. When the present studies began, little was known about slow moving protease. The work of Hirsch-Marie et al (1976) suggested that fetal stomach was rich in SMP and poor in pepsin and gastricsin. For this reason, fetal stomach was initially used as a source of SMP. Later, SMP, purified from human gastric mucosa, was gifted to us.

This chapter describes the purification of the various aspartic proteinases, the development of antisera to the enzymes and the tests performed to demonstrate the specificity of these antisera, which were to be used for subsequent immunohistological work. Although pepsin, gastricsin and cathepsin D have been relatively well characterised, little is known about SMP. In previous studies, there has been speculation that it is identical to cathepsin D (Chapter 1). Experiments were therefore carried out in an attempt to clarify the nature of this enzyme.

MATERIALS AND METHODS

Sources of enzymes and zymogens

The sources from which the aspartic proteinases and zymogens used in this study were obtained are listed in Table 2.1. Pig pepsinogen was further purified as described below. The human pepsin obtained from Dr. A.P. Ryle had been purified from gastric juice (Ryle, personal communication). It had been shown to migrate as a single band on SDS-polyacrylamide gel electrophoresis. The human gastricsin obtained from Professor J. Tang had been purified by the previously described method (Tang, 1970). It migrated as a single band on SDS-PAGE electrophoresis and on electrophoresis in 5.6% polyacrylamide gel. The human seminal progastricsin gifted by Professor Svasti had been prepared as described previously (Ruenwongsa & Chulavantnatol, 1975) and was activated at pH 2 to yield the enzyme, gastricsin (Surinrut, Svasti & Surarit, 1981). The human cathepsin D gifted by Dr. Barrett had been purified as previously indicated (Barrett, 1977a). Attempts were made to purify SMP from homogenates of fetal gastric mucosa. The supply of SMP gifted by Professor I.M. Samloff was purified from human gastric mucosa as described (Samloff et al, 1987).

TABLE 2.1

SOURCES OF ASPARTIC PROTEINASES AND ZYMOGENS

Enzyme/Zymogen	Tissue of origin	Source
pig pepsin (lyophilised)	pig gastric mucosa	Sigma Chemicals Ltd
pig pepsinogen "	" " "	" " "
human pepsin	human " "	Dr. A.P. Ryle, Edinburgh
human gastric gastricsin	human gastric mucosa	Prof. J. Tang Oklahoma
human seminal progastricsin	human seminal fluid	Prof. J. Svasti, Bangkok
human cathepsin D		Dr. A.J. Barrett, Cambridge
human cathepsin D	human spleen	purified de novo
human SMP	human gastric mucosa	Prof. I.M. Samloff, California

ENZYME ASSAY

Assay of pepsinogen, progastricsin and cathepsin D is based on the ability of the active enzymes to digest haemoglobin at optimal pH 2.0, 3.0 and 3.5 respectively. The methods described are for pepsinogen but, with appropriate pH adjustments, are suitable for estimation of the other two enzymes and zymogens.

1. Anson's method

The method was that described previously (Anson & Mirsky, 1932; Anson, 1937, 1938; Berstad, 1970). Duplicate samples of bovine haemoglobin were kept at 37°C in a water bath. Proteolysis was started by addition to each tube of either the unknown sample or a standard solution of pepsinogen, the mixture being buffered to pH 2.0. After 10 mins the reactions were stopped by addition of 5% trichloracetic acid (TCA). The TCA soluble split products formed were estimated by measuring the optical density of the filtrates at 280 nm against the blanks. The amount of enzyme in each tube was proportional to the optical density and could be estimated by comparison with the optical density of the known standards.

2. Radial diffusion assay

This is a convenient method for assay of large numbers of samples and is, like Anson's method, based on haemoglobin digestion. The samples were allowed to diffuse radially from circular wells in agar impregnated with haemoglobin buffered to the optimal pH of the enzyme under assay. The agar gels were prepared as described previously (Samloff & Kleinman, 1969). It was found convenient to use glass microscope slides (7.5 x 2 cm), on which a slab of gel 1 mm thick could be layered by addition of 1.8 ml of the agar mixture. The slides were stored in racks at 4°C in an airtight moist container. As originally described, the wells in the agar plate were 4 mm in diameter. It was found in the present studies, however, that wells as small as 2 mm could

be used with satisfactory results. In this way, up to 50 assays could be accommodated on one slide, although in practice about 30 per slide sufficed. Into each well was placed a sample, pig pepsin or pepsinogen being used as standard. The slides were incubated in a humid chamber for 1 hr at 37°C and fixed in 5% acetic acid in 50% ethanol to stop the reaction and precipitate undigested protein. Each slab was overlain with moist filter paper to prevent cracking of the agar during drying. Slides were then stained for protein with 1% amido black (Uriel and Grabar, 1956). A clear zone developed round each well where there had been proteolytic digestion of haemoglobin. The logarithm of the concentration of enzyme is linearly related to the diameter of the clear zone minus the diameter of the well (Mancini, Carbonara & Heremans, 1965). The concentration of enzyme in each sample could therefore be read off a series of known pepsinogen standards.

3. Milk clotting assay for pepsin

Pepsin was assayed by the milk clotting method at pH 5.3 (McPhie, 1976).

4. Assay of cathepsin D

During purification, cathepsin D activity was assayed by digestion of the synthetic substrate Pro-Thr-Glu-Phe-(NO_2Phe)-Arg-Leu (Dunn, Kammermann & McCurry, 1984).

PROTEIN ASSAY

Protein was assayed either by a standard Lowry method (Lowry et al, 1951) or by a Bio-Rad protein assay (Bio-Rad Laboratories - Technical Bulletin 1051E, April, 1977).

PREPARATION OF GASTRIC MUCOSAL EXTRACTS

Stomachs which had been resected for peptic ulceration or malignancy were collected fresh and washed in normal buffered saline.

The mucosa was scraped off with a glass microscope slide, homogenised in 0.1M phosphate buffer, pH 7.3 at 4°C and centrifuged at 18,000 rpm (Samloff, 1969). The supernatant was stored at -20°C.

ELECTROPHORESIS OF GASTRIC MUCOSAL EXTRACTS

Agar gel electrophoresis

Agar gel electrophoresis was carried out by the method described by Samloff (1969). 1.5% Noble agar (Difco) was made up in 0.05M Veronal buffer pH 8.3 and poured over levelled glass plates to a uniform thickness of 1 mm. When the agar had solidified samples were placed in slots 10 x 1 mm cut 2-3 cm from the cathodal end. The plates rested on a perspex box cooled by circulating water. Electrophoresis was carried out for several hours at 11 volts per cm (constant voltage).

After electrophoresis the plates were incubated in 0.65% bovine haemoglobin (Sigma) in 0.06 N HCl, pH 1.6 for 10 min. The excess fluid was poured off and the plates were incubated in a humid chamber at 37°C for 1 hr. The plates were then fixed for at least 1 hr in 5% acetic acid in 50% ethanol. Moist filter paper was laid on the agar surface to prevent cracking and the gels were dried and stained with 1% amido black, which stained the haemoglobin protein blue and left a clear zone where there had been proteolysis.

Polyacrylamide gel electrophoresis (PAGE)

This was carried out by three different methods:

1. PAGE in the presence of sodium dodecyl sulphate (SDS). This was performed by a standard method as described previously (Valler, 1986).
2. Horizontal slab PAGE. The separating gel consisted of 8% acrylamide and 0.25% bisacrylamide in 0.42% Tris base (finally 35 mM), pH 7.5. The electrode buffer consisted of barbitol 22.08g and Tris base 4.0g in 4 litres of water, pH 7.2-7.5. This was diluted 1:2 for the

cathode chamber. Electrophoresis was carried out at constant power 2.5W.

3. Discontinuous vertical thin layer PAGE. The stacking and separation gels were similar to those published (Taggart et al, 1978). The electrode buffers were the same as those used in the horizontal slab PAGE.

Proteolytic activity in the gels was demonstrated by incubation in haemoglobin solution followed by staining with Coomassie blue (Taggart et al, 1978).

PURIFICATION OF PIG PEPSINOGEN

Lyophilised pig pepsinogen (Sigma Chemical Co.) was further purified by ion exchange chromatography and gel filtration, as described by Ryle (1970) and finally by affinity chromatography. All the procedures were carried out at $4^{o}C$. Forty-five mg of pig pepsinogen in 0.02M phosphate buffer, pH 6.9 was loaded on to a DEAE-cellulose column (2.5 x 20 cm), which was then washed with 100 ml of buffer. The column was washed with an exponential gradient of 1M NaCl, 7.2 ml fractions being collected. The fractions containing the resultant optical density peak at E_{280} were assayed for proteolytic activity by the radial diffusion and Anson's haemoglobin methods. The fractions with maximal proteolytic activity were pooled and the total amount of pepsinogen estimated. The sample was concentrated, dialysed against phosphate buffer containing 0.1M NaCl and added to a G100 superfine Sephadex column (2.5 x 40 cm), which was washed with 0.02M phosphate buffer, pH 6.9, 8 ml fractions being collected. The fractions containing the resultant peak at E_{280} were assayed as before. The samples containing maximal proteolytic activity were pooled, dialysed against 0.05M phosphate buffer, pH 6.5 and loaded on to an affinity column (2 x 16 cm) of poly-L-lysine 1B (Sigma catalogue P1886) coupled to Sepharose 4B.

This was washed with 0.05M phosphate buffer pH 6.5 and a linear gradient with 120 ml 1M NaCl applied. The fractions of the resultant peak at E_{280} were assayed as before. The samples with maximal proteolytic activity were pooled, re-run on the washed poly-l-lysine column and collected, pooled and concentrated as before. The composition of the final sample was determined by amino acid analysis on a Locarte analyser following hydrolysis with 6M-HCl in vacuo for periods ranging from 24-72 hrs. The sample was also examined by SDS-polyacrylamide gel electrophoresis and was also tested for the presence of pepsin by milk clotting assay at pH 5.3. These analyses were undertaken by Dr. J. Kay & M.J. Valler, Biochemistry Department, University College, Cardiff.

PURIFICATION OF SEMINAL FLUID ACID PROTEINASE ZYMOGEN

Samples of seminal fluid, obtained from the infertility and vasectomy clinics, were pooled and stored at -20°C. The pooled seminal fluid was later thawed and centrifuged at 25,000 rpm at 4°C for 30 mins. The supernatant plasma was fractionated by addition of ammonium sulphate to 40% saturation. After centrifugation, the resultant supernatant was assayed for acid proteinase activity by Anson's method. The supernatant was purified by DEAE-cellulose by the method of Ruenwongsa & Chulavantnatol (1975) and then by affinity chromatography as described above for pepsinogen. The fractions collected were assayed for enzyme activity by radial diffusion assay and those with peak activity were pooled.

PURIFICATION OF CATHEPSIN D

Cathepsin D was purified from normal human spleen by a three step procedure as described by Afting & Becker (1981). Normal human spleen

(64.2g), obtained at splenectomy and stored at -20°C, was homogenised in 3 volumes of 30mM veronal buffer, pH 7.4. The homogenate was spun at 17,500 G for 15 min at 4°C and the supernatant retained. The pellet was resuspended in buffer (1 ml per gram of tissue), homogenised and spun (31,000g, 20 min). Both supernatants were filtered through glass wool to remove fat and were pooled and assayed for cathepsin D activity. Concanavalin-A-Sepharose CL4B (Pharmacia, 17-0440-01) was washed in a Buchner funnel with 30mM veronal buffer, pH 7.4 containing 1mM Mn^{++} and 1mM Ca^{++} and 0.6M NaCl. The combined supernatants were stirred gently at 10 mls per gram of the wet washed ConA-Sepharose for 30 mins and the gel was washed on a Buchner funnel with excess veronal/0.6M NaCl buffer containing 1mM mercaptoethanol. The washed ConA-Sepharose was packed into a column (30 x 1.5cm) and washed with veronal/NaCl/mercaptoethanol until the eluate had E_{280} = 0. The column was washed with 0.1M sodium phosphate buffer, pH 6.5 containing 0.6M NaCl/1mM mercaptoethanol and 0.2M alphamethyl mannoside (Sigma, M6882). Six bed volumes were collected, each volume being allowed to equilibrate in the column for 20 min at room temperature. The eluate fractions were assayed for cathepsin D activity as above. The active fractions were pooled and concentrated to 10-15 mls with a PM-10 Amicon membrane. The concentrate was applied to a Sephadex G100 column (2 x 60 cm) in 0.1M sodium phosphate buffer, pH 6.5/0.6M NaCl/1mM mercaptoethanol. The cathepsin D activity of the eluate fractions was assayed at pH 3.

Pepstatin-agarose was prepared as follows: isovaleryl pepstatin was covalently linked via its carboxyl terminal to aminohexyl Sepharose CL4B with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide by the method of Kay & Dykes (1976).

The pooled eluate from the Sephadex column was adjusted to pH 4.0 with 12N hydrochloric acid and passed through a column containing 5 ml of the pepstatin agarose, which had previously been equilibrated in

50 mM sodium citrate buffer, pH 4.0 containing 0.6M sodium chloride. The column was washed with 100 ml of this buffer containing 25 ml of 6M urea, followed again by 100 ml buffer. Finally, cathepsin D was eluted with 50mM $NaHCO_3$ solution, pH 8.2 containing 0.6M sodium chloride. Fractions displaying activity were pooled and dialysed overnight against 2mM sodium phosphate buffer, pH 6.5 and freeze dried. 40 µg samples were tested by SDS-PAGE as described above.

PURIFICATION OF SLOW MOVING PROTEASE

Attempts were made to purify slow moving protease from fetal gastric mucosa. Stomachs from fetuses of gestation age 21-36 weeks were collected at autopsy within 24 hours of death and stored at -20°C. The stomachs were thawed and the mucosa scraped off with a glass microscope slide and homogenised in phosphate buffer as follows:-

1. Mucosae from 14 stomachs were separately homogenised in 1:10 volumes of 0.005M phosphate buffer, pH 7.4 at 4°C at 16,000 rpm. The zymogens were extracted by the method of Hirsch-Marie & Touboul (1973), which entails acetone extraction at -35°C and ether precipitation at 4°C. The specific activity of the resultant precipitate was calculated from the relation of acid proteinase activity as measured by radial diffusion assay to the protein content by Lowry assay. Agar gel electrophoresis of 4 of the extracts was carried out, normal adult gastric mucosal extract being used as control. Five extracts were selected for immunisation of rabbits.
2. Pooled mucosae from 8, 5, 8 and 24 fetal stomachs were homogenised in 5 vol/g of 20mM sodium phosphate buffer, pH 7.2 and centrifuged at 20,000 rpm. The supernatants were assayed for proteolytic activity by Anson's method and the specific activity calculated. A

sample of the fourth homogenate of 24 fetal gastric mucosae was tested by agar gel electrophoresis with normal adult gastric mucosa as control.

PREPARATION OF ANTISERA

All the polyclonal antisera were prepared by injection of rabbits with enzyme or zymogen emulsified in complete Freund's adjuvant and boosted with subsequent injections. Finally the rabbits were bled out. Sodium azide was added to the serum to inhibit bacterial growth and the serum was stored in aliquots at -20°C. The antisera were tested for immunohistochemical reactivity by a peroxidase-antiperoxidase (PAP) method on sections of paraffin-embedded gastric mucosa or, in the case of anticathepsin D, spleen (for details, see Chapter 3).

Pepsin and Pepsinogen

Anti-pig pepsin

Three rabbits were injected intramuscularly with 1 mg of pig pepsin and boosted with injections of 0.5 mg at 2 week intervals. After a further 2 weeks, the rabbits were bled out.

Anti-pig pepsinogen

Antiserum to the highly purified pig pepsinogen was prepared in a similar way to anti pig pepsin except that there were 7 boosts at 10 day intervals.

Anti-human pepsin

This was prepared similarly to anti pig pepsin except that the boosts were at monthly intervals. Serum from 2 of the rabbits labelled normal gastric mucosa by the PAP method; the rabbits with the higher titre was bled out.

Gastricsin

Antiserum to seminal fluid acid proteinase

Three rabbits were injected with seminal fluid acid proteinase,

50 µg in 2 doses, 1 intradermally, the other intramuscularly. Two weeks later a similar dose was given. A test bleed 3 weeks later showed no evidence of antibody to gastricsin in serum from any of the rabbits and in view of the small amount of acid proteinase in the remaining extract (154 µg) no further rabbits were immunised.

Anti-human gastric gastricsin

Three rabbits were immunised by intradermal and intramuscular injection of 50 µg (total) of human gastric gastricsin. This was repeated after 4 weeks and after a further 4 weeks a similar injection of 37 µg was injected intramuscularly. Two weeks later a test bleed gave positive labelling of gastric body mucosal sections by the PAP method with serum from 2 rabbits, the third having died. After a further week, another intramuscular boost was given and the rabbits were bled out after a further week.

Anti-human seminal gastricsin

Three rabbits were immunised with 25 µg each of seminal gastricsin and boosted monthly for 2 months. Serum from all 3 rabbits was tested by the PAP method on normal human gastric antrum. Only 1 serum labelled the glands and the rabbit was boosted twice more at monthly intervals before being bled out.

Cathepsin D

Anti-cathepsin D

Three rabbits were injected intradermally and intramuscularly with a total dose of 50 µg of cathepsin D each. One month later a similar intramuscular dose was given. Two further intramuscular boosts of 37 µg were given at 2 week intervals. Test bleeds gave positive labelling of paraffin embedded sections of human spleen by the PAP method with serum from 2 rabbits. One of these died and the other was bled out.

SMP

Antiserum to fetal gastric zymogen

Extracts of 5 fetal gastric mucosae, prepared as above, were

injected intradermally and intramuscularly into separate rabbits (total protein dose 5-7 mg) and boosted at 2 week intervals up to 8 injections. The animals were bled out.

Anti-SMP

Rabbit anti-SMP was a gift from Professor I.M. Samloff, VA Medical Center, Sepulveda, California, USA and had been prepared by injecting New Zealand white rabbits subcutaneously with 100 µg of SMP which had been dissolved in phosphate buffered saline and emulsified in complete Freund's adjuvant. Booster injections in incomplete adjuvant were given at 4, 8 and 12 weeks.

CHARACTERISATION OF ANTISERA

All rabbit sera were initially tested for immune reactivity with paraffin-embedded histological sections. Subsequently, certain antisera were further tested by double diffusion (Ouchterlony) and immune (Western) blotting.

1. Immune labelling by antisera

Immune labelling was performed by the peroxidase-antiperoxidase (PAP) method, described in detail in Chapter 3. The antisera were tested on formalin fixed paraffin embedded sections of appropriate control tissues as follows:

Normal human gastric body mucosa - anti-pig pepsin, anti-pig pepsinogen, anti-human pepsin, anti-human gastric gastricsin, anti-human seminal fluid zymogen, anti-human seminal gastricsin, anti-human fetal gastric zymogen, anti-human SMP.

Normal human spleen - anti-cathepsin D.

The antisera to fetal gastric zymogen were also tested on sections of fetal stomach and the following adult tissues:

Normal gastric mucosa, colon, pancreas and endometrium.

Carcinomas of stomach, breast, colon, endometrium, ovary and pancreas.

An attempt to block non-specific labelling was made by preincubating antifetal gastric zymogen with lyophilised umbilical cord serum (1 and 5 mg/ml) before incubation with sections of normal and carcinomatous stomach.

2. Double Diffusion (Ouchterlony)

Immunodiffusion was attempted between pepsin and pepsinogen and their antisera and between fetal zymogen extracts and their antisera but not for other aspartic proteinases, as only limited quantities of these enzymes were available in pure form.

Immunodiffusion was performed on glass microscope slides layered

with a 1 mm thick slab of 1.2% Noble agar (DIFCO) in 0.15M phosphate buffered saline, pH 7.3, containing 4% polyethylene glycol. Wells, 4 or 8 mm in diameter, were cut. Commercial pig pepsin, pure pepsinogen and the pure pepsin prepared from it by acid activation were tested against rabbit antisera to pig pepsinogen and pig pepsin.

3. Immune (Western) blotting

Extracts of normal human gastric body mucosa (Samloff, 1969) were run by discontinuous vertical thin layer PAGE, as described above. Two lanes of the gel were detached and enzyme activity demonstrated as before (Taggart et al, 1978). Protein was transferred to nitrocellulose sheets by a Western blot method (Towbin, Staehelin & Gordon, 1979). Strips corresponding to samples were incubated with antisera to pig pepsin, human gastric gastricsin or human cathepsin D, all at 1:20 and labelled by an indirect alkaline phosphatase technique (Symington, 1984).

COMPARATIVE STUDIES OF CATHEPSIN D AND SMP

The properties of these two enzymes were compared by several methods: 1. Enzyme kinetics, 2. Immune blotting, 3. Electrophoresis The first two were undertaken by Drs. M.J. Valler and J. Kay, Biochemistry Department, University College, Cardiff.

1. Kinetic studies

The hydrolysis of two chromogenic substrates, Pro-Thr-Glu-Phe-(NO_2)Phe-Arg-Leu and Lys-Pro-Ala-Glu-Phe-(NO_2)Phe-Arg-Leu, at their Phe-(NO_2)Phe bonds was followed spectrophotometrically at 300 nm as described previously (Dunn et al, 1985; Dunn et al, 1986).
The substrates were kindly supplied by Dr. B.M. Dunn, University of Florida College of Medicine. Kinetic parameters were obtained using ranges of substrate concentrations appropriate for both enzymes. In all cases, Michaelis-Menten kinetics were observed and plots of S/v against

S permitted the fitting (by the method of unweighted sum of least squares) of unambiguous straight lines. For each determination, initial rates (v) were measured with at least five values of initial substrate concentretion (S).

The active concentration of each enzyme was determined by titration against a solution if isovaleryl-pepstatin (kindly supplied by Professor H. Umezawa, Institute of Microbial Chemistry, Tokyo, Japan). The concentration of isovaleryl-pepstatin had been determined by amino acid analysis.

Kinetic constants (K_i) for the interaction of the inhibitor from Ascaris lumbricoides with SMP and cathepsin D were determined as described previously (Valler et al, 1985). The inhibitor was the kind gift of Dr. T. Hofmann, University of Toronto and Dr. R. Peanasky, University of South Dakota.

2. Immunodot blotting

Immunological cross-reactivity between SMP and cathepsin D was examined using the immunodot-blot procedure described previously (Heath, Lewis & Kay, 1985). The antigens in a range of concentrations from 5 to 60 g/ml were applied to nitrocellulose strips and reacted with 1:500 and 1:1000 dilutions of their respective primary antisera, heterologous antisera, or with non immune rabbit serum for 2 hrs at room temperature. After washing, the strips were incubated with second antibody (sheep anti-rabbit IgG conjugated to horseradish peroxidase; Serotec Ltd., Bicester, U.K.) for 1 hr at room temperature and then developed with diaminobenzidine.

3. Electrophoretic studies

Cathepsin D and SMP were examined by horizontal slab polyacrylamide gel electrophoresis (PAGE). The gels were prepared as described above.

Samples were run after incubation with their primary antisera, heterologous antisera or normal rabbit serum. Enzyme activity was demonstrated by incubating the gels in haemoglobin solution and staining with Coomassie blue (Taggart et al, 1978).

RESULTS

PURIFICATION OF PIG PEPSINOGEN

The optical density peaks from the chromatography columns are shown in Fig 2.1. The proteolytic activity as estimated spectrophotometrically is also plotted and illustrates that maximal activity corresponded to the optical density peaks. Fractions from the DEAE cellulose and Sephadex G100 columns were also estimated for proteolytic activity by the radial diffusion assay (Fig 2.2a and b) and the results were in agreement with those shown in Fig 2.1a and b. Of the 45 mg of pepsinogen in the original sample, the amount remaining after each stage was as follows: DEAE cellulose, 9 mg, Sephadex G100 3.25 mg and affinity chromatography 1.2 mg.

The amino acid composition of the sample was as follows:

	Residues per mole of protein
Arginine	4
Lysine	10
Histidine	3
Phenylalanine	16
Tyrosine	15

This is in agreement with the calculated composition of the major pig pepsinogen A. No pepsin activity was detected by milk clotting at pH 5.3. The sample yielded a single line by SDS-PAGE.

PURIFICATION OF SEMINAL FLUID ACID PROTEINASE ZYMOGEN

In 98 ml of pooled seminal plasma the estimated acid proteinase content was 12 mg. Following purification, an estimated 450 µg was retrieved.

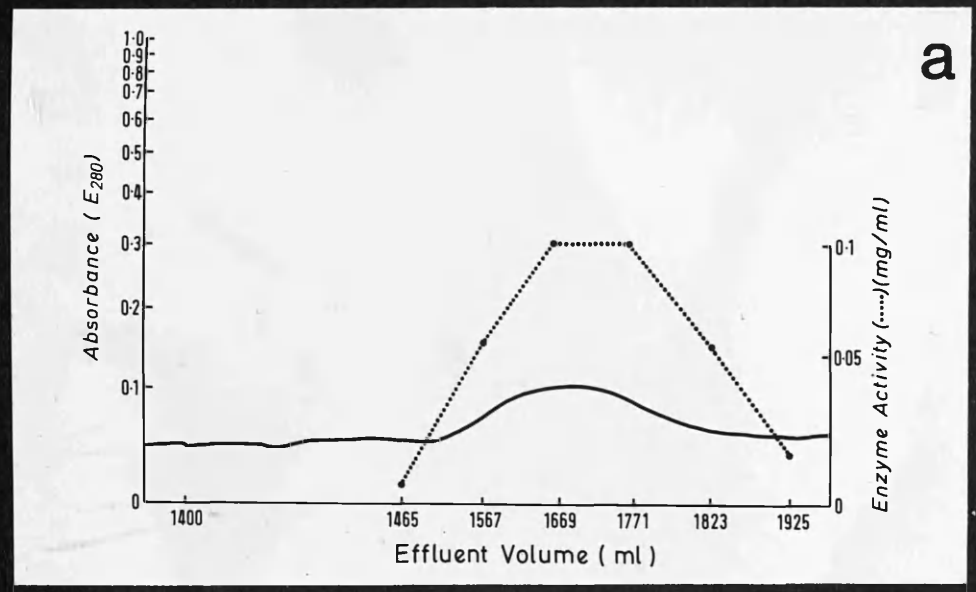
FIG. 2.1 PLOTS SHOWING RESULTS OF FURTHER PURIFICATION OF COMMERCIAL PIG PEPSINOGEN

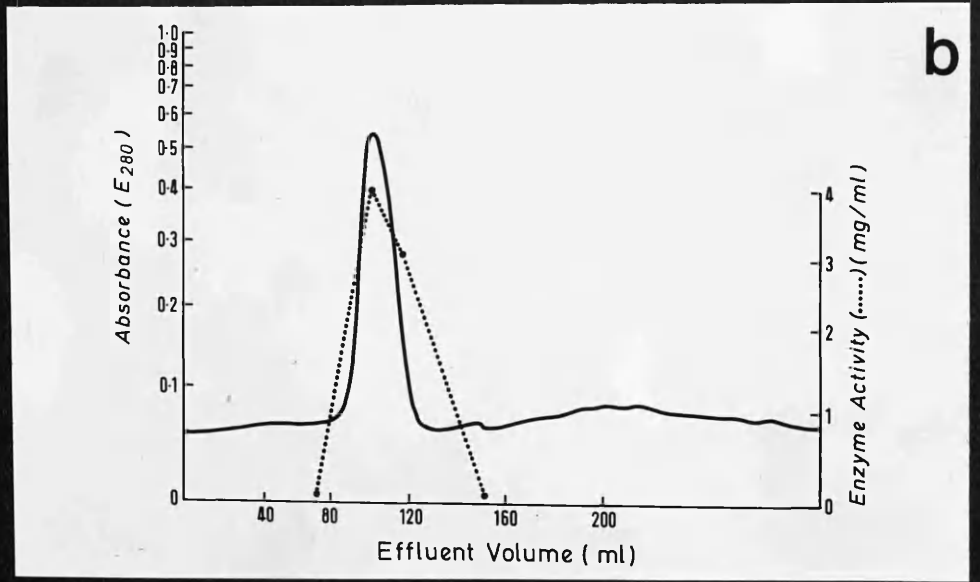
a. Ion exchange chromatography on DEAE cellulose. The volume of the mixing vessel was 2 litres and the gradient 0-0.45M NaCl. The results of radial diffusion assay on the fractions of the optical density peak are shown in Fig. 2.2a.

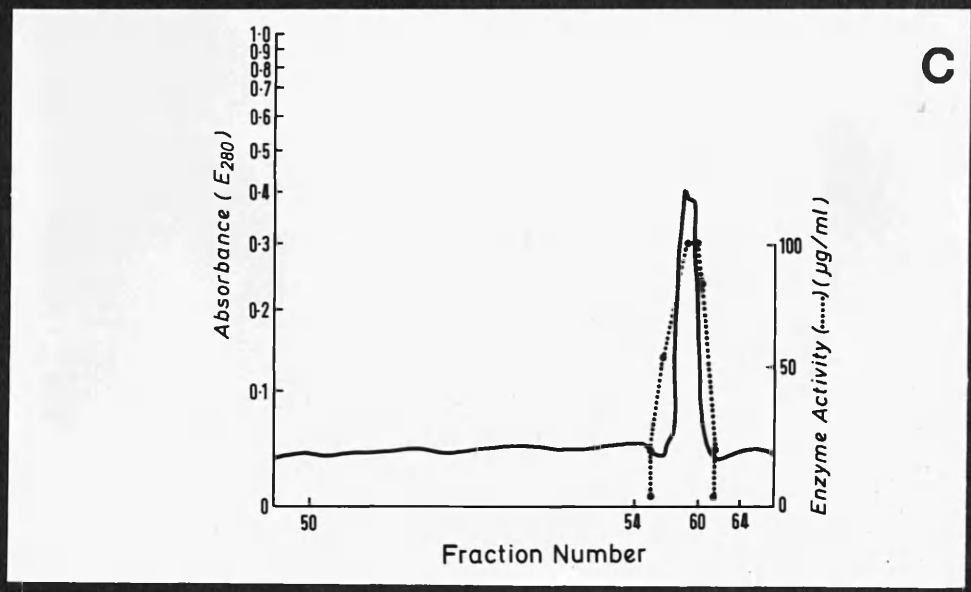
b. Gel filtration on Sephadex G100. The results of radial diffusion assay on the fractions of the optical density peak are shown in Fig. 2.2b.

c. Affinity chromatography on poly-l-lysine Sepharose 4B first run.

d. Affinity chromatography on poly-l-lysine Sepharose 4B second run.


a
Absorbance (E_{280})
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
Enzyme Activity (.....)(mg/ml)
0·1
0·05
0
1400
1465
1567
1669
1771
1823
1925
Effluent Volume (ml)


b
Absorbance (E_{280})
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
Enzyme Activity (.....)(mg/ml)
4
3
2
1
0
40
80
120
160
200
Effluent Volume (ml)


c
Absorbance (E_{280})
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
Enzyme Activity (.....)(µg/ml)
100
50
0
50
54
60
64
Fraction Number

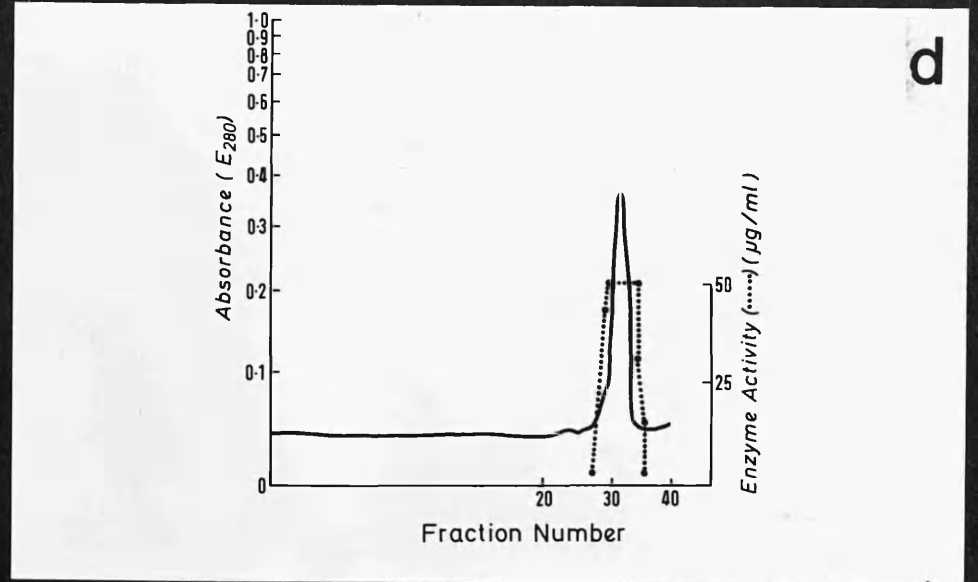

d
Absorbance (E_{280})
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
Enzyme Activity (.....)(µg/ml)
50
25
20
30
40
Fraction Number

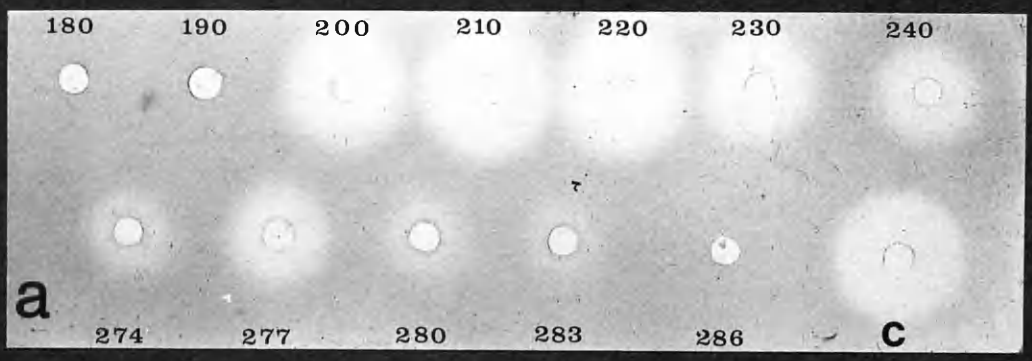
FIG. 2.2 Radial diffusion assay on agar gel of fractions (indicated by numbers) which yielded the optical density peaks shown in Fig. 2.1.

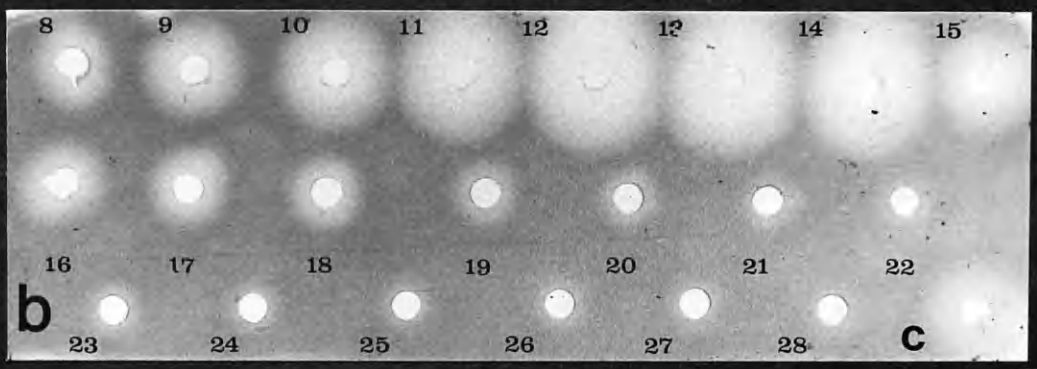
a) Optical density peak from Fig. 2.1a.

b) Optical density peak from Fig. 2.1b.

Note the lytic zone around each well, the diameter being proportional to the log (concentration of enzyme). c) is the pepsinogen control.

FIG. 2.3 Antiserum to pig pepsin (8) shows a dense precipitin line with both samples (large and small wells) of pure pig pepsin (1 and 11) and both samples of commercial pepsin (3 and 10); one of the latter (10) yields two precipitation lines, the denser of which shows identity with that of pure pepsin (1). Antipepsinogen (9) precipitated with pure pepsinogen (5), pure pepsin (6 and possibly 11) and commercial pepsin (4 and possibly 12). There is partial identify between pepsinogen (5) and pepsin (6 and 4).


180
190
200
210
220
230
240
a
274
277
280
283
286
c


8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
b
23
24
25
26
27
28
c

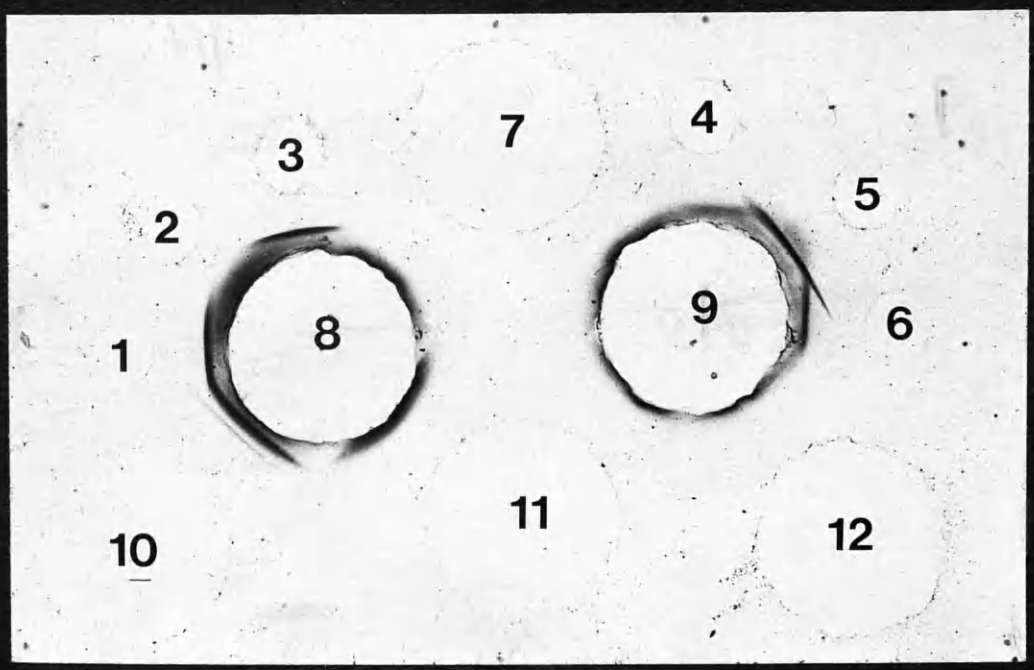

1
2
3
4
5
6
7
8
9
10
11
12

FIG. 2.4 IMMUNE (WESTERN) BLOT OF ZYMOGEN FRACTIONS OF GASTRIC MUCOSAL EXTRACT

Lanes 1 and 2 are zymograms (on PAGE) of gastric mucosal extract, neat (lane 1) and 1:5 (lane 2). The lane at the top (arrow) is the marker dye, towards the anode, and the origin is aligned with the arrow at the lower right (lane 5). The fastmoving pepsinogen fractions (1-5), slower progastricsin bands (6 and 7) and SMP (8) show as clear lytic zones. There is a protein deposit just anodal to SMP. Lanes 3, 4 and 5 are strips of nitrocellulose, blotted from similar gel runs to that in lane 2. They have been labelled respectively with antipig pepsin (3), antigastric gastricsin (4) and anticathepsin D (5). In 3, there is labelling of pepsinogen fractions 2, 3, 4 and 5. The eccentric deposit between 7 and 8 may be artefactual. In 4 there is labelling of progastricsin fraction 7 and, apparently, of the SMP zone (8). In 5, there is a faint deposit near the origin; this is equivocal, but further evidence (Fig 2.6) suggests that it may genuinely be cathepsin D.


1
2
3
4
5
6
7
8
1
2
3
4
5

FIG. 2.5 Immunodot-blot. Upper panel. Samples (0.5 µl) of cathepsin D (strips 1 and 2) and SMP (strips 3 and 4) were applied to nitrocellulose strips at concentrations (from top to bottom) of 0, 60, 30, 15 and 5 µg/ml. The strips were immersed in antiserum to cathepsin D at dilutions of 1:500 (strips 1 and 3) and 1:1000 (strips 2 and 4). After washing, the strips were incubated with a second antibody (peroxidase-conjugated sheep anti-rabbit IgG) and stained with diaminobenzidine. Staining is observed with cathepsin D but not with SMP. Lower panel. Same as in upper panel except that the strips were immersed in antiserum to SMP at dilutions of 1:500 (strips 1 and 3) or 1:1000 (strips 2 and 4). Staining is observed only between anti-SMP and SMP.

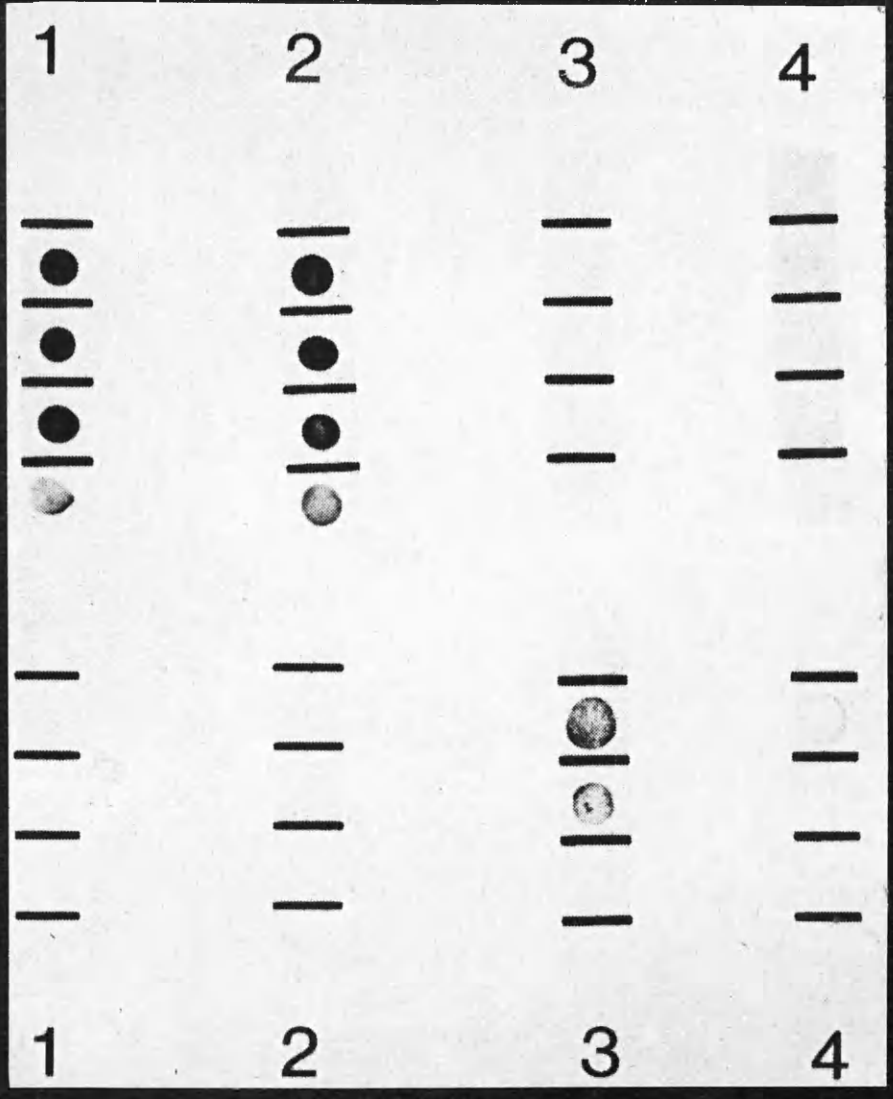
FIG. 2.6 Zymogram run in 5% polyacrylamide gel (horizontal) at pH 7.5 of the following samples:

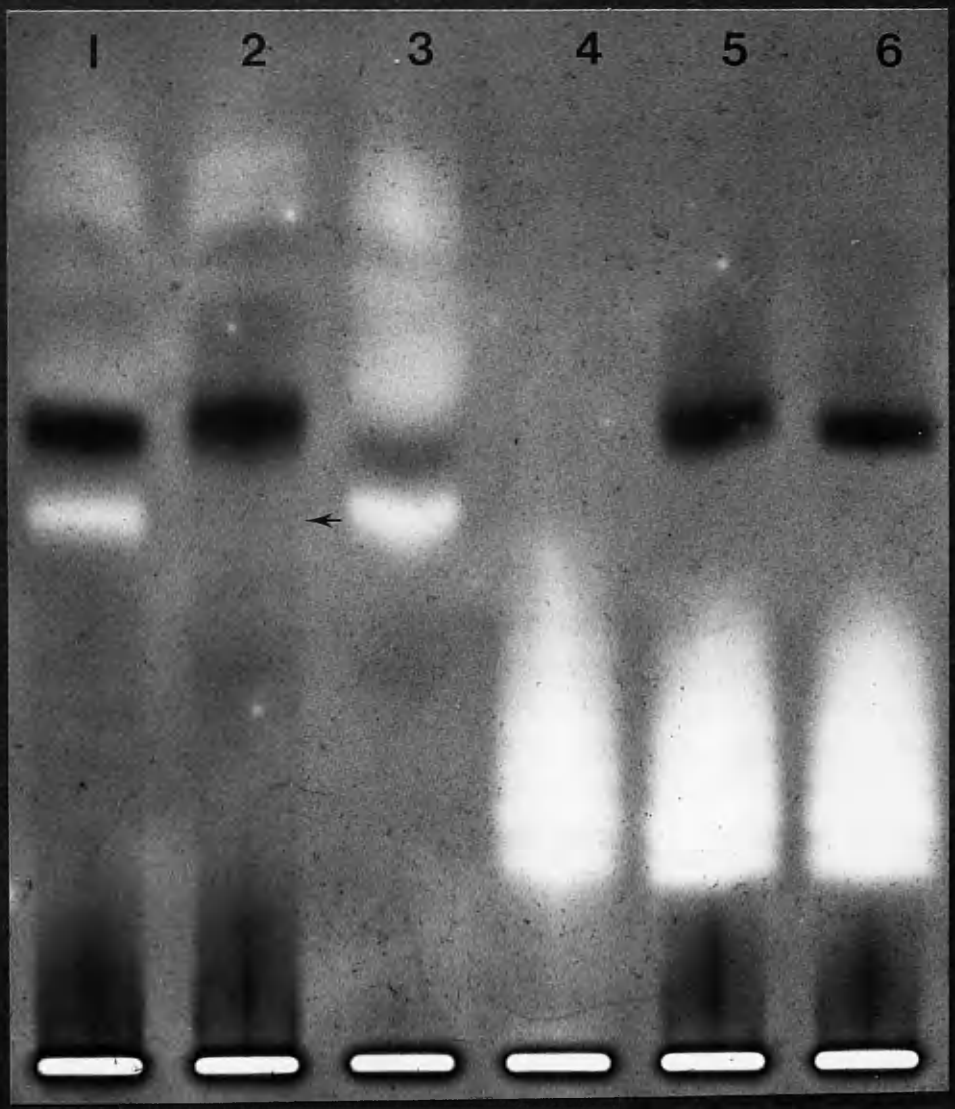
1. Gastric mucosal extract and normal rabbit serum
2. Gastric mucosal extract and anti-SMP
3. Gastric mucosal extract and distilled water
4. Cathepsin D and distilled water
5. Cathepsin D and anti-SMP
6. Cathepsin D and normal rabbit serum

Cathepsin D is more slow-moving than SMP and is split into 6 or 7 bands.

The anti-SMP abolishes the SMP band in lane 2 (arrow) (compare controls 3 and 1) but not the cathepsin D bands in 5 (compare 6 and 4).

The fast moving pepsinogen and progastricsin bands are fainter than in Fig. 2.4 because the gel was developed in haemoglobin at pH 3.5 to show SMP and cathepsin D optimally. Pepsinogen and progastricsin are less active at this pH than at 2.0.


1
2
3
4
1
2
3
4


1
2
3
4
5
6

PURIFICATION OF CATHEPSIN D

The homogenate of spleen contained 16.85 mg of cathepsin D in 700 ml. Of 11.28 mg bound to Con-A Sepharose, 4.63 mg was eluted and of 4.52 mg bound to pepstatin agarose, 1.69 mg was eluted. The final recovery of cathepsin D was 743 µg.

SDS-PAGE showed the enzyme to be pure and composed of two subunits of molecular weights 28 and 14 Kd.

EXTRACTION OF ZYMOGEN FROM FETAL GASTRIC MUCOSAL HOMOGENATES

1. Electrophoresis of the 4 samples which contained most enzyme demonstrated that in 2 cases there was a band in the position of slow moving protease while in the other 2 there was no proteolytic band. The 2 former samples were used as antigens for immunisation of rabbits.

2. The total amount of zymogen in the four extracts of pooled fetal gastric mucosae was 2.6, 8.1, 5.2 and 79.8 mg respectively. The amount in the first 3 was considered too low for the intended further purification on DEAE cellulose to be worthwhile. Electrophoresis of the fourth pooled homogenate yielded lytic bands that corresponded to those present in the adult stomach control. As zymogens or enzymes other than SMP were present, it was considered that further attempts to purify SMP from the sample were not worthwhile.

CHARACTERISATION OF ANTISERA

1. Immune labelling by antisera

Antipepsin and antipepsinogen

Rabbit antisera to pig pepsin and to the purified pepsinogen gave strong labelling of the glands of human gastric body mucosa, the strongest of each group of rabbit sera being used subsequently. As the antiserum to pig pepsin gave stronger labelling, it was used in most subsequent experiments, rather than anti pepsinogen.

The antiserum to human pepsin from one rabbit gave stronger labelling of gastric mucosal glands than the others, although still weaker than the antisera to pig pepsin and pepsinogen.

Dilution experiments showed that all three antisera at 1:100 for 30 mins gave satisfactory labelling of sections. Trypsinisation of sections markedly reduced or abolished labelling and was not performed.

Antigastricsin

Antiserum to seminal fluid acid proteinase

All three sera labelled weakly the connective tissue of the gastric mucosa and prostate. The glands were, however, negative. It was concluded that the antibodies raised were against some connective tissue element which was present in the extract used for immunisation. These sera were not used for further experiments.

Antihuman gastricsin

Antiserum to human gastric gastricsin gave labelling of human gastric mucosa, the strongest antiserum being used in subsequent studies. One rabbit antiserum to human seminal gastricsin also labelled gastric mucosa satisfactorily.

Anticathepsin D

The rabbit whose serum gave strongest labelling of human spleen unfortunately died, and the second strongest antiserum was used subsequently.

AntiSMP

Antisera to fetal gastric zymogen

Antisera to fetal gastric zymogen all gave strong non-specific labelling of connective tissue and epithelium in all the tissues tested.

Normal stomach, colon and pancreas and carcinomas of these sites and of endometrium all showed strong labelling in the cytoplasm and cell membranes of normal and malignant epithelial cells. Pre-incubation of antisera with lyophilised umbilical cord serum reduced labelling of connective tissue but not that of cells. The antisera were considered not specific and were not further used.

AntiSMP

The antiSMP donated by Professor Samloff gave labelling of gastric body mucosa, the details of which will be described in Chapter 3.

2. Double Diffusion (Ouchterlony)

The results of double diffusion between the preparations of pepsin and pepsinogen and their antisera are shown in Fig 2.3. The antiserum to pig pepsin contains an additional antibody which, however, does not appear to crossreact with pepsin or pepsinogen.

The fetal gastric zymogen all yielded several precipiting lines with their corresponding antisera, which were, therefore, considered not to be specific.

3) Immune (Western) blotting

The results of Western blotting are shown in Fig 2.4. Antipepsin reacted with the fast moving pepsinogen fractions and antigastricsin with the zones corresponding to progastricsin and, apparently, with a zone in the SMP position. The anticathepsin D result was equivocal.

COMPARATIVE STUDIES OF CATHEPSIN D AND SMP

1. Kinetic studies

Kinetic parameters (k_{cat} and K_m) for the hydrolysis of

Lys-Pro-Ala-Glu-Phe-(NO_2)Phe-Arg-Leu and Pro-Thr-Glu-Phe-(NO_2)Phe-Arg-Leu were obtained for SMP and cathepsin D. Both enzymes gave comparable values for the turnover number (k_{cat}) of each substrate, but SMP exhibited considerably lower K_m values than did cathepsin D (Table 2.2).

An even greater distinction between the two enzymes was observed with the protein inhibitor from Ascaris lumbricoides. The activity of SMP was inhibited very strongly, whereas that of cathepsin D was not affected by concentrations of inhibitor as high as 0.6 M (Table 2.2).

2. Immunodot blotting

With this procedure (Fig. 2.5) as little as 5 µg/ml of cathepsin D was detected readily with the anti-cathepsin D antiserum. In contrast, as much as 60 µg/ml of SMP exhibited no reactivity. Reciprocally, no cross-reactivity was observed between anti-SMP and cathepsin D in amounts up to 60 µg/ml, while SMP was detectable with anti-SMP in amounts down to 30 µg/ml. Control incubations with normal rabbit serum gave negative results with both cathepsin D and SMP (not shown).

3. Electrophoretic studies

The results of zymography are shown in Fig. 2.6. The antiserum to SMP abolished the SMP band in normal gastric mucosal extract but did not affect the cathepsin D.

TABLE 2.2

KINETIC CONSTANTS FOR THE HYDROLYSIS OF TWO SYNTHETIC CHROMOGENIC SUBSTRATES BY SMP AND CATHEPSIN D AND FOR THEIR INHIBITION BY THE PROTEIN FROM ASCARIS LUMBRICOIDES

	Substrate I[a]		II[b]		Ascaris inhibitor[c]
	K_m	k_{cat}	K_m	k_{cat}	K_i
Enzyme	(μM)	(sec^{-1})	(μM)	(sec^{-1})	(nm)
SMP	160 $\pm$ 30	135 $\pm$ 10	145 $\pm$ 35	27 $\pm$ 3	5 $\pm$ 1
Cathepsin D	550 $\pm$ 75	175 $\pm$ 20	840 $\pm$ 300	60 $\pm$ 18	No inhibition at 600 nM

a - Lys-Pro-Ala-Glu-Phe-(NO_2)Phe-Arg-Leu.

b - Pro-Thr-Glu-Phe-(NO_2)Phe-Arg-Leu.

c - The Ascaris inhibitor was preincubated with each enzyme for 5 min at 37°C before adding substrate I to initiate the catalytic reaction. All reactions were in 0.1 M sodium formate buffer, pH 3.1 at 37°C.

DISCUSSION

The purification of pig pepsinogen and human cathepsin D appears to have been successful. Preliminary attempts to purify the seminal fluid zymogen were not satisfactory, judging by the results of immune labelling with antiserum to it, but a supply of pure enzyme and zymogen was gifted to us before further attempts were made. Fetal stomach was expected to be a rich source of SMP. However, it appeared that the stomachs in early gestation contained so little that purification was not worthwhile, while stomachs in late gestation contained other aspartic proteinases, as will be more fully discussed in Chapter 7. Fetal stomach was therefore not a practicable source of SMP. The anti SMP gifted to us was prepared by rabbit injection of SMP. This SMP was purified from adult gastric mucosa on an affinity column, on which was adsorbed monoclonal antibody to SMP (Samloff et al, 1987).

The results of Western blotting support the view that antipepsin and antigastricsin react with the expected components on the zymograms. Later immunolocalisation studies did not support the suggestion that antigastricsin was contaminated with anti SMP. Despite the additional antibody on double diffusion, anti pig pepsin was used in subsequent work, but the specificity was confirmed by absorption controls and by labelling with anti human pepsin on anti pig pepsinogen.

The studies of SMP and cathepsin D show conclusively that they are separate enzymes. This is supported by work which shows that their distribution in tissues is different (see Chapters 3, 6, 8 and 10). Since this work was completed, further investigations by others have shown that SMP has properties similar to those of cathepsin E (Tarasova, Sczecsi & Foltmann, 1986).

CHAPTER 3

THE DISTRIBUTION OF ASPARTIC PROTEINASES IN NORMAL HUMAN STOMACH AND DUODENUM

INTRODUCTION

Normal structure of gastric and duodenal mucosa

The morphology of the normal gastric and duodenal mucosa is well documented at light microscopic and ultrastructural level (Rubin et al, 1968).

The surface epithelium is similar to that lining the gastric pits. In the gastric body, the crypts contain, in order downwards, mucous neck, undifferentiated neck, parietal and chief cells. Some parietal cells are intermingled with chief cells deeper down the crypts. Occasional argyrophil cells are also present. It is thought that all these cell types evolve from the undifferentiated neck cells. In the antrum the glands are not as straight as in the body, but are coiled and lined by mucous cells, scattered among which are variable numbers of parietal cells (Tominaga, 1975). The glands of the cardia are similar to those in the antrum. The duodenal mucosa is similar to that in other parts of the small bowel, although Paneth cells are uncommon. The cells of the submucosal Brunner's glands are of similar morphology to those of the gastric, antral and cardiac glands.

Ultrastructurally, there are six cell types (Rubin et al, 1968);

1) chief cells, which contain characteristic 2 μ m diameter zymogen granules, most prominent apically,
2) parietal cells, which include intracellular canaliculi and abundant mitochondria,
3) mucous neck cells, which contain mucous granules 0.2-2 μm in diameter,
4) pyloric gland cells, which resemble the mucous neck cells but include, in addition, granules which resemble the zymogen granules of the chief cells,
5) surface and pit cells, with prominent microvilli and
6) undifferentiated neck cells, in which organelles are less

prominent than in the other cell types.

The Brunner's glands are ultrastructurally similar to the cells of the antral glands (Leeson & Leeson, 1968).

Distribution of aspartic proteinases

Pepsinogen

The main zymogen of the human stomach, pepsinogen, has long been known to be a product of the chief cells (Heidenhain, 1870), in which it has been demonstrated biochemically (Holter & Linderstrøm-Lang, 1934) and by immunofluorescence (Samloff, 1971b). Its presence in the mucous neck cells was first demonstrated by immunofluorescence in the pig (Yasuda, Suzuki & Takano, 1966) and then in man (Samloff, 1971b; Hirsch-Marie et al, 1976). It was found in the apical region of the cytoplasm of the mucous neck cells in granules which have non-fluorescent centres. Pepsinogen has also been demonstrated in granules of both chief and mucous neck cells by an indirect immunoperoxidase method (Wurster, Kuhlmann & Rapp, 1978). There has been no evidence of pepsinogen in the other cell types of the gastric body and it has not been demonstrated in the gastric antrum or in the duodenum. The sub-cellular location of pepsinogen has not been demonstrated in humans and while it appears likely that pepsinogen is synthesised in the chief cells, it is not clear whether its presence in mucous neck cells reflects synthesis or uptake from the lumen of the glands.

Progastricsin

Progastricsin has been shown by immunofluorescence to co-localise with pepsinogen in the cells of the gastric body, in cytoplasmic granules with non-fluorescent centres (Samloff & Liebman, 1973).

Several electrophoretic studies (Seijffers, Segal & Miller, 1963a; Seijffers, Segal & Miller, 1963b; Kushner, Rapp & Burtin, 1964; Hanley, Boyer & Naughton, 1966; Hirsch-Marie, 1968; Cheret & Bonfils, 1968; Samloff, 1969) have demonstrated acid proteinase activity in extracts of

antral and duodenal mucosa. This appears to be due not to pepsin but to gastricsin, the zymogen of which has been localised by immunofluorescence in the cells of the deep antral glands, duodenal Brunner's glands and ducts which open into the crypts of Lieberkühn (Samloff & Liebman, 1973). The surface epithelial cells appear not to contain progastricsin. Progastricsin is probably located in the homogeneous granules demonstrable by electron microscopy in the antral and Brunner's glands, although this has not been proven by immunolocalisation studies. Hirsch-Marie et al (1976) found progastricsin and pepsinogen in only occasional fundic-type glands in the antrum and not in the typical antral deep glands. Their work is, however, open to criticism because of the somewhat crude methods they used to purify the zymogens to which they raised antisera. The localisation of progastricsin in the gastric antral glands has been confirmed by the immunoperoxidase method (Wurster, Kuhlmann & Rapp, 1978), although the findings were only briefly described. Progastricsin has also been demonstrated in the cells of the cardiac glands (Weinstein et al, 1977).

Thus, progastricsin appears to be more widely distributed than pepsinogen, according to the published results with immunofluorescence techniques. It appears not to have been localised at subcellular level.

Cathepsin D

Early literature on gastric juice cathepsin is confused by the fact that some early workers were clearly discussing the enzyme now termed gastricsin (Willstätter & Bamann, 1929; Freudenberg, 1940; Buchs, 1953; Buchs, 1954; Taylor, 1959). Recent evidence suggests that cathepsin D does indeed occur in the gastric juice (Barrett, 1977b) and mucosa (Pohl, Bureš & Slavík, 1981). Cathepsin D is commonly supposed to be a lysosomal enzyme and it would be of interest to know whether its

presence in gastric juice reflects passive release from cell breakdown or active secretion, possibly in the form of a zymogen (Puizdar & Turk, 1981). Although limited work on the localisation of cathepsin D in other sites has been undertaken in human (Matthews, Decker & Knight, 1981; Whitaker, Bertorini & Mendell, 1983) and animal (Poole, Dingle & Barrett, 1972; Bird, Schwartz & Spaniar, 1977) tissues, there appears to be no information on the sites in which cathepsin D occurs in the gastric mucosa.

Slow moving protease (SMP)

Slow moving protease (SMP) has been located by immunofluorescence (Hirsch-Marie et al, 1976) by which there was weak reactivity in the gastric body in the surface epithelium and strong reactivity in the antrum, in surface epithelium and superficial glands. The fluorescence was often perinuclear but sometimes also present in the apical region of the cells. As noted above, the purity of the SMP and, therefore, of the antiserum used in this study is open to doubt. There appears to be no other published work on the localisation of SMP and its function, too, is unknown.

Aim of present study

The work to be described in the present chapter was initiated in order to localise the above four enzymes or zymogens in formalin-fixed paraffin embedded sections of stomach and duodenum by the peroxidase-antiperoxidase (PAP) method (Sternberger, 1970; Burns, 1978).
The aim was to compare the distribution of pepsinogen and progastricsin with that obtained previously by immunofluorescence and to localise for the first time cathepsin D and SMP. In addition, immunolocalisation studies were undertaken at ultrastructural level in an attempt to identify the subcellular sites in which the enzymes or zymogens occur. Their presence on endoplasmic reticulum or in vesicles would indicate synthesis or storage respectively and it would be of interest to

determine whether the parietal cells play a role in zymogen or enzyme production. Ultrastructural identification of cathepsin D in secretory vacuoles would suggest a role as an extracellular enzyme, whereas its presence in lysosomes would indicate that it is implicated in intracellular proteolysis. The role of SMP might likewise be elucidated by determination of its cellular and subcellular location.

MATERIALS AND METHODS

Antisera

The antisera and dilutions used are shown in Table 3.1, the optimal for each being determined by titration. The secondary antibodies, swine anti-rabbit immunoglobulin (SAR) and peroxidase-antiperoxidase (PAP) were purchased from Dakopatts A/S, Denmark.

Immune-labelling procedure (Peroxidase-antiperoxidase (PAP) method)

Sections were deparaffinised and endogenous peroxidase blocked by incubation in methanol containing fresh 0.5% hydrogen peroxide and 0.2% hydrochloric acid for 30 minutes. Sections were then washed in tap water. Subsequently, all dilutions were in 0.05 M Tris-HCl buffer, pH 7.6 (TBS) and all washings were in 10% TBS. Sections were washed in 10% TBS and background staining was reduced by incubation with 20% normal human serum. Sections were then overlain with rabbit antiserum, appropriately diluted. Excess antiserum was washed off and sections overlain with 1:40 swine anti-rabbit immunoglobulin (SAR) for 30 minutes. This was washed off and the sections overlain with 1:100 rabbit peroxidase-antiperoxidase (PAP) complex for 15 minutes. The dye reaction was developed with 0.03% 3,3' diamino-benzidine tetrahydrochloride solution containing 0.05% hydrogen peroxide for 10 minutes. Sections were counterstained with haematoxylin or methyl green.

Slide rack

In the course of the work, large numbers of sections had to be labelled with antisera. The tedium this entailed stimulated a search for a way of simplifying the procedure. This led to the design of a slide rack which was subsequently used in most of the immune labelling work described in this thesis (Reid et al, 1985) (Fig. 3.1).

TABLE 3.1

ANTISERA AND DILUTIONS USED IN IMMUNOPEROXIDASE (PAP) METHOD

Antiserum to	Dilution
Pig pepsin	1 : 200
Pig pepsinogen	1 : 100
Human pepsin	1 : 100
Human gastricsin	1 : 100 (in 20% normal human serum)
Human progastricsin	1 : 100
Human seminal gastricsin	1 : 100
Cathepsin D	1 : 100
SMP	1 : 100
Lysozyme	1 : 1000
SAR	1 : 40 (in 20% normal human serum)
PAP	1 : 100

Electron microscopic immunolocalisation

Sections were overlaid with neat normal swine serum (NSS) for 30 mins and incubated in antiserum to pig pepsin, gastricsin or cathepsin D at 1:20, for either 1 hr at room temperature for the frozen sections or 24 hrs at $4^{o}C$ for the resin embedded sections. Sections were then washed and incubated for 2 hrs in goat anti rabbit immunoglobulin (G20) at 1:20 in 20mM TBS with 1% bovine serum albumin and azide, pH 8.3. After further washes in TBS and distilled water, sections were counterstained with aqueous uranyl acetate/lead citrate and coated with 3% methyl cellulose. All antisera were microcentrifuged and the Tris was filtered with millipore.

Tissues

Preliminary experiments with the PAP technique on paraffin embedded sections of gastric mucosa showed that labelling of pepsinogen, progastricsin and cathepsin D was strongest with Bouin's fixative and only slightly less so with 10% formol saline. SMP was most strongly labelled in tissue fixed in 10% formol saline, while Bouin's fixative gave weaker results. Other fixatives proved generally less satisfactory and for this reason Bouin's fixative or 10% formol saline were used.

Gastrectomy specimens, removed because of peptic ulceration or malignancy, were obtained fresh from the operating theatres of Glasgow Royal Infirmary or the General Infirmary at Leeds. The stomachs were opened along the greater curvature and the mucosal surface was washed with normal phosphate buffered saline, pH 7.6. Full thickness slices approximately 10 x 1 mm of stomach or duodenum were fixed in Bouin's fixative or 10% formol saline. The tissues were paraffin embedded and 6 μm sections cut.

A section, stained with haematoxylin and eosin, of each tissue sample was examined to check for its suitability for immune labelling. Samples which included malignant tumour or inflammation were excluded.

For the studies on cathepsin D and SMP and those on normal duodenum fixed tissue from the files was used.

For electron microscopic studies, small pieces of mucosa were removed from fresh gastrectomy specimens and fixed immediately in 3% glutaraldehyde and after 3 hrs were transferred to 10% formol saline.

For localisation of pepsinogen and progastricsin in parietal cells, strips of gastric wall, obtained as above from fresh gastrectomy specimens, were snap frozen in solid carbon dioxide/alcohol. Serial frozen sections were cut, fixed in Bouin's fixative and labelled with anti-human pepsin, the alternate sections being stained H&E to facilitate identification of the parietal cells.

Absorption controls

Anti-pig pepsin

Anti-pig pepsin was incubated with serial dilutions of pig pepsin from 1 mg/ml to 0.5 µg/ml and used in the PAP method to label gastric mucosa. As controls, anti-pig pepsin was also incubated with bovine trypsin, ribonuclease and diastase, all at 1 mg/ml. Similar experiments were performed by pre-incubation of anti-pig pepsin with serial dilutions of purified pig pepsinogen at 1:2 - 1:10 (600-120 µg/ml). In parallel experiments, pepsinogen was activated by addition of a calculated volume of HCl, so that the pH would fall to 2.0, thus activating the zymogen to pepsin. After 15 min the acid was neutralised by addition of sufficient NaOH to raise the pH to 7.3, at which pepsin has no enzymic activity. The dilutions with Tris buffer were such that identical amounts of pure pepsinogen and pepsin were used for incubation with the anti-pepsin. Serial dilutions of pepsinogen and pepsin were used at a range of concentrations between 1:5 and 1:100 (240 and 12 µg/ml). After incubation for 15 mins at room temperature, the solutions were used to label normal gastric mucosa by the PAP method.

An attempt was made to block immunoreactivity by pre-incubating

anti-pig pepsin (1:200) with pig intrinsic factor (Armour Pharmaceutical Co.) 2 mg/ml.

In addition, sections of gastric mucosa were labelled with anti-pepsin, pre-incubated with serial solutions of pepsin from 1 mg/ml - 0.5 µg/ml in order to determine if labelling of chief and parietal cells was blocked at different dilutions.

Anti-pig pepsinogen

Blocking experiments similar to those with anti-pig pepsin were performed.

Anti-human pepsin

Because pure human pepsin was scarce, only limited blocking experiments were performed with anti-human pepsin (1:200) and human pepsin (0.5 mg/ml).

Anti-gastricsin

Blocking of anti-gastricsin 1:100 was performed with gastricsin (100 µg/ml) and the various pepsinogen and pepsin preparations. Experiments similar to those with zymogen and enzyme were performed with seminal fluid progastricsin and the gastricsin prepared from it by acidification to pH 3 followed by neutralisation to pH 7.

Anti-cathepsin D

The cathepsin D gifted by Dr. Barrett was used for blocking experiments, as even less of the cathepsin D purified by us was available. The cathepsin D was used at 1 µg/ml - 0.1 µg/ml and the antiserum to cathepsin D was diluted at 1:200. Sections of normal spleen were used as positive controls.

Anti-SMP

Blocking experiments could not be performed as insufficient pure SMP was available to us for this purpose.

RESULTS

GASTRIC BODY

Pepsinogen

The pattern of labelling in the gastric body was similar with the antisera to pig pepsin, pig pepsinogen and human pepsin, although anti-pepsinogen gave weaker results than anti-pepsin, both in tissue fixed in Bouin's fixative and in that fixed in formol saline. In all 10 cases there was strong labelling of all the chief cells in the gastric crypts (Fig. 3.2). Reaction product appeared granular and was present throughout the cytoplasm of the cells and on high power examination was noted to be in vesicles with clear centres (Fig. 3.4). Marked granular reactivity for pepsinogen was also present in the cytoplasm of the mucous neck cells, although the intensity and distribution was less constant than in the chief cells. In some cases the surface epithelial cells appeared to contain pepsinogen. In most cases the majority of the parietal cells were negative. However, some specimens included a few parietal cells in which there was definite reactivity. Usually this was fainter than in the chief cells but in some parietal cells it was marked. Although some positive parietal cells were adjacent to strongly labelled chief or mucous neck cells, in some cases these parietal cells were negative while isolated parietal cells higher up the crypt were positive. In four cases the parietal cells were strongly labelled, even in the absence of adjacent positive chief cells in the sections. The pattern of labelling in the cytoplasm of the parietal cells was not granular, but was either diffuse or in the shape of tubular structures (Fig. 3.5), the latter being thought most likely to be the intracellular canaliculi. Frozen sections of fresh stomach obtained at gastrectomy showed definite labelling of some parietal cells for pepsinogen in 3 of 4 cases studied.

Progastricsin

Labelling for progastricsin appeared in all 10 cases to be similar in intensity and distribution to that for pepsinogen (Fig. 3.2). The parietal cells were similarly labelled.

Cathepsin D

Cathepsin D was strongly labelled in nearly all the parietal cells in the 26 cases studied (Fig. 3.6a and b). In most specimens the chief cells were negative but in occasional cases there was diffuse positivity which was weak compared with that in the parietal cells. In the mucous neck cells there was a granular reaction, most marked on the luminal side of the nucleus, but often deep to the mucus globule (Fig. 3.7). A few mononuclear cells, probably macrophages, in the lamina propria were also strongly positive.

Slow moving protease

In all 7 cases there was weak or moderate cytoplasmic labelling of the surface epithelial cells (Fig. 3.8). There was weak reactivity with mucous neck cells in 1 case and some parietal cells in 2 cases, but in all specimens the chief cells were negative. In all cases there was strong labelling of red blood cell membranes.

GASTRIC ANTRUM

Pepsinogen

The coiled deep antral glands of 10 cases studied were mainly pepsinogen negative, although occasional cells in some glands labelled strongly. Similarly, although occasional mucous neck cells contained pepsinogen, most were negative.

Progastricsin

The cells of the deep antral glands in 10 cases labelled strongly for progastricsin (Fig. 3.9). The apparent granular distribution was in part due to coexistence in the cytoplasm of droplets of mucin. Progastricsin appeared also to be present in the mucous neck cells.

Cathepsin D

In the 14 cases studied, the epithelial cells of the deep antral glands showed strong granular reactivity for cathepsin D (Fig. 3.10). Labelling of mucous neck, parietal and mononuclear cells was similar in intensity and distribution to that in the body of the stomach.

Slow moving protease

The antral glands were weakly positive in 4 specimens out of 11 In 2 specimens there was faint labelling of some parietal cells and in 2 cases the surface epithelial cells were weakly positive.

DUODENUM

Pepsinogen

In 3 fresh cases and 7 from the files, pepsinogen was absent from most sections of duodenum. However, occasional cells in the mucosal crypts and in the submucosal Brunner's glands were strongly positive (Fig. 3.11).

Progastricsin

Three fresh cases and 7 from the files were examined. There was a strong reaction for progastricsin in occasional cells in the mucosal crypts and in most cells of Brunner's glands (3.12).

Cathepsin D

In the duodenum, cathepsin D was predominantly localised to the cytoplasm of mononuclear cells, probably macrophages, of the lamina propria in the 10 cases examined (3.13).

Slow moving protease

In 8 cases studied, the surface epithelial cells were in 7 specimens weakly positive. The mucosal crypts were weakly reactive in 1 case only, and the Brunner's glands were in all cases negative.

ABSORPTION CONTROLS

Antipepsin

In all cases labelling of gastric mucosa was abolished by prior incubation of anti pig-pepsin 1:200 with pig pepsin at 40 µg/ml. When the concentration of pepsin was lowered to 4 µg/ml the chief cells became positive, but the parietal cells remained unlabelled until the pepsin was diluted to 1 µg/ml. Intrinsic factor, trypsin, ribonuclease and diastase all failed to block labelling by antipepsin. Labelling was only slightly weakened by incubation of anti pig pepsin with pure pepsinogen at 600 µg/ml. By contrast, pure pepsin was effective in blocking immunoreactivity even when diluted to 24 µg/ml. Labelling by anti human pepsin was only partially blocked by prior incubation with human pepsin at 100 µg/ml.

Antigastricsin

Immune labelling by antigastricsin 1:100 was completely blocked by prior incubation with gastricsin at concentrations of 5 µg/ml or more but not by any of the preparations of pepsinogen or pepsin. Antigastricsin was also blocked by seminal gastricsin or progastricsin at 10 µg/ml. However, progastricsin at 1 µg/ml failed to reduce the intensity of labelling, whereas gastricsin at the same concentration completely blocked reactivity.

Anticathepsin D

Cathepsin D at 1 mg/ml blocked almost all immune reactivity of anti cathepsin D with spleen, whereas 100 µg/ml reduced labelling only slightly.

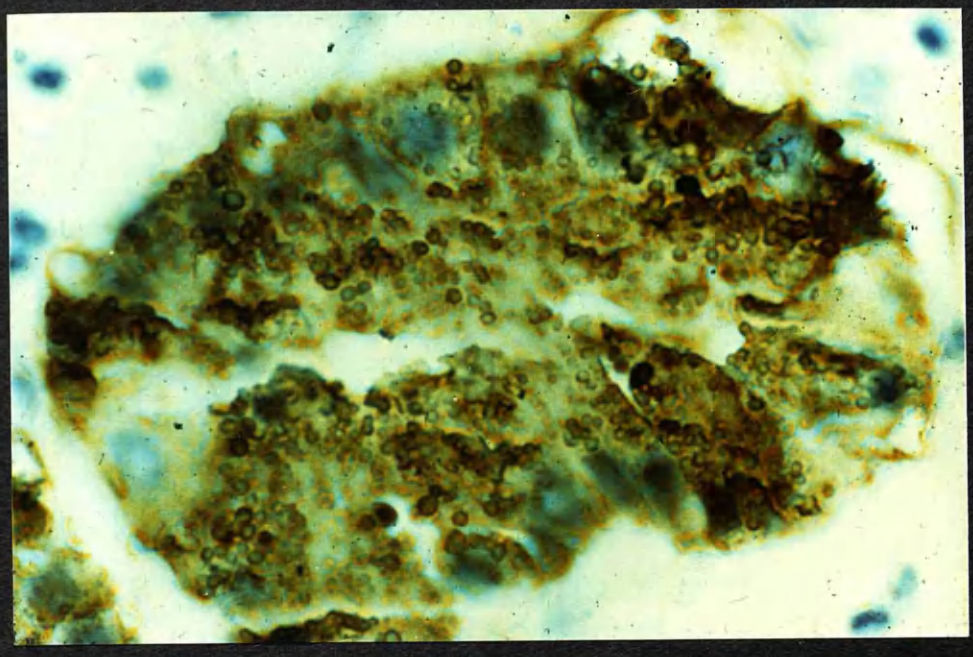
ELECTRON MICROSCOPIC IMMUNOLOCALISATION

The chief cells of the gastric body were shown to contain pepsinogen and progastricsin within the secretory vacuoles and rough endoplasmic reticulum, but the zymogens could not be convincingly

demonstrated in the parietal cells (Figs 3.14, 3.15 and 3.16). Cathepsin D was localised to the parietal cells and appeared to be in intracellular organelles, possibly lysosomes (Fig 3.17).

FIG. 3.4 Pepsinogen in vesicles of chief cells in normal human gastric mucosa. Each vesicle has a clear centre and a labelled rim.
Antipig pepsin, PAP method. x1600.

FIG. 3.5 Pepsinogen in parietal cell, apparently within a tubular structure (T), thought to be the intracellular canaliculus. Most of the pepsinogen is in chief cells, but at least one other parietal cell is labelled (P), while another (N) is negative.
Antipig pepsin, PAP method. x1600.



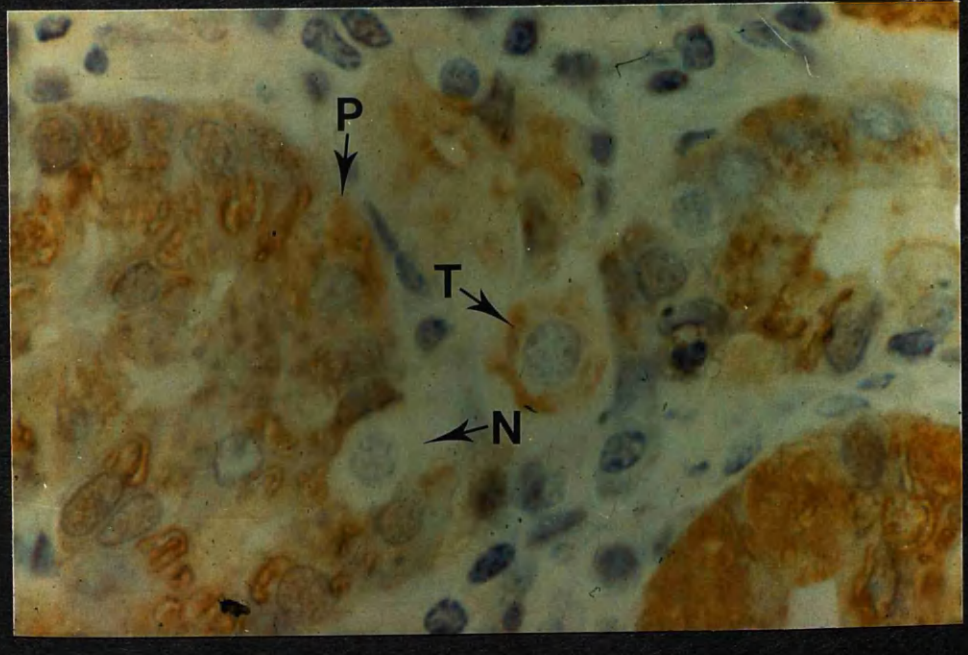
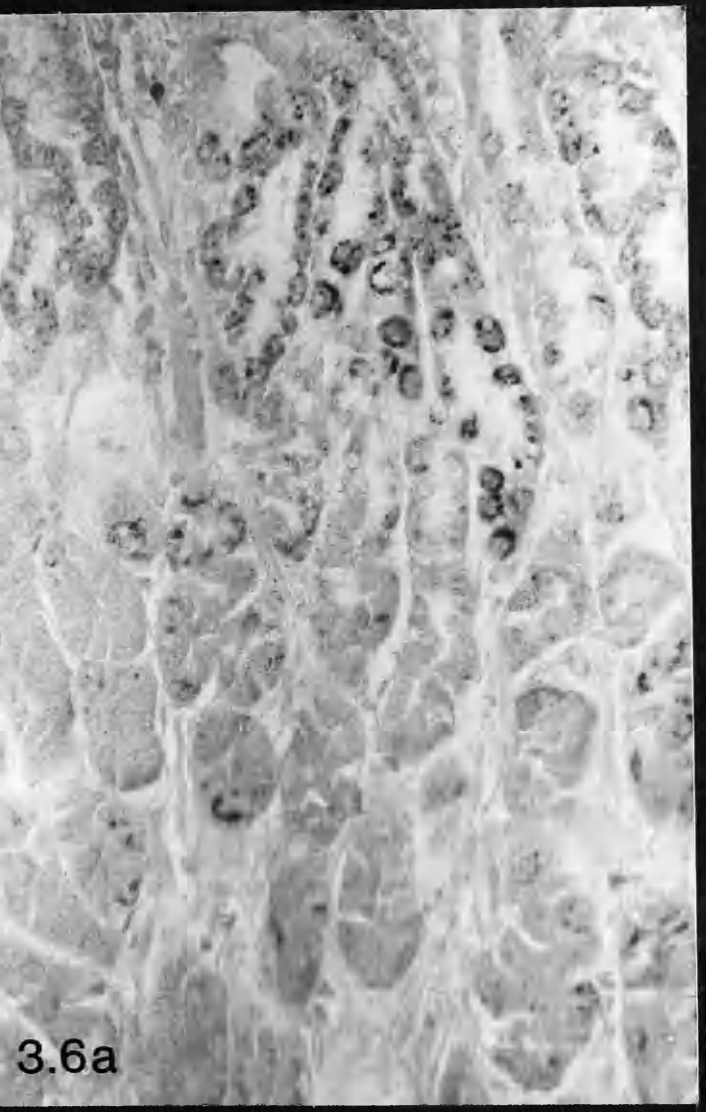

P
T
N

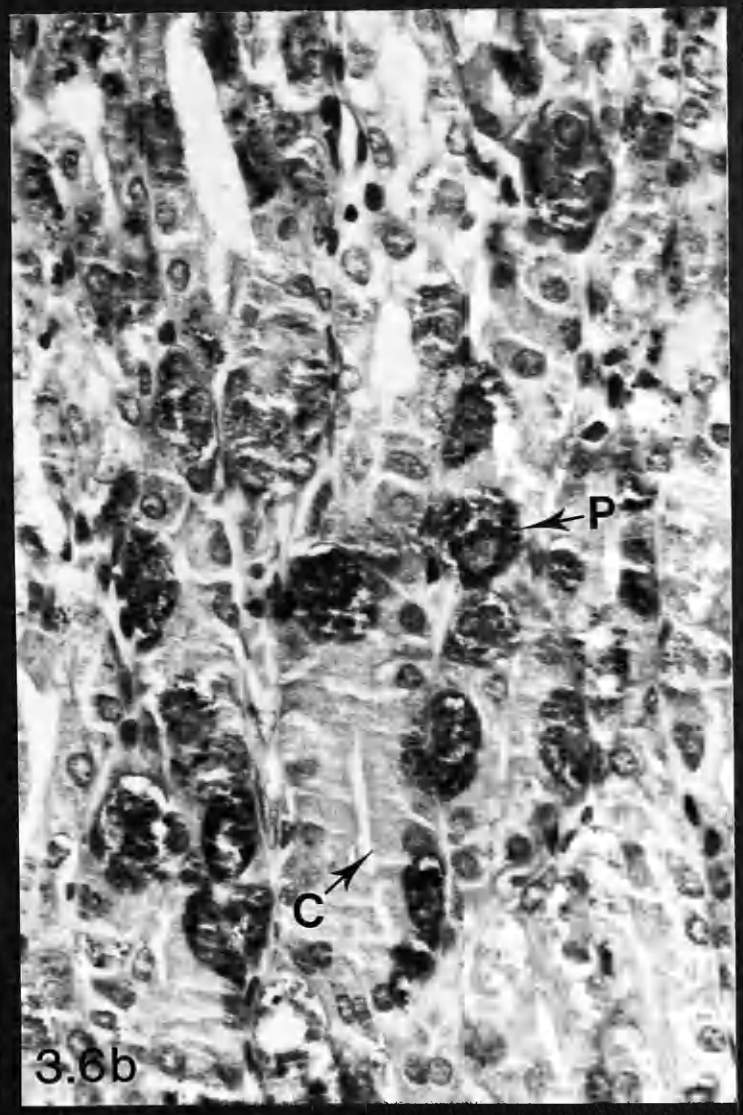
FIG. 3.6 a) Cathepsin D in normal gastric body mucosa. There is granular labelling of parietal cells but the chief cells are negative.
Anticathepsin D, PAP method. x180.

b) Cathepsin D in normal gastric body mucosa. The cytoplasm of the parietal cells (P) is strongly positive but the chief cells (C) are negative.
Anticathepsin D, PAP method. x520.

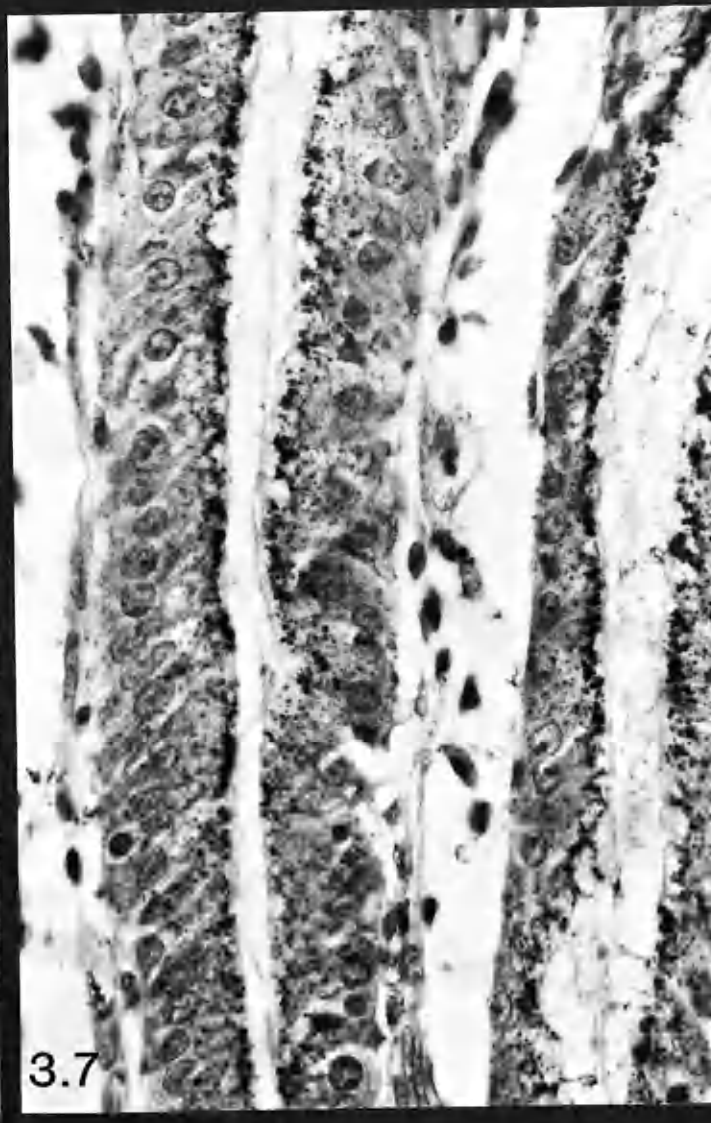
FIG. 3.7 Cathepsin D in mucous neck cells of gastric body mucosa. Labelling is cytoplasmic and granular and predominantly supranuclear.
Anticathepsin D, PAP method. x520.



3.6a



3.6b

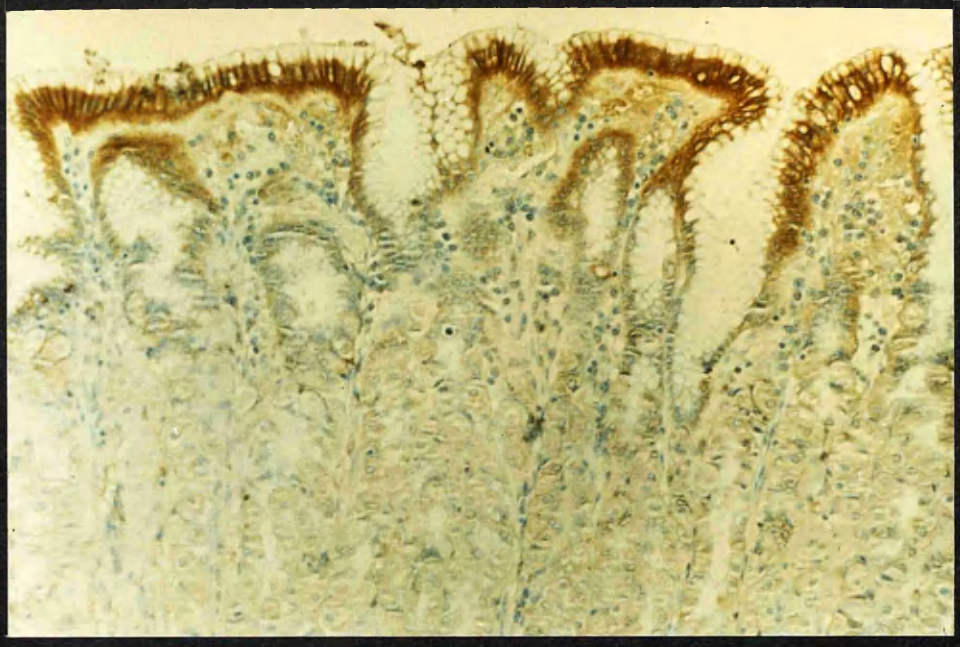


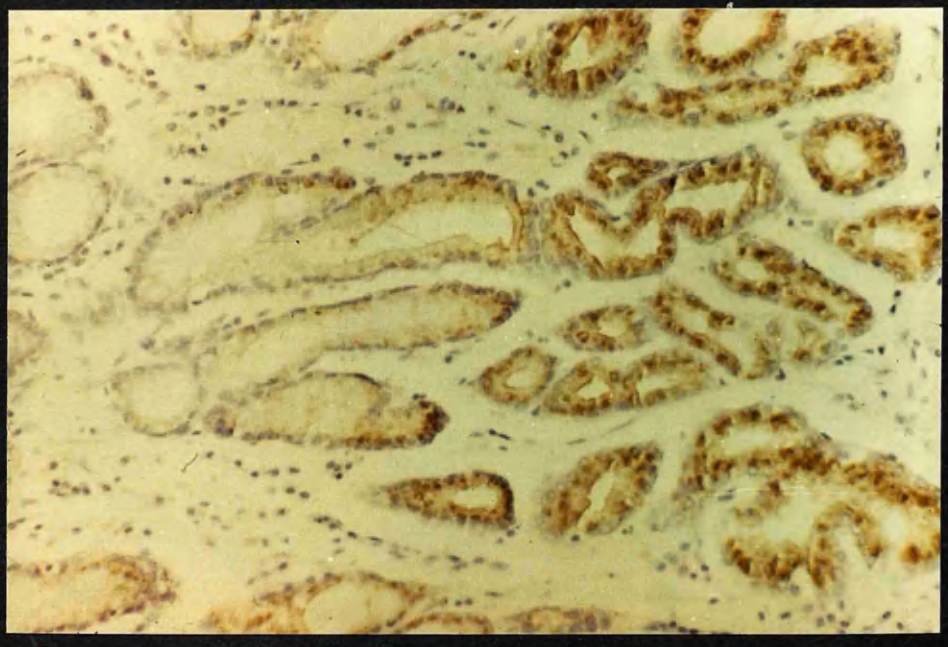
3.7

FIG. 3.8 SMP in the normal gastric body. It is localised in the surface epithelium and in the epithelium of the gastric pits. The mucus globule towards the lumen is, however, negative.
Anti SMP, PAP method. x180.

FIG. 3.9 Progastricsin in the gastric antral glands The upper part of the glands (to the left of the picture) is negative.
Antigastricsin, PAP method. x180.

FIG. 3.10 Cathepsin D in the deep glands of the gastric antrum. Note the granularity of labelling.
Anticathepsin D, PAP method. x520.





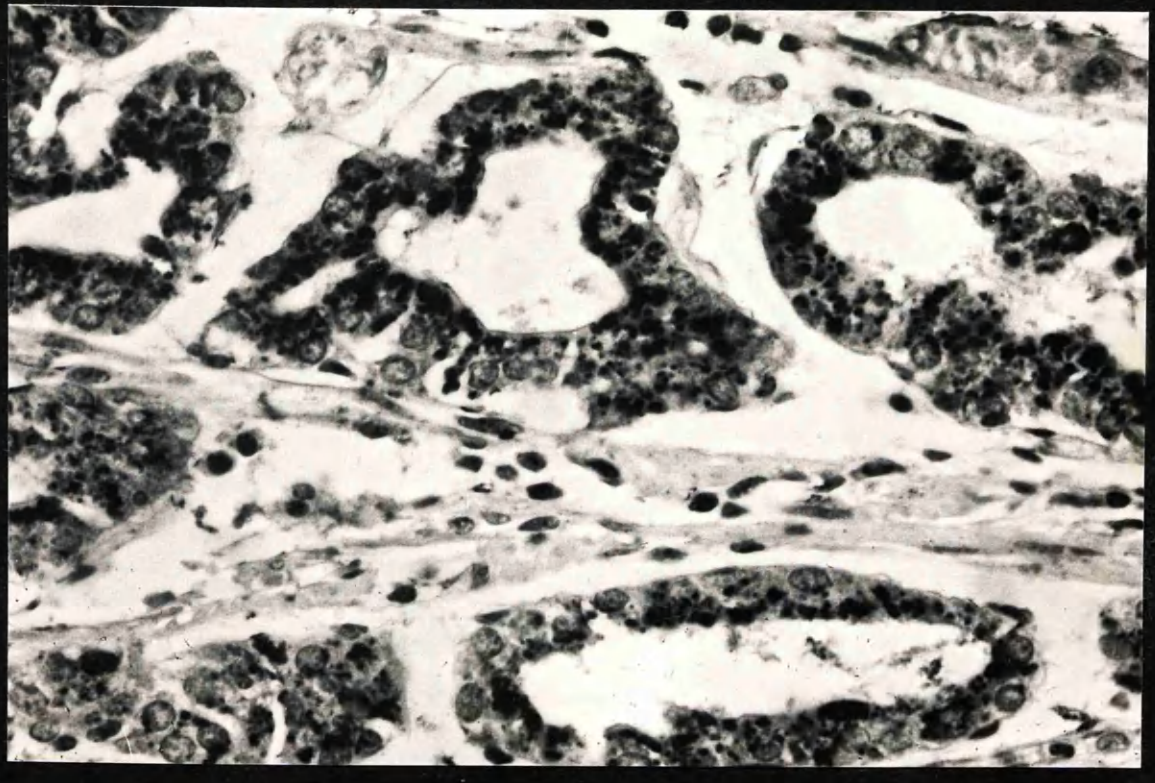
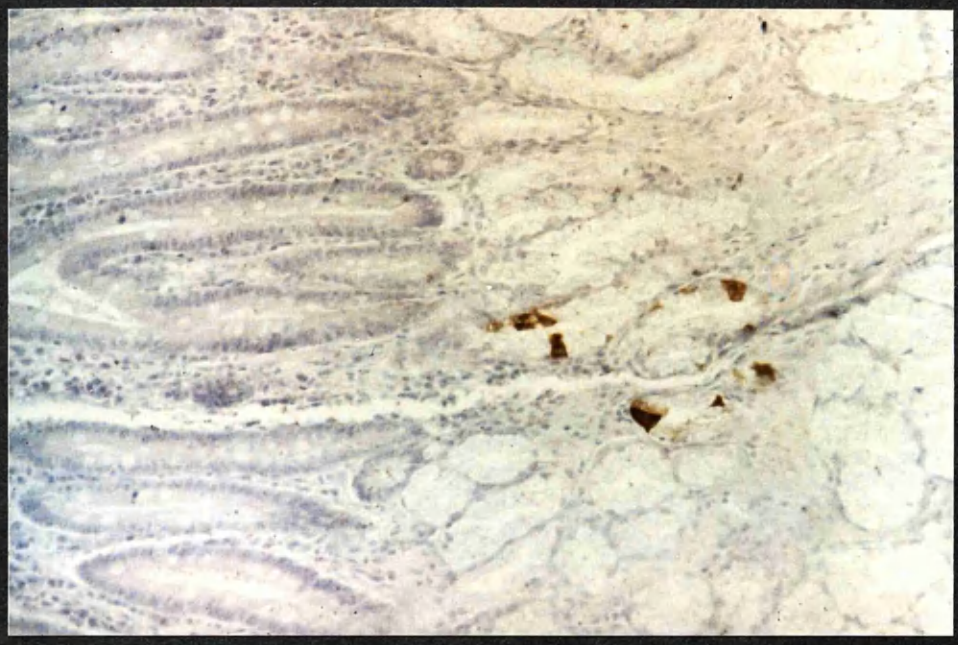
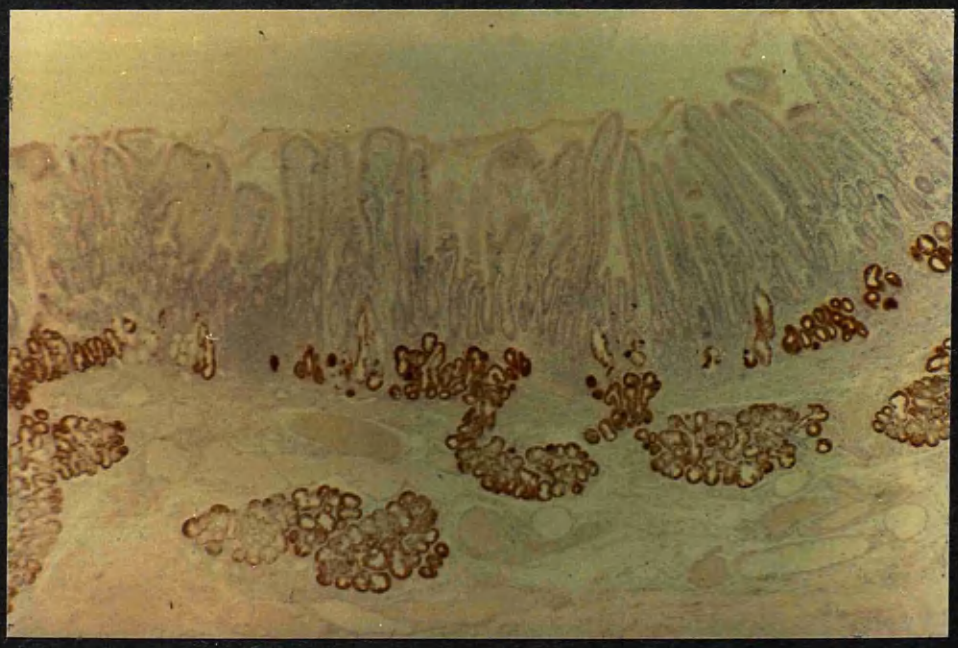


FIG. 3.11 Pepsinogen in occasional cells of duodenal Brunner's glands. (Luminal surface to left.)
Antihuman pepsin, PAP method. x188.

FIG. 3.12 Progastricsin in duodenum, mainly in Brunner's glands but also in mucosal glands.
Antigastricsin, PAP method. x70.

FIG. 3.13 Cathepsin D in mononuclear cells of the lamina propria, duodenum.
PAP method. x288.





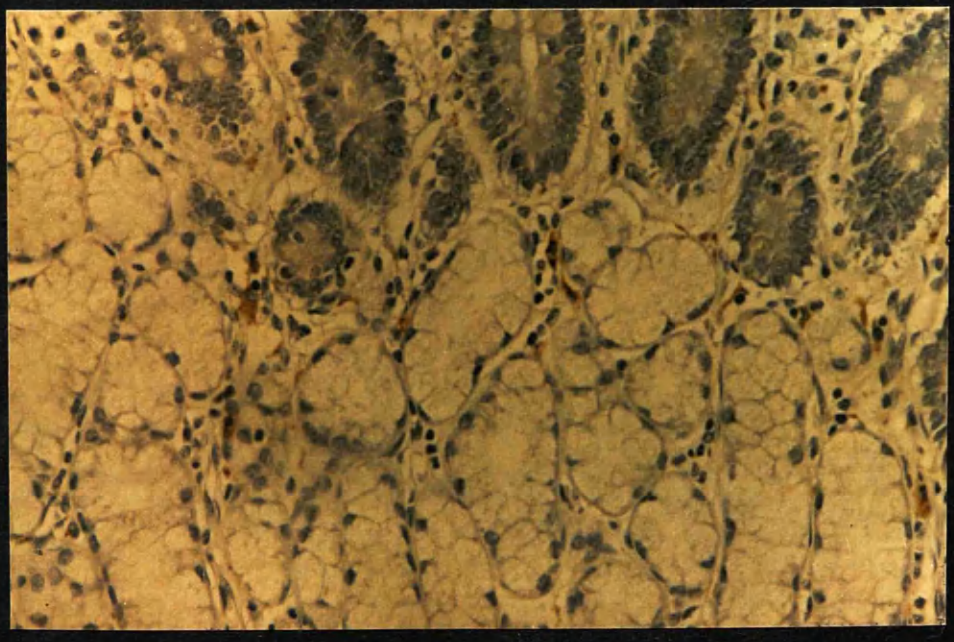
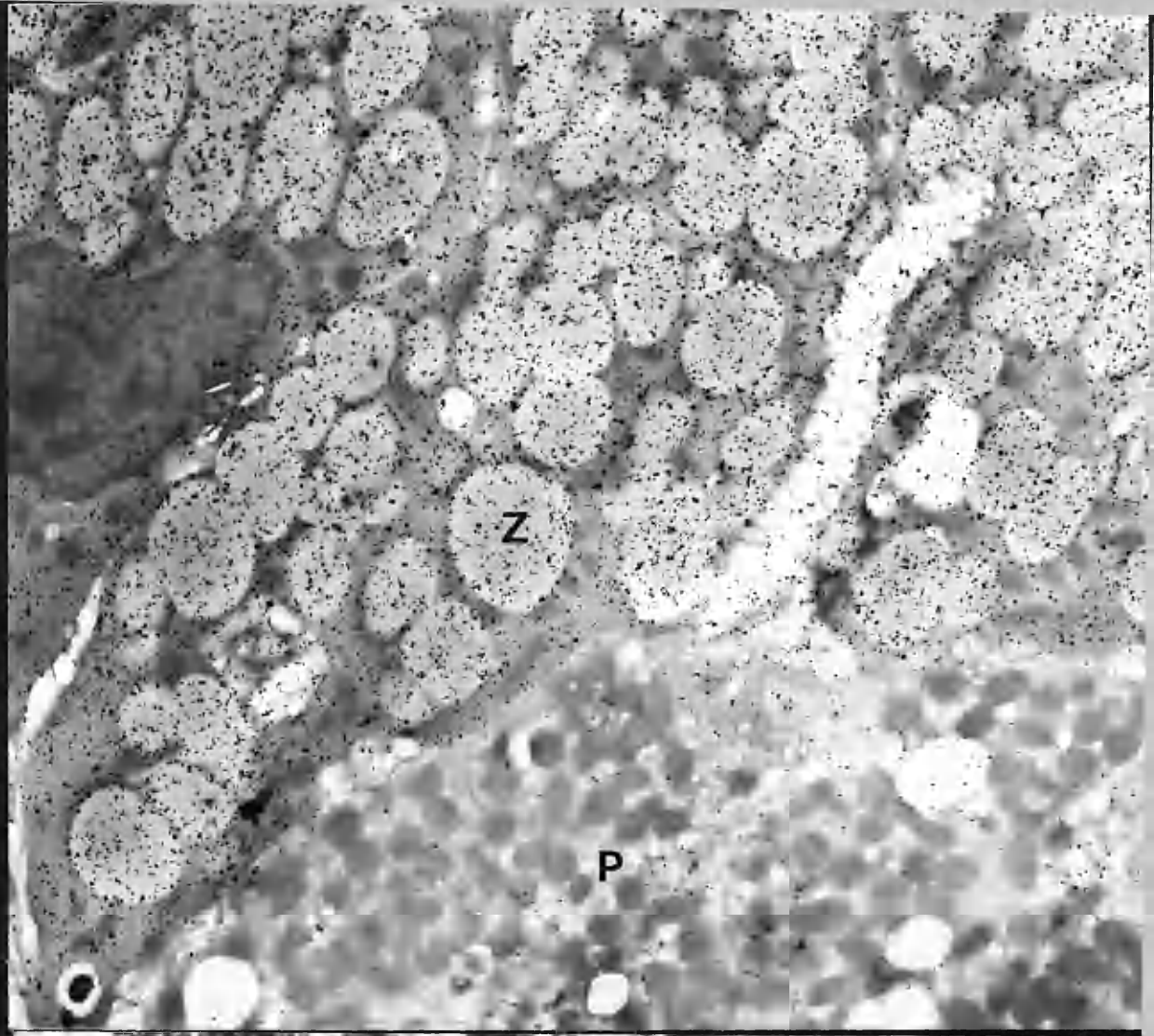


FIG. 3.14 Pepsinogen in zymogen vacuoles (Z) of gastric mucosal chief cell. The gold reaction product is seen as black granules. Note that some granules are present over the parietal cell (P), possibly in this case due to diffusion of zymogen.
Indirect immunogold method, ultracryostat frozen section, labelled with antipig pepsin, electron micrograph x3000.

FIG. 3.15 Progastricsin gastric chief cells. The gold reaction product is seen as black granules in the secretory vacuoles (Z) and rough endoplasmic reticulum (R) but not in the nucleus (N) or in the adjacent parietal cell (P).
Embedded in LR white, labelled with antigastricsin, indirect immunogold method, electron micrograph x7500.


Z
P

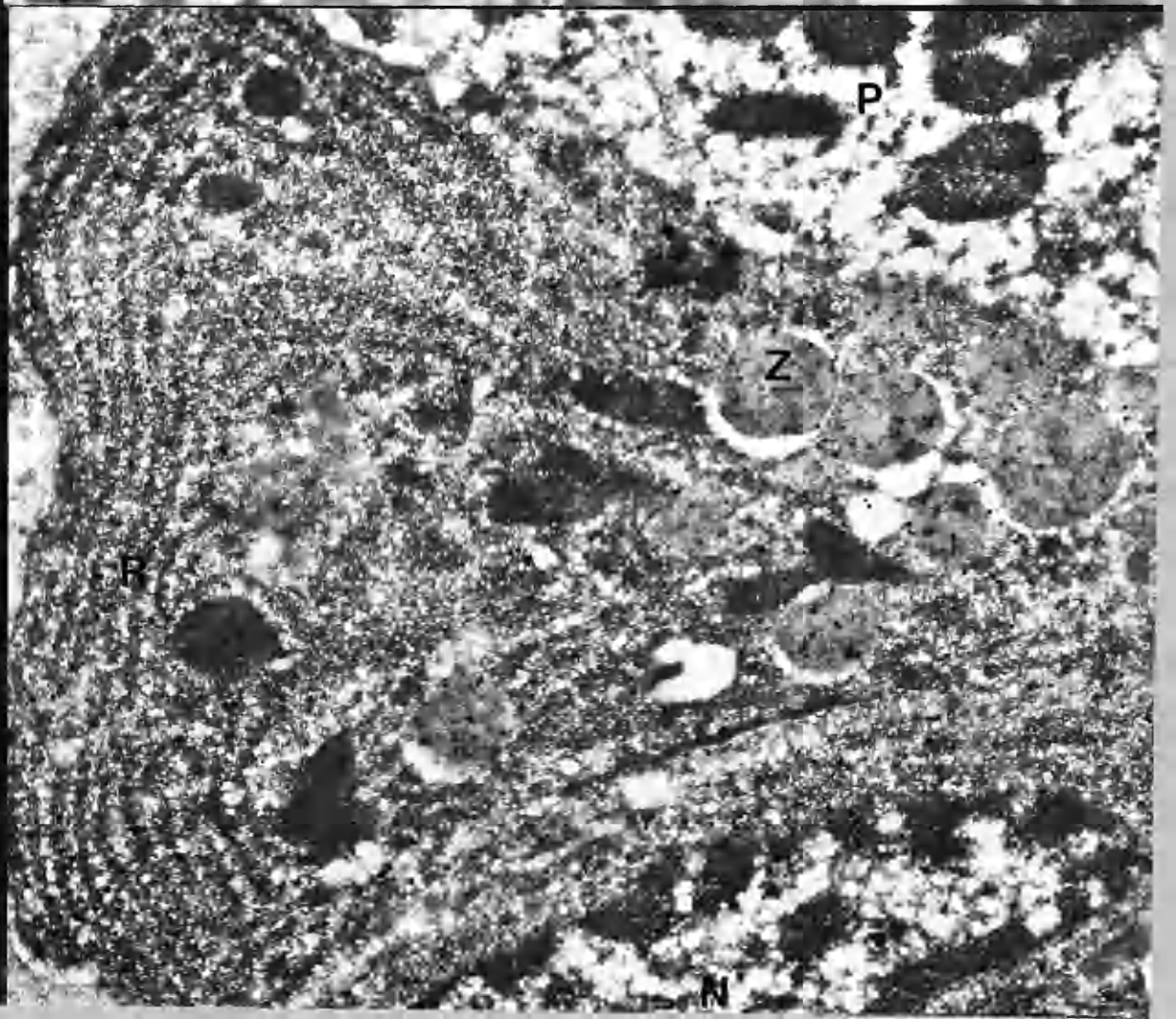
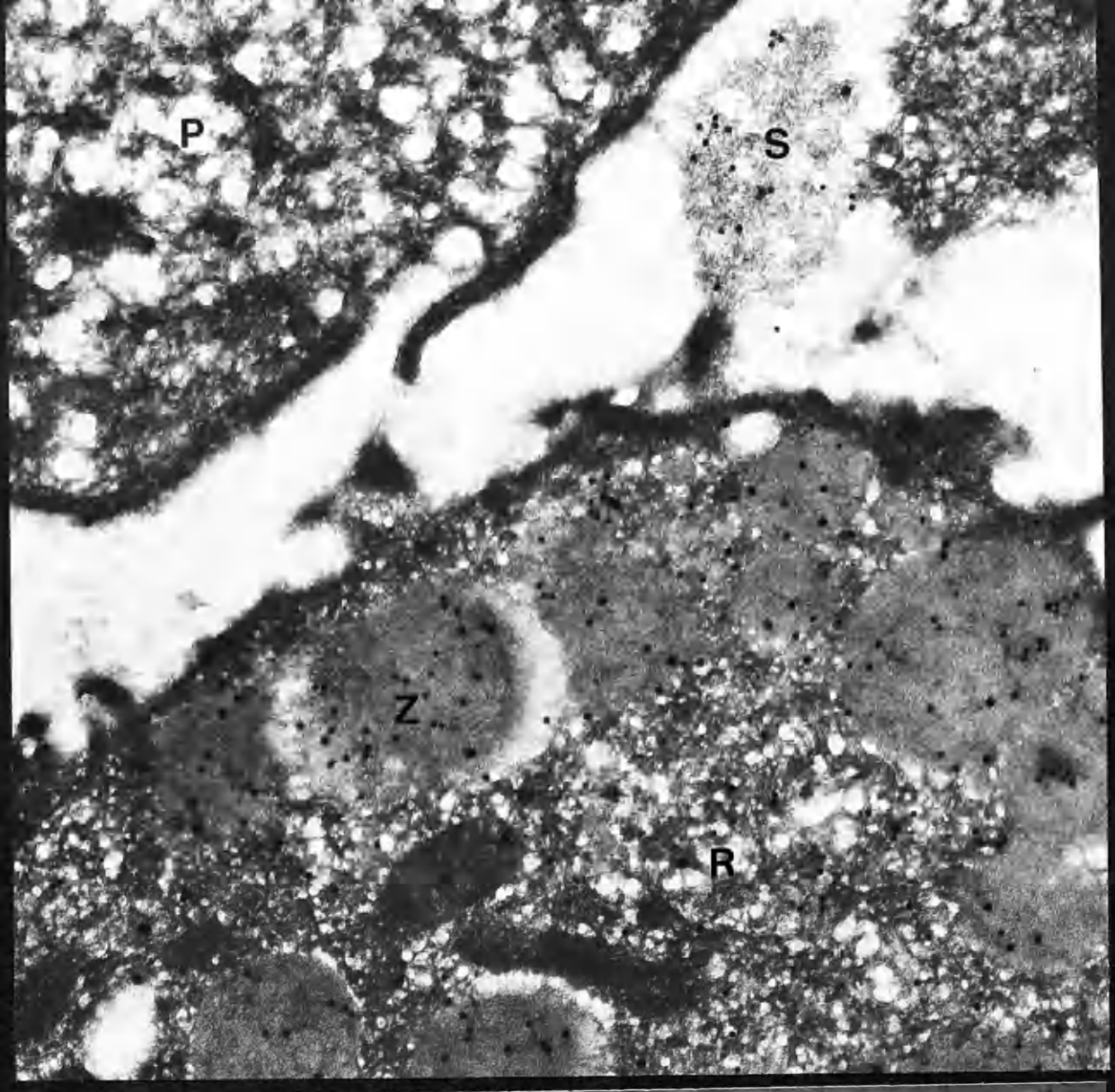
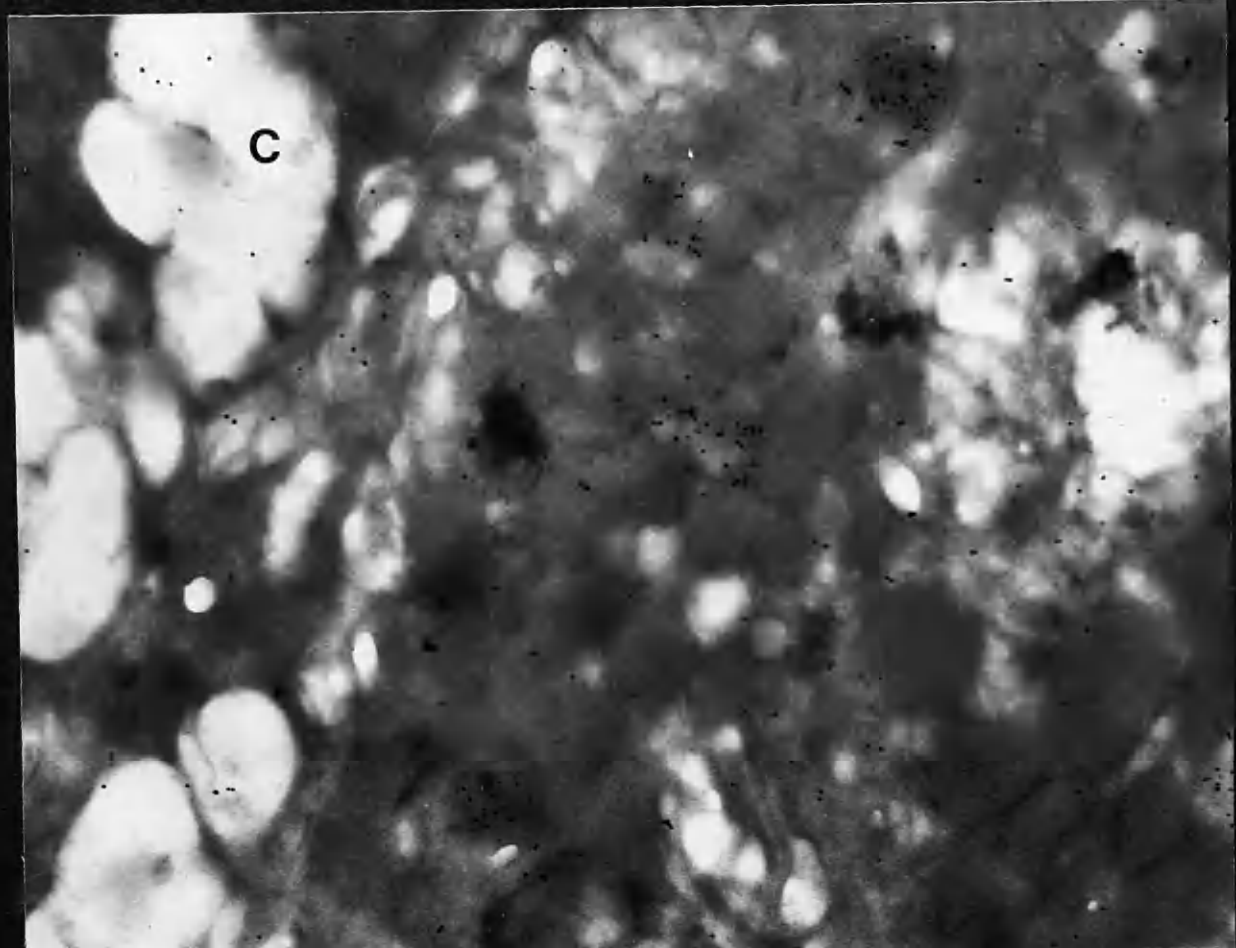

P
Z
R
N

FIG. 3.16 Electron micrograph including parts of a gastric chief cell (lower) and parietal cell (upper). Progastricsin is labelled with 20 nm gold particles, which are located with secretory zymogen vesicles (Z) and rough endoplasmic reticulum (R) of the chief cell and in luminal secretion (S). Note that the parietal cell (P) is negative.
Embedded in LR white, labelled with antigastricsin, indirect immunogold method, electron micrograph x15000.

FIG. 3.17 Cathepsin D in gastric body mucosal parietal cell. The black gold particles appear to be localised over electron dense organelles, possibly lysosomes. Part of the intracellular canaliculus (C) is present in this section.
Ultracryostat frozen section, labelled with anticathepsin D, electron micrograph x8200.


P
S
Z
R


C

DISCUSSION

The distribution of pepsinogen and progastricsin in stomach and duodenum obtained with the immunoperoxidase method appears similar to that demonstrated by immunofluorescence (Samloff, 1971; Samloff & Liebman, 1972a, 1973). The labelling of both zymogens in some parietal cells has not previously been described. One obvious possibility is that the antisera contained more than one antibody. However, the zymogens, especially pepsinogen and progastricsin and at least some of the enzymes, such as gastricsin, to which antisera were raised, were highly pure and any contaminant could have been present in only small amounts. Moreover, the contaminant would have to be closely related to the enzymes or zymogens, as five different enzyme or zymogen preparations all yielded antisera which gave similar labelling of parietal cells.

The possibilities of cross-reaction between antigastricsin and SMP, or of contamination of antigastricsin by anti SMP were raised in Chapter 2 on the basis of the Western blot result, but immunolocalisation sites differ; antigastricsin did not react with, for example, red blood cell membranes.

Another explanation for the staining of parietal cells is diffusion of zymogen or enzyme from the chief cells. This might explain labelling of parietal cells adjacent to the chief cells, but not of those some distance away from pepsinogen-producing cells. As parietal cells were still labelled in frozen sections of fresh tissue, diffusion seems not to be a complete explanation. It is, however, possible that the antisera were bound by a cross-reacting tissue constituent other than the antigen, but this applies to all immunohistology and is a theoretical limitation of the technique.

Finally, it may be that zymogen or even active enzyme is present within the parietal cells, possibly within the intracellular canaliculus.

Electron microscopic studies have so far failed to demonstrate progastricsin in the parietal cells, except when diffusion from adjacent chief cells could explain its presence.

The distribution of cathepsin D in the stomach favours its being a lysosomal enzyme and this is supported by the electron microscopic findings. It is difficult to exclude fully the possibility that it is secreted as an extracellular enzyme, but its absence from most chief cells does not support this, and it has not been located ultrastructurally in secretory granules and on rough endoplasmic reticulum. Unlike pepsinogen, progastricsin and SMP, cathepsin D is present in cells of the lamina propria. Morphologically, these are most probably histiocytes but further studies with known so-called markers of such cells have not been undertaken.

The possibility that diffusion of SMP from gastric juice could account for its demonstration in surface epithelium (see Chapter 4) seems unlikely, because pepsin and gastricsin, which account for about 99% of acid proteolytic activity in the gastric juice (Samloff, personal communication) were not so localised. Indeed, there may be no SMP in the gastric juice (Kay, personal communication). SMP, like cathepsin D, could be lysosomal, but electron microscopic immunolocalisation has not yet confirmed this and its presence on red blood cell membranes might suggest some other role.

The possibility was considered that unbuffered fixatives such as Bouin's fixative could, because of the low pH, activate pepsinogen in the gastric mucosa to active pepsin. As pepsinogen and pepsin have immunologically only partial identity (Chapter 2), the antipepsin would be expected to react better with the latter. If this were the case, antipepsinogen should give poorer results with tissue fixed in Bouin's fixative than those fixed in non-acid fixatives, as the zymogen would

have been activated to pepsin. Repeat labelling with antipepsinogen however, showed a similar pattern of results to those with antipepsin. Zymogen activation could not, therefore, explain the different results with different fixatives.

CHAPTER 4

THE EFFECT OF DIFFUSION ON THE IMMUNOLOCALISATION OF ANTIGEN

INTRODUCTION

A well-recognised problem with any immunohistochemical technique is that false positive results may be generated during the staining procedure (Burns, 1978) and appropriate controls are generally included with each batch of slides stained. False positivity may, however, also occur if the antigen is correctly labelled in the section but has moved from the site of its normal location *in vivo* into other areas prior to fixation of the tissue. Although such a cause of false immunolocalisation has been recognised previously (Isaacson et al, 1980; Mason et al, 1980; Isaacson et al, 1981), the extent to which it occurs in practice has received scant attention. In the investigations discussed in Chapter 3, it was noted that antibody to pepsin sometimes labelled connective tissue and cells adjacent to those in which pepsinogen was known to occur *in vivo*; diffusion was suggested, without evidence, as a possible explanation. In the present study the extent to which the zymogens pepsinogen and progastricsin can diffuse from their normal sites in gastric mucosa prior to fixation has been examined. The diffusion hypothesis has been further tested by introduction of a mixture of antigens into tissues in which they do not normally occur.

MATERIALS AND METHODS

Human Gastric Mucosa

Five gastrectomy specimens, three of which had been removed for benign conditions and two for carcinoma, were obtained fresh from the operating theatre. From the gastric body distant from any obvious lesion, full thickness strips approximately 10 mm long and 2 mm wide were cut. One piece was fixed immediately in Bouin's fixative. Each of the others was placed in a universal container, along with a small piece of moist filter paper to prevent dessication. The lids were fitted and the specimens were kept at room temperature. Bouin's fixative was added to the containers in turn after 10, 20, 30, 40, 50, 60, 90 minutes and 2, 3, 4, 6, 12, 24, 48 hours.

After addition of fixative to the final specimen, all pieces of tissue were left to fix for a further 48 hours and then embedded in paraffin wax. Sections (6μm) were labelled by the PAP method with antisera to pig pepsin or to human gastricsin as described previously.

Mouse Tissues

Fresh murine small intestine was washed through with 0.05M TRIS-HCL buffer pH7.6 and then a solution of pig pepsin (10 mg/ml) (obtained from Sigma Chemical Co.,Poole, UK) in normal human serum was instilled into the lumen. The bowel was tied off into 2 cm segments. One segment was removed immediately and fixed in 10% formol saline. The other segments were removed and fixed after 1, 6, 12 and 24 hours. 2 mm cubes of mouse spleen and liver were placed in a similar mixture in universal containers, a piece of each being removed for fixation at the same time as the bowel segments. Controls with TRIS buffer in place of

the serum mixture were performed for all tissues. After fixation, sections of liver, spleen and transverse sections of small bowel were embedded in paraffin wax. 6μm sections were labelled by the PAP method with antisera to pepsin, IgG, lambda and kappa chains. Antipepsin was used as described above and the other primary antibodies, obtained from Dakopatts A/S, Denmark, were used at 1:1000.

Assessment of Diffusion

Each section of stomach was examined microscopically to ascertain that it was orientated perpendicular to the gastric mucosal surface and included a suitable amount of connective tissue surrounding gastric glands which contained labelled chief cells. Sections which did not fulfil these criteria were deemed uninterpretable and excluded. The maximum distance between the chief cells and the edge of the zone labelled for pepsinogen in the lamina propria was measured with an eyepiece micrometer. The presence of labelling of connective tissue cells was also noted. Sections labelled for progastricsin were similarly examined but not measured.

In the experiment on murine tissues,the extent to which antigens had diffused from the surface into the liver, spleen and small intestine was noted.

RESULTS

Human gastric mucosa

As described previously (Chapter 3), pepsinogen and progastricsin were strongly labelled in the chief, mucous neck and occasional parietal cells of the gastric glands in tissues that had been immersed in fixative immediately (Fig 4.1a). Labelling of the lamina propria extended progressively further from the chief cells as the duration of fixation delay increased (Figs 4.1b-c), the distance by 3 hours usually being more than 180 μm (Table 4.1). After longer time intervals, the edge of the zone became less easy to define and the distance sometimes appeared less than in specimens fixed earlier. Cells in the lamina propria, including lymphocytes, plasma cells and macrophages, became strongly positive, often to an extent greater than that observed in the surrounding connective tissue (Fig 4.1d). By 24-48 hours, considerable autolysis had occurred in most cases and the intensity of the immune reaction product was weaker than at 12 hours.

Mouse Tissues

Antigens were also demonstrated in sections of the mouse tissues with which they had been left in contact. In tissues fixed immediately, there was labelling of the outer surface of the liver, spleen and intestinal mucosa with antisera to pepsin, IgG, kappa and lambda chains (Fig 4.2a). In all tissues there was a progressively widening zone of labelling (Fig 4.2b-d). This was of simlar extent with each antiserum and by 12-24 hours the tissues were diffusely labelled.

All antisera failed to label sections of control tissue in contact with TRIS buffer.

TABLE 4.1

DISTANCE DIFFUSED BY PEPSINOGEN AFTER DIFFERENT PERIODS OF AUTOLYSIS OF GASTRIC MUCOSA

PERIOD OF AUTOLYSIS (hrs)	DISTANCE (μm)				
Case:	1	2	3	4	5
0	-	90	90	0	45
1	135	90	315	270	90
3	180	90	270	270	450
6	360	225	-	135	225
12	-	135	-	90	315
24	-	135	-	225	540

Several slides in Case 1 could not be satisfactorily assessed (see Materials and Methods). Case 3 was studied up to 3 hours only.

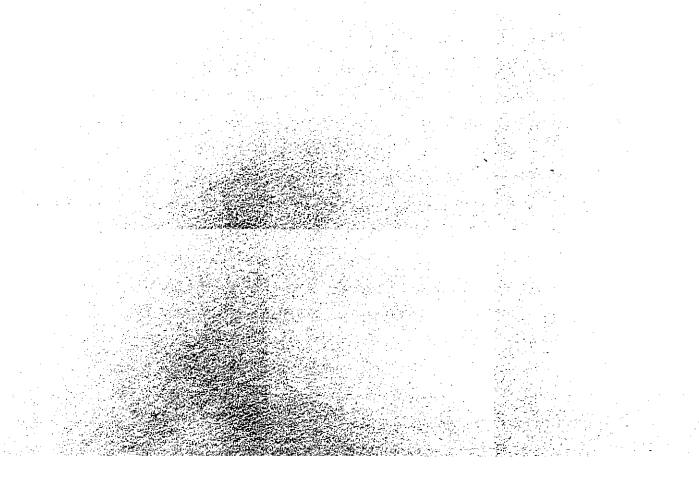
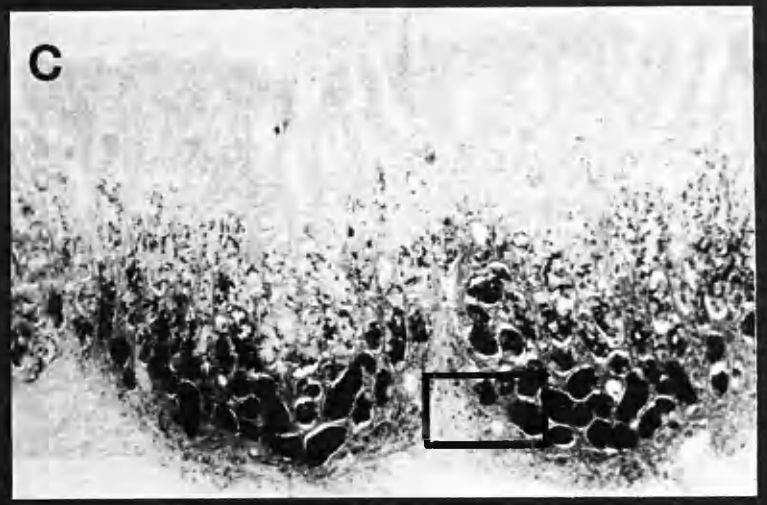


FIG. 4.1 Pepsinogen in normal gastric mucosa fixed immediately (a) and after autolysis of 1 hour (b) and 4 hours (c). Note the halo of pepsinogen in the lamina propria around the crypts in b and c. 1d is a higher power view of the area of Fig 4.1c marked and shows focal distribution of pepsinogen in lymphoid cells adjacent to intensely positive chief cells. (PAP method; a x60, b & c x 53; d x 350)


a


b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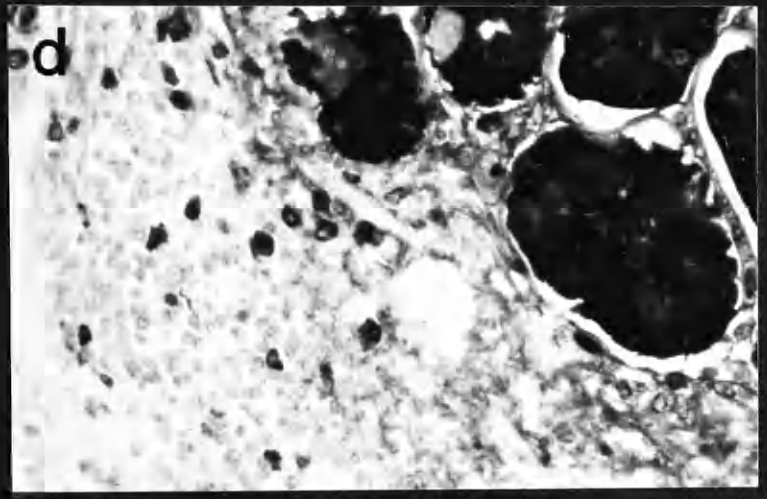
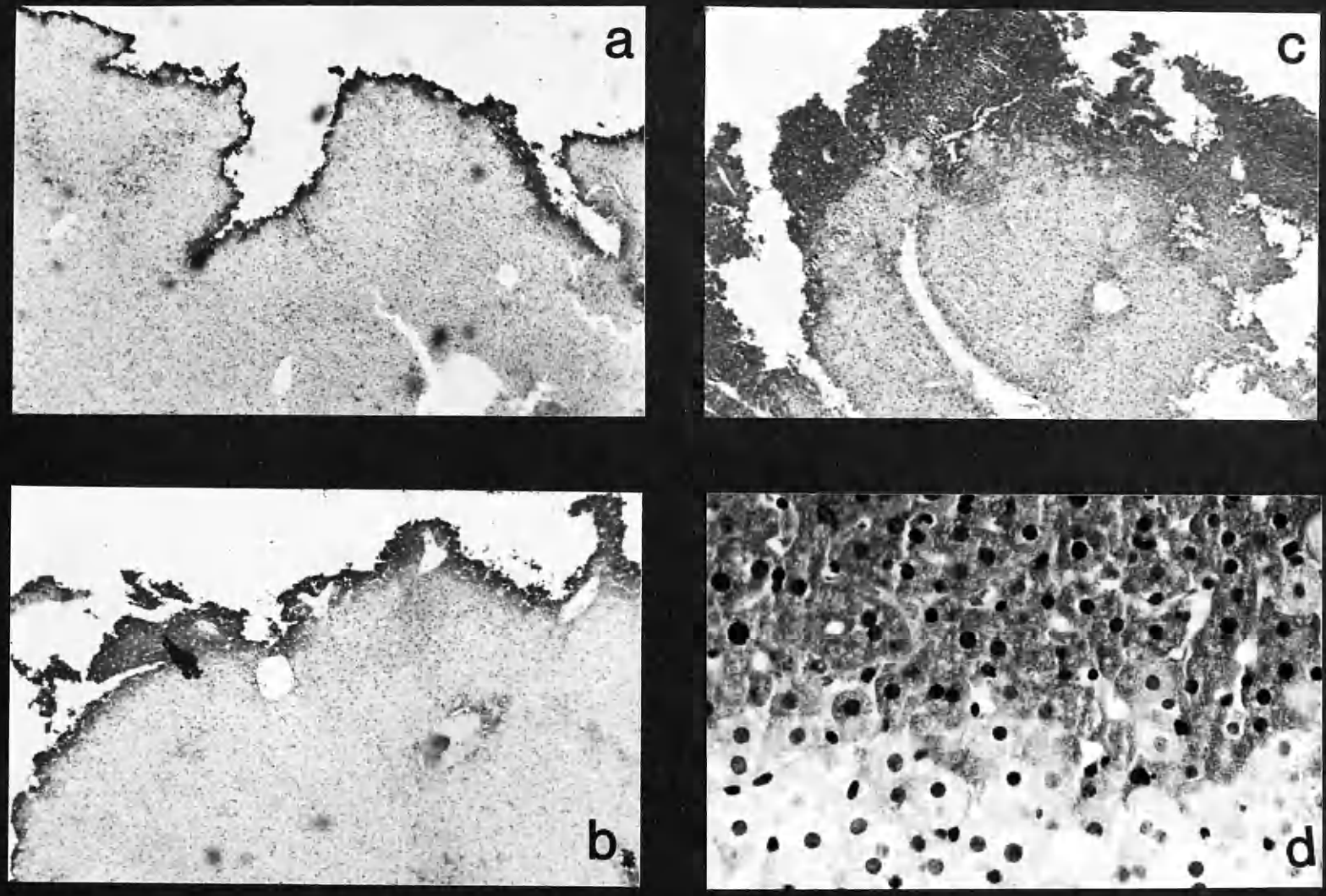

d

FIG. 4.2 IgG in mouse liver fixed immediately (a) and after 1 hour (b) and 12 hours (c) autolysis. There is progressive diffusion of immunoglobulin into the liver. Higher power (d) shows IgG at the edge of the diffusion rim at 12 hours. Note the granularity of labelling. (PAP method; a, b & c x 53; d x 540)


a
c
b
d

DISCUSSION

In human stomach there was a time-dependent increase in the distance between the chief cells and the edge of labelling of connective tissue and cells of the lamina propria for both pepsinogen and progastricsin. This is consistent with the hypothesis that after removal of tissue from the body, the zymogens diffuse into the surrounding tissues from their normal site. The appearances seen in the autolysing mouse bowel, liver and spleen can only be explained by diffusion of serum immunoglobulins and pepsin into the tissues since the controls not in contact with these antigens were negative.

The distance between the normal site of an antigen and the edge of the diffusion zone in surrounding tissues is dependent upon 2 factors:

1. The rate of diffusion of fixative into the tissues, which is approximately proportional to the square root of time (Bancroft & Stevens, 1982).

2. The rate of diffusion of antigen through the tissues, which decreases with time, although in a more complex way than (1) (Harris, 1972).

The increasingly long time intervals chosen reflected the expected decreasing rate of diffusion of antigen. The apparent diminution in the distance diffused after autolysis of 6 hours or more in some samples probably reflects difficulty in defining the diffusion edge clearly and differences in concentration of pepsinogen in chief cells, plane of section and fixation times.

An additional factor which might accelerate diffusion is tissue digestion. This might be significant if an antigen were an enzyme or colocalized with an enzyme. This was considered unlikely to be the case with pepsin and gastricsin which are inactive at the physiological pH at which the above experiments were carried out.

It is important to note the short time, often less than 1 hour, in which a diffusion effect may be noted. This has implications for immune localisation of antigens in tissues not fixed immediately. Moreover, most fixatives in common use penetrate tissues at less than 1 mm per hour (Bancroft and Stevens, 1982). In large pieces of tissue, complete fixation of antigens may be delayed for several hours and hence significant diffusion may occur.

Falsely localised antigen due to diffusion is likely to occur adjacent to sites containing high antigen concentration, as in Fig 4.1d. However, diffusion will occur not only from tissue but also from bathing fluid such as plasma, and cells may take up tissue fluid during autolysis or even during life. This problem has been noted by Mason et al, 1980 and by Isaacson et al (1980, 1981), who, in complementary experiments to those described above, demonstrated synthesis of antigen by cells in which it had previously been localised immunohistologically. Their criteria for recognising uptake of antigen, in this case immunoglobulin and albumin from permeating plasma were that reaction product in the cells was diffuse, non-granular and often in the nucleus as well as in the cytoplasm. While this may often be true, it can been seen from Fig 4.2d that diffusion cannot be ruled out because labelling is granular, presumably because prominent cytoplasmic organelles such as mitochondria would still cause such a pattern on light microscopy, even if antigen had diffused into the cell.

The focal distribution of labelling seen in Fig 4.1d is of interest as it could readily be mistaken for genuine <u>in vivo</u> localisation. There are several possible explanations for such a pattern: firstly, uptake of antigen by cells may vary, as differences in the survival time of cells in tissue removed from the body could lead to active ingestion of antigen by live cells or passive uptake by dead ones. Moreover, some antigens could be imbibed or bound selectively, although in the case of

pepsinogen and progastricsin this is unlikely as there are no naturally occurring intracellular protease inhibitors with which these zymogens might form complexes (Kay, 1985). Secondly, antigen may be preferentially washed out of extracellular tissue during processing. Thirdly, there may be masking of extracellular antigen; with formalin fixation, formation of cross-links between immunoglobulin and other extracellular proteins may reduce the antigenicity of the immunoglobulins and a similar mechanism might operate in the present experiments (Brandtzaeg, 1982). Whatever the explanation it is important to recognise that focality of immunostaining does not necessarily exclude diffusion of antigen.

The degree to which a substance diffuses depends, amongst other things, on its molecular weight, which in the above experiments varied from 42 kd for pepsinogen and progastricsin to 150 kd for IgG. Other factors such as charge and site of attachment in tissue will vary and some antigens will remain in their original site even after considerable autolysis has occurred. However, in localisation studies the possibility of artefactual labelling due to diffusion should always be taken into account, especially when autopsy material is used or when the fixation time of surgical biopsy material is not known. As immunohistochemical and other localisation techniques become more sensitive, such artefacts are likely to lead to an increased number of false positive results.

CHAPTER 5

IDENTIFICATION AND ORIGIN OF SEMINAL FLUID ACID PROTEOLYTIC ACTIVITY

INTRODUCTION

A zymogen, activated at optimal pH 2.5, was first demonstrated in human seminal plasma by Lundquist & Seedorff (1952). Later, the characteristics of this zymogen were shown by chromatography, electrophoresis and immunochemistry to be similar to those of a zymogen found in extracts of gastric and duodenal mucosa (Seijffers, Miller & Segal, 1965; Hirsch-Marie & Conte, 1967; Hirsch-Marie, 1968). These authors used different terminologies but all appeared to be describing progastricsin. Samloff & Liebman (1972a) purified two zymogen fractions from seminal fluid and demonstrated that they were electrophoretically and immunochemically indistinguishable from the two progastricsin fractions known to be present in gastroduodenal mucosa. The zymogen was also purified by Ruenwongsa & Chulavantnatol (1975), who found that its catalytic activity on some substrates, such as haemoglobin, resembled that of pig pepsin, but that it was less effective than pepsin in hydrolysing synthetic substrates. The seminal fluid zymogen is irreversibly denatured at neutral pH; so it cannot be cathepsin D or E (Barrett, 1977a).

Thus, although the seminal fluid zymogen is similar to progastricsin of gastroduodenal mucosa, definite proof of identity has still not been furnished. Moreover, the origin of the zymogen is not clear. Lundquist & Seedorff (1952) claimed that it originated in the seminal vesicles. The process of ejaculation has been studied by x-ray cinematography (Mitsuya et al, 1960) and the findings, combined with those of biochemical marker studies of epididymis, prostate and seminal vesicle appeared to show that acid proteolytic activity was confined to the later part of the ejaculate, which originated in the seminal vesicle, while there was no activity in the early ejaculate, which came from the prostate (MacLeod & Hotchkiss, 1942; Glezerman & Lunenfeld,

1975). The zymogen was therefore considered to be derived from the seminal vesicle and not the prostate. On the other hand, Hirsch-Marie & Delafontaine (1978) found equal amounts of activity in fractions of split ejaculates and concluded that the zymogen originated in both seminal vesicle and prostate. The most convincing evidence that prostate is one, if not the only, source is that a zymogen, considered to be progastricsin, was isolated from homogenates of prostate (Chiang et al, 1981). Thus, while biochemical data favour an origin in prostate and perhaps in seminal vesicle, other sources in the male genital tract have not been excluded. The precise cellular localisation of the seminal fluid zymogen is unknown.

In the present study, the biochemical properties of the seminal fluid enzyme were compared with those of gastricsin and pepsin from human gastric mucosa and the site of origin of the zymogen was determined immunohistochemically.

MATERIALS AND METHODS

IMUNOHISTOCHEMICAL STUDIES

Antisera

The rabbit antisera and human enzymes and zymogens used are shown in Table 5.1. All antisera were diluted in Tris buffered saline (TBS) except for antigastric gastricsin which was diluted in 10% normal human serum in TBS. The antiserum to seminal gastricsin was raised in rabbits by serial injection of the enzyme in Freund's adjuvant (Chapter 2).

Absorption control experiments were performed by incubation of antisera with enzyme or zymogen for 1 hr at room temperature before use.

Tissues

All tissues were from the routine surgical histology files of the Pathology Department, Glasgow Royal Infirmary and had been fixed in 10% formol-saline and paraffin-embedded. 6μm sections were cut. Ninety-six cases of benign prostatic enlargement were studied. In 85 the prostate had been removed transurethrally as curettings and in the other 11 cases by suprapubic enucleation. Other normal male genital tract tissues studied (number of cases in brackets) were testis (16), epididymis (12), vas deferens(11), seminal vesicle(3) and bladder transitional mucosa (10).

A selected group of 19 benign prostates was also labelled with antipepsin.

Alternate serial sections from 10 of the cases of benign nodular hyperplasia were labelled for progastricsin and prostatic acid phosphatase. The extent of colocalisation was determined by examination with a comparison microscope.

Controls

In all staining runs a section of normal gastric body mucosa

TABLE 5.1

ANTISERA AND ZYMOGENS OR ENZYMES

Antisera to:	Source	Dilution
Gastric gastricsin	See Chapter 2	1:100 in 10% normal human serum in TBS
Human pepsin	See Chapter 2	1:100 in TBS
Prostatic acid phosphatase	Miles Lab. Ltd.	1:500
Seminal gastricsin	See Chapter 2	1:100

Blocking controls		
Seminal progastricsin	Prof. J. Svasti, Bangkok	10 µg/ml
Seminal gastricsin	"	"
Gastric gastricsin	Prof. J. Tang, Oklahoma	"
Pig pepsin	Sigma Chemicals Ltd.	"
Pig pepsin	"	1 mg/ml

labelled with antigastric gastricsin was included as a positive control. Similar sections were labelled with antiserum to gastric gastricsin and seminal gastricsin and controls in which both antisera were preincubated with seminal gastricsin (10 μg/ml) for 1 hr at room temperature were included.

The specificity of immune labelling of the prostate was also demonstrated by attempts to block staining by prior incubation of antigastricsin with gastricsin (10 μg/ml) or pig pepsin (10 μg/ml or 1 μg/ml) for 1 hr. For this purpose the most strongly positive 13 cases were selected. As human pepsin was in short supply, pig pepsin, which is immunologically similar, was used for absorption experiments.

BIOCHEMICAL STUDIES

These experiments were carried out by Drs. M.J. Valler and J. Kay, Biochemistry Department, University College, Cardiff. The inhibitors isovaleryl- and lactoyl-pepstatins were gifts from Professor T. Aoyagi, Institute of Microbiological Chemistry, Tokyo. The preparation of the latter has been described previously (Kay et al, 1982). The synthetic peptide substrate Pro-Thr-Glu-Phe-(NO_2)Phe-Arg- Leu was a gift from Dr. B.M. Dunn, University of Florida. The hydrolysis of this substrate by the various acid proteinases was followed at 300 nm in formate buffer, pH 3.1 at 37°C as described previously (Kay, Valler & Dunn, 1983). Km values for the enzymes were obtained with substrate concentrations of 10-100 μm for pepsin and 50-300 μm for gastricsin. Michaelis-Menten kinetics were observed in all cases and straight lines were fitted to plots of S/v vs S by the method of unweighted sum of least squares. For each determination initial rates v were measured with at least five values (estimated to be precise ±5%) of the initial substrate concentration S within these ranges.

With Pro-Thr-Glu-Phe-(NO_2)Phe-Arg-Leu at concentrations between 40

and 130 μM as substrate, kinetic constants (Ki) for the inhibition of the enzymes by isovaleryl- and lactoyl-pepstatins were determined. The inhibitors were used at 0.03-1 μm and 2-20 μM respectively with the seminal and gastric gastricsin. Both inhibitors were added at concentrations between 5-25 nM to pepsin. For tight-binding inhibitors Ki values were obtained by the method of Goldstein (1943), while for weaker-binding inhibitors plots of 1/v vs inhibitor at different substrate concentrations were used.

RESULTS

IMMUNOHISTOCHEMICAL STUDIES

Sixty-six of the 96 cases of benign prostatic enlargement gave a positive reaction for progastricsin in the cytoplasm of the cells lining the acini. Labelling was in most cases confined to a few acini but was invariably strong (Fig. 5.1 and 5.2). In some cases labelling was widespread, but even then was focal. Often the supranuclear secretion in the tips of cells was positive (Fig. 5.3 and 5.4). There was no difference in labelling between prostates removed intact and those removed by transurethral resection. There was no evidence of progastricsin in any of the sections of normal testis, epididymis, vas deferens, or bladder transitional mucosa. Spermatozoa in the testis were negative. The epithelium of the seminal vesicle was rich in lipofuscin which, being brown and granular, could have been mistaken for reaction product. However, the granules were no more intensely brown in sections labelled for progastricsin than in control sections stained only with haematoxylin and were therefore considered to be progastricsin negative.

Serial sections from 10 cases of progastricsin positive prostates were labelled for acid phosphatase, which gave a strong reaction in nearly all acinar cells. Comparison microscopy showed that none of the few acid phosphatase negative cells contained progastricsin. All 19 prostates labelled for pepsin were negative.

Controls

There was strong labelling of prostate with antigastricsin preincubated with pepsin at both concentrations used, but not when preincubated with gastricsin (Fig 5.5a and b).

Preincubation with seminal gastricsin abolished staining of gastric mucosa by antigastric gastricsin but not by antipepsin (Fig 5.6a-d).

FIG. 5.1 Progastricsin in the acinar epithelium of the benign prostate.
Note that progastricsin appears to be focally distributed even within individual acini.

Labelled with antigastricsin, PAP method. x76.

FIG. 5.2 Progastricsin in the acinar epithelium of the benign prostate. In this case most acinar epithelial cells are stained.

Labelled with antigastricsin, PAP method. x76.

FIG. 5.1 Progastricsin in the acinar epithelium of the benign prostate.
Note that progastricsin appears to be focally distributed even within individual acini.

Labelled with antigastricsin, PAP method. x76.

FIG. 5.2 Progastricsin in the acinar epithelium of the benign prostate. In this case most acinar epithelial cells are stained.

Labelled with antigastricsin, PAP method. x76.

FIG. 5.3 Progastricsin in the acinar cells of the benign prostate. Labelling is most intense towards the luminal end of the cells.

Labelled with antigastricsin, PAP method. x350.

FIG. 5.4 Progastricsin in prostatic acinar epithelium, in which it is located at the luminal end of the cells and in secretion (S).

Labelled with antigastricsin, PAP method. x420.

S

FIG. 5.5 Prostate labelled with antigastric gastricsin (a) without and (b) with prior incubation of antiserum with seminal gastricsin (10 µg/ml) for 1 hr at room temperature.

PAP method. x90.

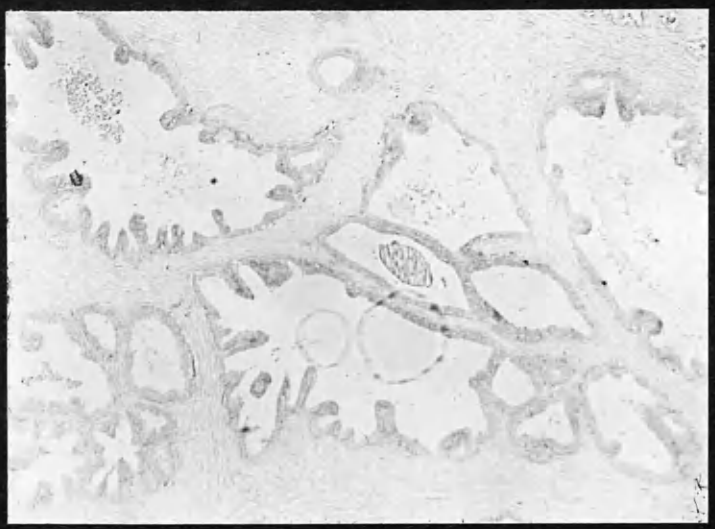
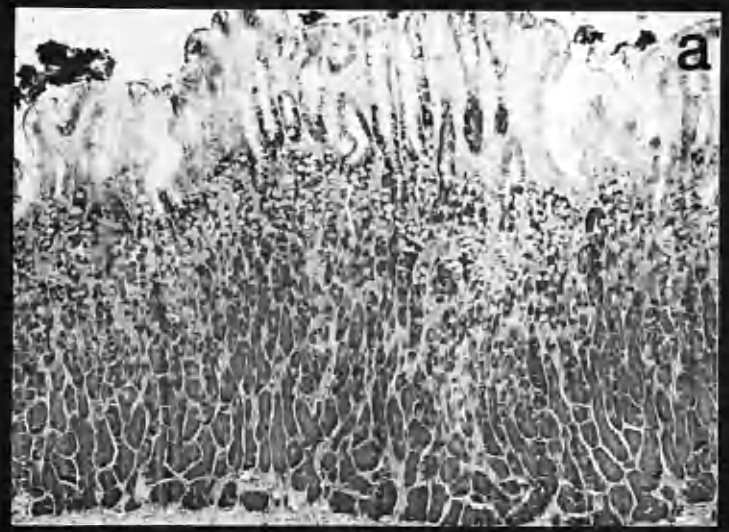
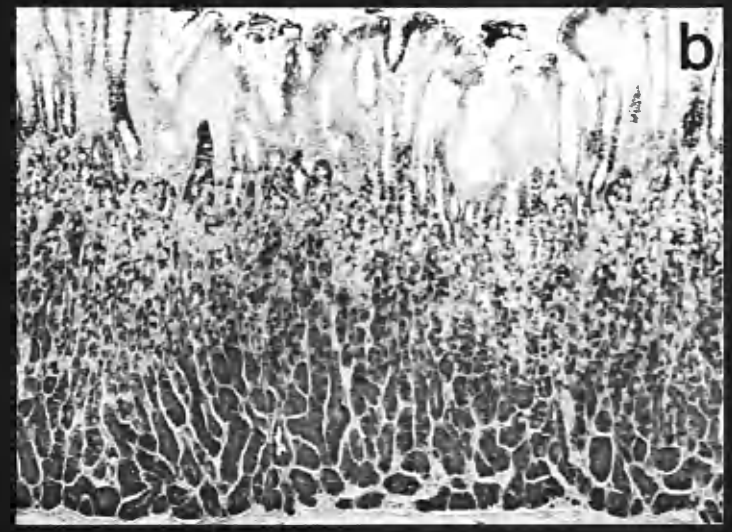


FIG. 5.6 Stomach labelled with antihuman pepsin (a and b) or antihuman gastric gastricsin (c and d). In b and d each antiserum was preincubated with seminal gastricsin (10 µg/ml) for 1 hr at room temperature.

PAP method. x90.


a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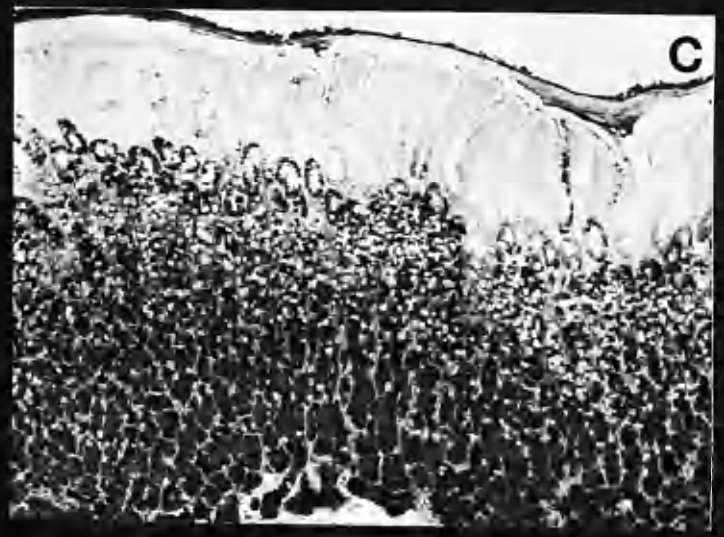

c


d

BIOCHEMICAL STUDIES

It can be seen from the data in Table 5.2 that Pro-Thr-Glu-Phe-(NO_2)Phe-Arg-Leu has a lower Km value with human pepsin than for gastric gastricsin. It would appear that the seminal enzyme behaves more like a gastricsin than a pepsin. This conclusion is confirmed by the inhibition constants obtained with the two pepstatins (Table 5.2). In keeping with previous observations with animal pepsins (Kay, Valler & Dunn, 1983) lactoyl-pepstatin is equally as effective an inhibitor of human pepsin as is the isovaleryl derivative. By contrast, lactoyl-pepstatin inhibits gastric gastricsin very weakly.

TABLE 5.2

KINETIC CONSTANTS FOR THE HYDROLYSIS OF PRO-THR-GLU-PHE-(NO_2)PHE-ARG-LEU BY HUMAN PEPSIN, GASTRIC GASTRICSIN AND THE SEMINAL ENZYME, AND FOR THE INHIBITION OF THESE REACTIONS BY ISOVALERYL-AND LACTOYL-PEPSTATINS

	Substrate	Isovaleryl-pepstatin	Lactoyl-pepstatin
	Km (mM)	K_i ($x10^8$)M	
Human pepsin	0.11	<0.05	0.04
Gastric gastricsin	0.42	10	580
Seminal enzyme	0.60	16	400

All reactions were in 0.1M sodium formate buffer, pH 3.1 at 37°

DISCUSSION

The immunohistochemical results demonstrate clearly that progastricsin is present in and presumably synthesised and secreted by the cells lining the prostatic acini. This is supported by the observation that the zymogen was located in the tips of cells. There is no evidence of its presence in seminal vesicle epithelium, although, as this tissue was obtained postmortem, it is possible that loss of zymogen due to autolysis could explain the negative results. There was no evidence of progastricsin in any of the other tissues studied from the male genito-urinary tract. The spermatozoa are known to contain an acrosomal acid proteinase (Polakoski, Williams & McRorie, 1973) but showed no evidence of progastricsin.

As labelling with antigastricsin was blocked by prior incubation with gastricsin of gastric origin and by gastricsin and progastricsin of seminal fluid origin, but not by pepsin, even at high concentration (1 mg/ml), the zymogen labelled must be progastricsin and not pepsinogen. This is supported by the finding that the prostate did not label with antipepsin.

The biochemical data, too, show that the seminal fluid zymogen behaved more like a gastricsin than a pepsin. Firstly, the Km value of the seminal zymogen for hydrolysis of the synthetic substrate Pro-Thr-Glu-Phe-(NO_2)Phe-Arg-Leu is closer to that of gastricsin than of pepsin, which is much lower. Secondly, both pepstatins, especially lactoyl-pepstatin, are weak inhibitors of gastricsin and the seminal enzyme and strong inhibitors of pepsin.

Biochemically and immunohistochemically, therefore, the seminal enzyme behaves like gastricsin and not like pepsin and appears to be produced by the epithelial cells which line the prostatic acini.

It is tempting to speculate on its function. As progastricsin is not activated to gastricsin at the pH of semen, normally around 7.4,

it cannot have any proteolytic role before ejaculation (Polakoski & Zaneveld, 1976). In the vagina, however, the pH can fall to 4, low enough to activate the zymogen and within the range of proteolytic activity of gastricsin, which may be implicated in digestion of vaginal protein or mucoprotein after ejaculation (Seijffers, Miller & Segal, 1965; Hirsch-Marie & Delafontaine, 1978). The presence of other acid proteinases in acrosomal extracts of spermatozoa from various mammals, including humans, supports this suggestion (Polakoski, Williams & McRorie, 1973). Presumably pepsin, optimally active at even lower pH than gastricsin, is less suited to such a function.

Efforts have been made to correlate the concentration of seminal gastricsin with sperm count and a tendency towards an inverse relationship was found, but proved not to be statistically significant (Hirsch-Marie & Delafontaine, 1978).

The findings in the above study have since publication (Reid et al, 1984, 1985) been confirmed (Reese et al, 1987) and progastricsin demonstrated mainly in the central zone of the prostate, with a sharp boundary at the junction with the peripheral zone. This is the first evidence that these two zones may serve different biological functions. In the same study, progastricsin was also found in seminal vesicle epithelium; the discrepancy between this observation and that of the present study could be due either to differences in the avidity of the antisera or to erroneous interpretation of brown lipofuscin granules as positive staining.

CHAPTER 6

DISTRIBUTION OF ASPARTIC PROTEINASES IN NORMAL TISSUES OTHER THAN STOMACH

INTRODUCTION

It has been established in Chapter 3 that pepsinogen is normally present and presumably produced in stomach and progastricsin in stomach, duodenum and prostate. In this chapter, the distribution of cathepsin D and SMP in other sites in the body will be considered.

Although cathepsin D is known to be widely distributed, the sites from which it originates have not been determined. Biotin-labelled pepstatin has been used to localise cathepsin D in cultured human synovial cells (Matthews, Decker & Knight, 1981) and the same cells in rabbit have been immunocytochemically labelled (Poole, Dingle & Barratt, 1972). Cathepsin D has also been localised in rat skeletal muscle by electron microscopic detection of enzyme reaction product (Bird, Schwartz & Spanier, 1977) and in human skeletal muscle and phagocytes by electron microscopic immunocytochemistry (Whitaker, Bertorini & Mendell, 1983). Cathepsin D has, however, been purified from human gastric mucosa as described in Chapter 1, although the cells which produce it were not then known.

The distribution of slow moving protease has been even less clearly defined. The tissues in which it occurs have not been determined, apart from gastric mucosa (Chapter 3) and its role is unknown. The present study was undertaken to localise cathepsin D and SMP in normal human tissue by the immunoperoxidase (PAP) method.

MATERIALS AND METHODS

Antisera

Anticathepsin D was used at 1 : 100 for 1 hr. Sections were counterstained with methyl green or haematoxylin but the Prussian blue reaction was performed on the lung sections instead of a counterstain, so that the brown haemosiderin granules commonly seen in alveolar macrophages would not be confused with the brown reaction product of the PAP technique.

Rabbit antilysozyme (Dakopatts A/S, Denmark) at 1 : 1000 in 0.05M TBS, pH 7.6 was used on selected tissues so that the distribution of lysozyme might be compared with that of cathepsin D.

Anti-SMP was used at 1 : 100 as in labelling experiments on gastric mucosa.

Tissues studied

Tissues studied were from the routine surgical files of the Pathology Departments, Glasgow Royal Infirmary and the General Infirmary at Leeds. All tissues were fixed in 10% formol saline and paraffin embedded. 6 μm sections were used in the immunoperoxidase PAP method.

Tissues examined for cathepsin D (number of cases in brackets) were:- spleen (7), liver (5), small bowel (5), large bowel (6), lung (6), prostate (7), lymph nodes (6), brain (5), joint synovium (2), kidney (5) and placenta (7).

Tissues stained for lysozyme included normal spleen (7), liver (5), colon (7).

Tissues examined for SMP were jejunum (5), colon (6), liver (7), spleen (6), bone marrow (5), pancreas (5), prostate (6), kidney (5), brain (11), lung (12), breast (6), endometrium (6), fallopian tube (6), testis (7), vas deferens (6), placenta (6). Six cases of nasal polyps were also examined so that plasma cells could be studied for SMP.

RESULTS

Cathepsin D

Spleen

In the spleen cathepsin D was shown in the littoral cells lining the sinusoids (Fig. 6.1a and c). There was also intense reactivity with single large cells in the Malpighian bodies; these had abundant cytoplasm, often containing vacuoles, and appeared morphologically to be macrophages (Fig. 6.1a). The pattern observed with anticathepsin D in spleen was different from that obtained with antilysozyme, which reacted strongly with numerous cells in the red pulp (Fig. 6.1b and d) but with only occasional littoral cells and with very few cells in the white pulp.

Liver

Liver showed widespread labelling of cathepsin D in hepatocytes but in only a small number of Kupffer cells (Fig. 6.2a). In contrast, lysozyme was present in most Kupffer cells and a few hepatocytes (Fig. 6.2b).

Lymph nodes

Tingible body macrophages within germinal centres were consistently strongly positive for cathepsin D (Fig. 6.3), as were occasional stellate cells, which seemed to be dendritic reticulum cells. Sinus lining cells showed a similar but weaker reaction pattern to those in the spleen. Most sinus histiocytes were weakly positive.

Bowel

In the duodenum and small and large bowel cathepsin D was most strongly labelled in the cytoplasm of mononuclear cells, probably macrophages, of the lamina propria. In the colon these cells containing cathepsin D tended to lie just below the surface epithelium (Fig. 6.4a), whereas those containing lysozyme were widely distributed

throughout the lamina propria (Fig. 6.4b). There was weak labelling of the surface epithelium for cathepsin D in some cases, but the cells in the crypts were negative, although in three cases the duodenal Brunner's glands showed weak positivity.

Lung

Of the sections from the six lungs studied, five contained abundant alveolar macrophages, all of which were rich in cathepsin D (Figs. 6.5 and 6.8). Counterstaining of PAP labelled sections with Prussian blue showed haemosiderin in the cells containing cathepsin D; at least some of the haemosiderin was in different granules, although the intense brown reaction of the DAB may have obscured some of the blue staining haemosiderin. Less commonly, alveolar lining cells were positive. Where present, the respiratory epithelium lining the bronchioles showed granular cytoplasmic labelling near the luminal aspect (Fig. 6.7).

Brain

The mononuclear phagocytes of the brain (the microglia) were negative, as were the astroglia. Neurones of the cerebral and cerebellar cortex, however, showed granular cytoplasmic reactivity for cathepsin D (Fig. 6.6). Two cases of brain included choroid plexus, the surface epithelium of which gave a positive reaction for cathepsin D.

Other tissues

In the four cases of normal kidney cathepsin D was mainly confined to proximal and distal tubular epithelium, although in two cases there was also focal glomerular reactivity. Prostate has already been shown to contain gastricsin, and although cathepsin D was present in several cases, labelling was weak and was shown in only a small proportion of acinar lining cells and occasional stromal cells. The syncytiotrophoblast of the placental chorionic villi exhibited granular

cytoplasmic reactivity (Fig. 6.9), but the Hofbauer cells, which are the mononuclear phagocytes of the placenta, were negative. Both specimens of joint synovium were negative for cathepsin D. In the bone marrow labelling was confined to non-haemopoietic cells, apparently histiocytes, with no cathepsin D in megakaryocytes, or in erythroid, or myeloid cells. In particular, neutrophils were negative.

SMP

The presence of SMP in red blood cell membranes (Fig. 6.10) acted as a useful internal control, as they were found to label strongly in most sections.

Bowel

In 4 of the 5 samples of jejunum and 3 of 6 colons the surface epithelium stained but the pattern resembled that noted in diffusion. The crypt epithelium was focally positive.

Lymph nodes

Occasional cells, apparently macrophages, were weakly positive in 4 cases.

Brain

Weak staining of neurones was noted in 2 cases only (Fig. 6.11). In 1 case the choroid plexus was labelled (Fig. 6.12).

Lung

In 3 cases the tips of the cilia of bronchial respiratory epithelium were focally positive, where they were in contact with red blood cells (Fig. 6.13). Alveolar macrophages were weakly positive in 3 cases.

Placenta

The apical microvillous membrane of the syncytiotrophoblast was labelled in 2 cases (Fig. 6.14).

Prostate

The surface membranes of a few acinar cells were stained in 5 of the 6 cases.

Kidney

Occasional tubules were positive in 1 of the 5 cases studied.

Nasal polyps

Plasma cells and respiratory epithelium appeared weakly labelled in 2 cases but were otherwise negative.

There was no convincing evidence of SMP in liver, spleen, bone marrow, endometrium, fallopian tube, vas deferens, testis or breast (including apocrine epithelium).

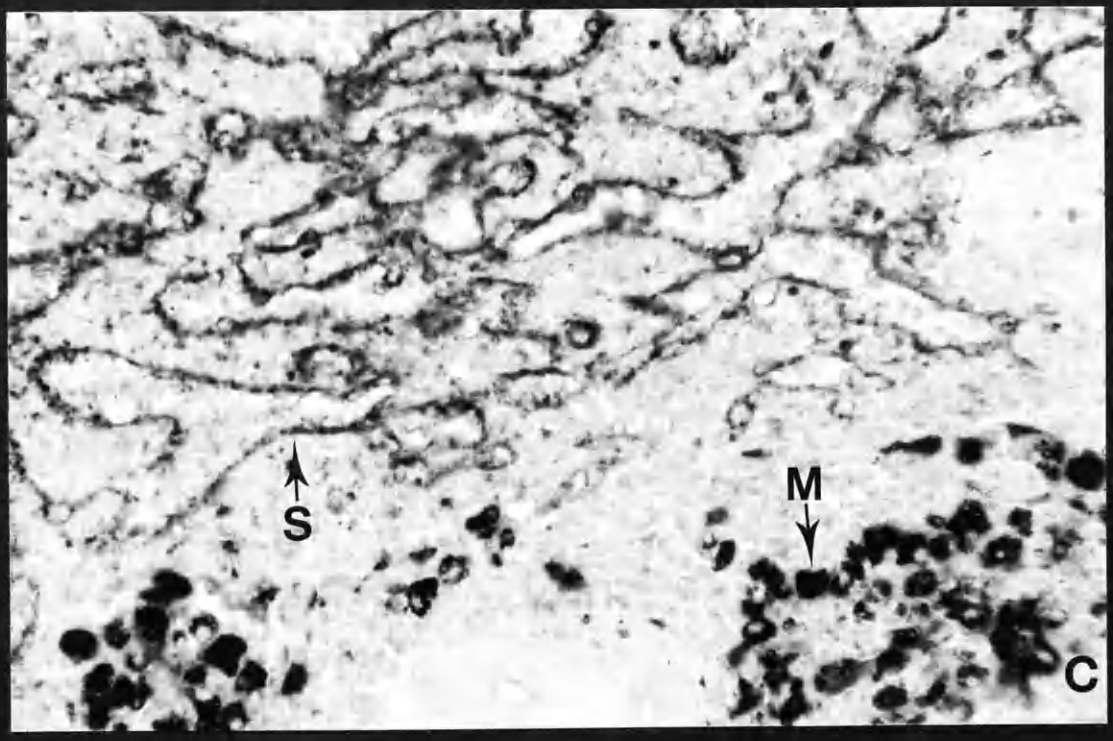
FIG. 6.1 a and b.
Normal spleen labelled for (a) cathepsin D and (b) lysozyme. Cathepsin D is present in littoral cells and in the cells of the Malpighian bodies. Lysozyme is widely distributed in the red pulp and is generally absent from the white pulp.
PAP method. x47.

a

b

FIG. 6.1 (c) Normal spleen labelled for cathepsin D. Cathepsin D is present in littoral cells (S) lining the sinusoids and in cells (M) in the white pulp.
PAP method. x340.

(d) Normal spleen labelled for lysozyme. Lysozyme is absent from white pulp (W) and littoral cells (S) although present in many other cells of the red pulp.
PAP method. x340.


S
M
C

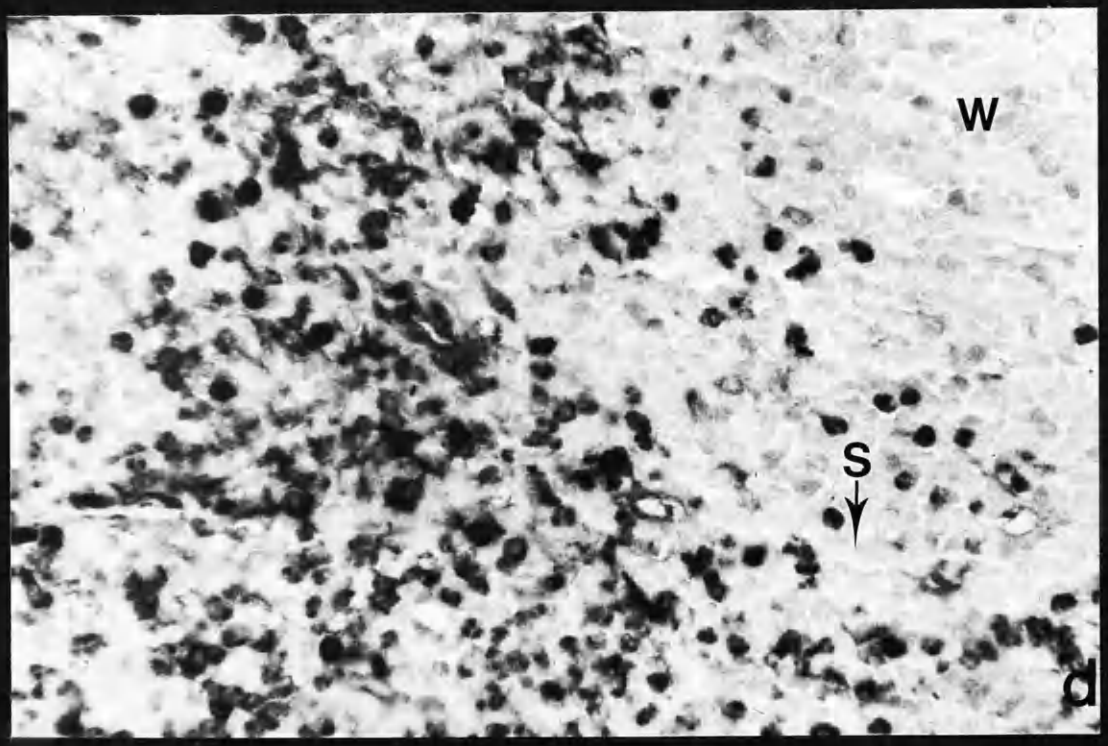
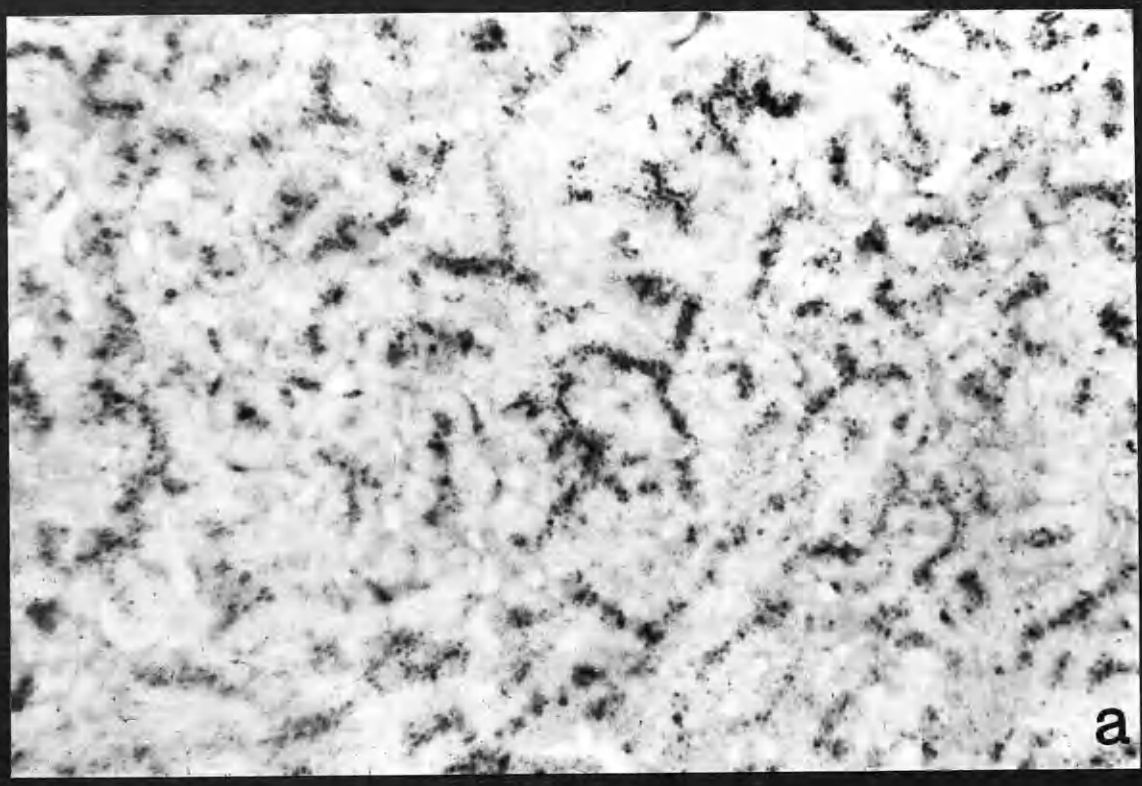

W
S
d

FIG. 6.2 (a) Normal liver labelled for cathepsin D, seen mainly in the hepatocytes.
PAP method. x340.

(b) Normal liver labelled for lysozyme, which is present mainly in the Kupffer cells.
PAP method. x340.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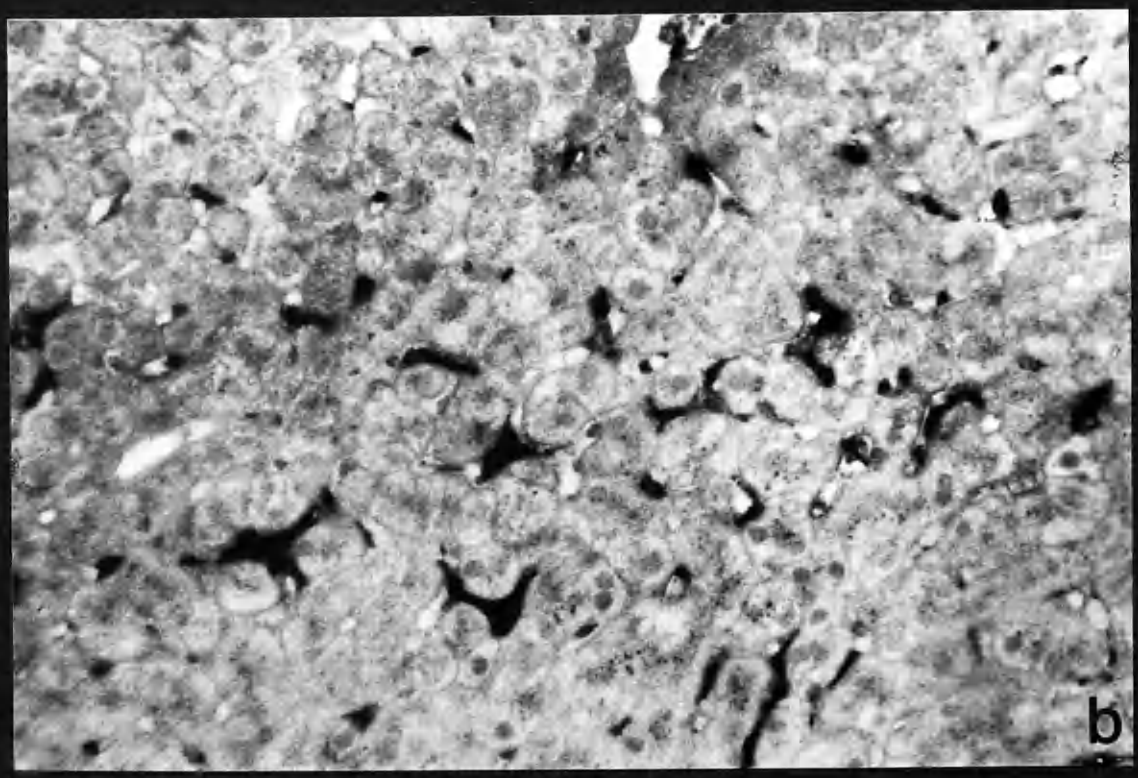

b

FIG. 6.3 Cathepsin D in the germinal centre of a reactive lymph node, where it is mainly in tingible body macrophages and dendritic reticulum cells.
PAP method. x340.

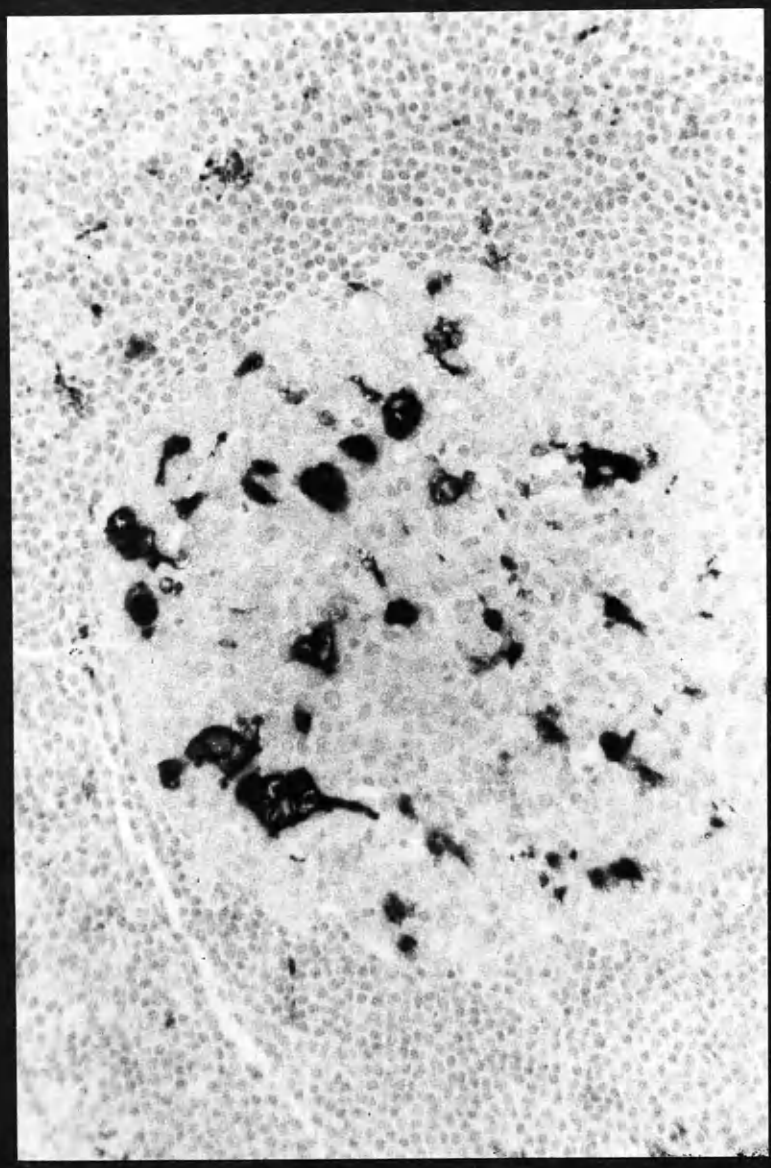
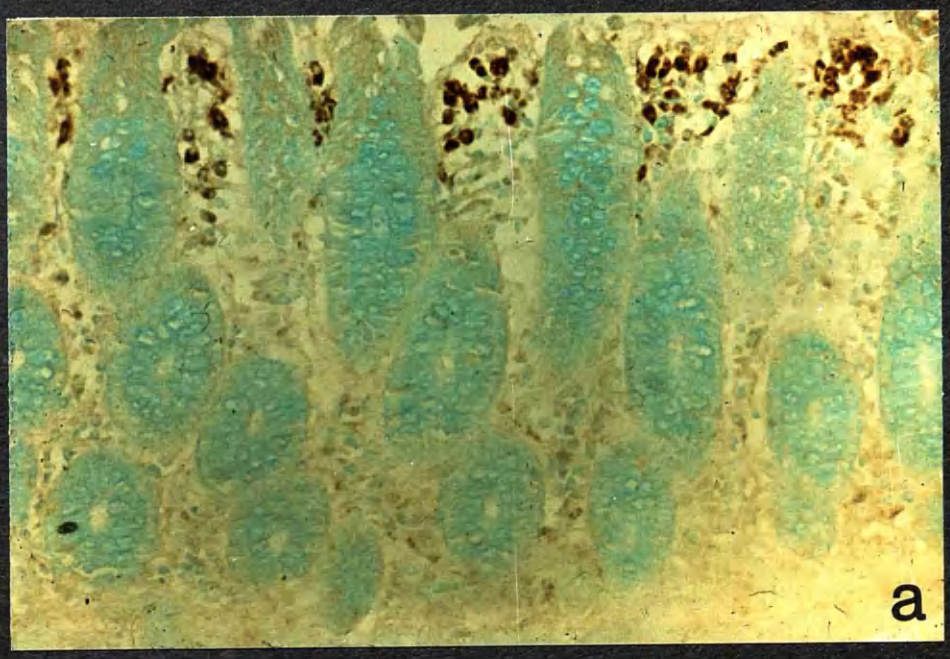


FIG. 6.4 a) Cathepsin D in colonic mucosa, in which it is located in mononuclear cells in the lamina propria near the luminal surface.
PAP method. x72.

b) Lysozyme in colonic mucosa, in which it is located in mononuclear cells throughout the lamina propria.
PAP method. x72.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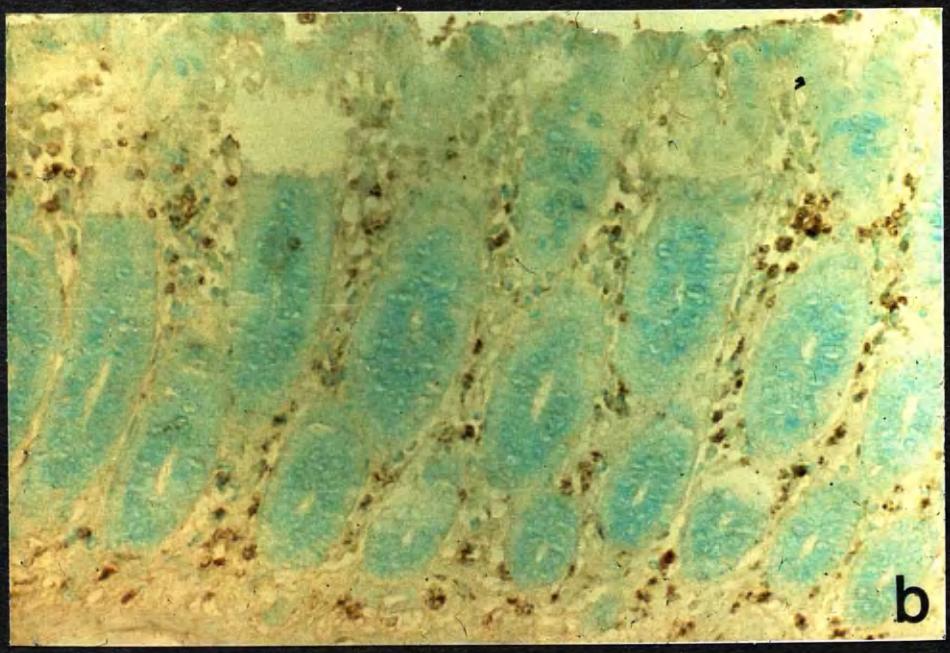

b

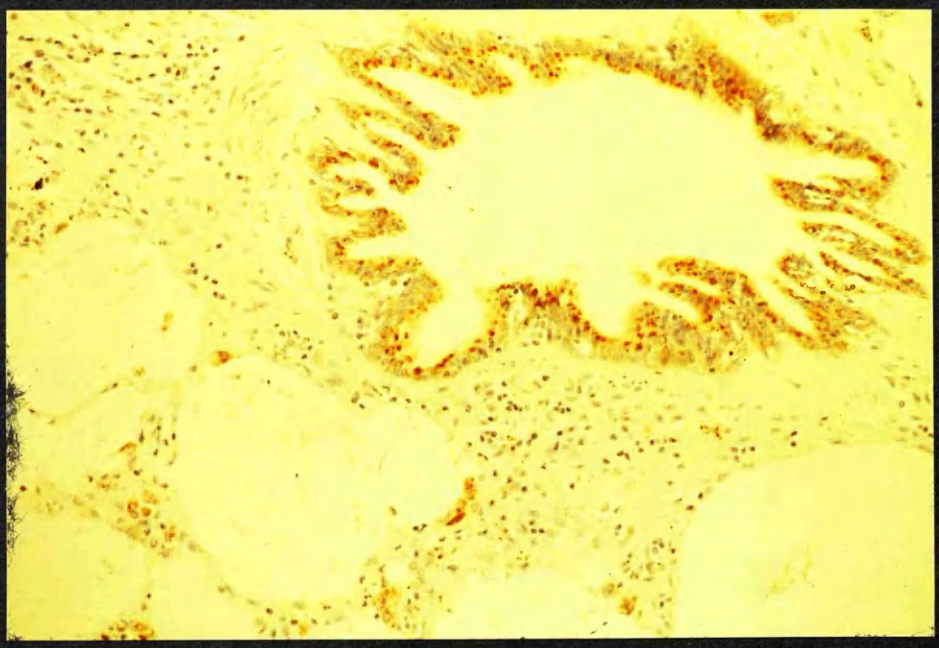
FIG. 6.5 Cathepsin D in alveolar macrophages of lung. PAP method. x210.

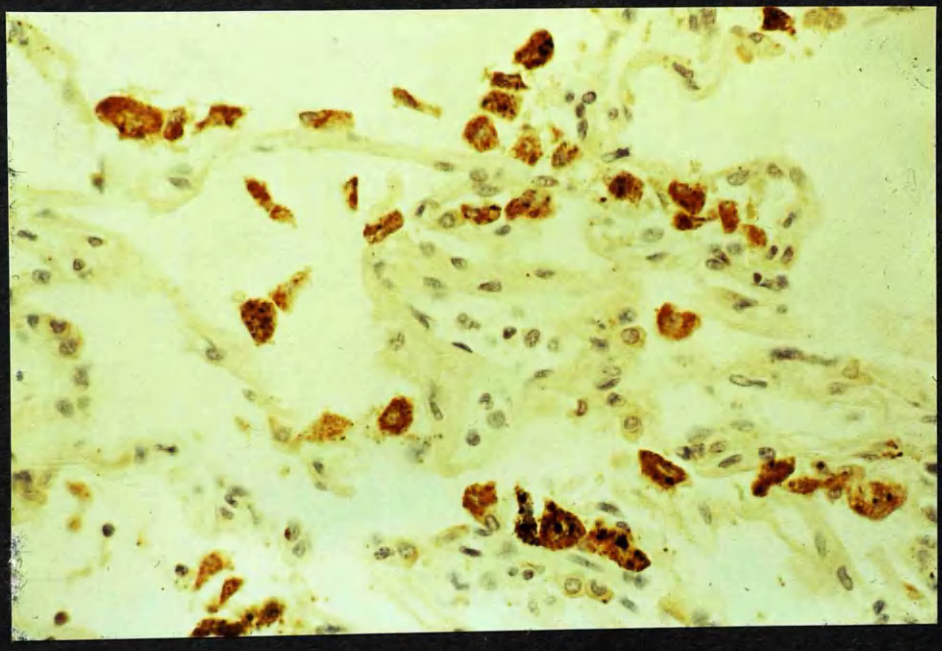
FIG. 6.6 Cathepsin D in neurones of normal brain. PAP method. x210.

FIG. 6.7 Cathepsin D in bronchiolar epithelium, lung. Reaction product is visible as brown granules towards the luminal side of the nuclei.
PAP method. x72.

FIG. 6.8 Cathepsin D in lung. Brown reaction product and black carbon particles are present in the same alveolar cells, which are therefore macrophages.
PAP method. x188.

FIG. 6.9 Cathepsin D in syncytotrophoblast of chorionic villi.
PAP method. x72.





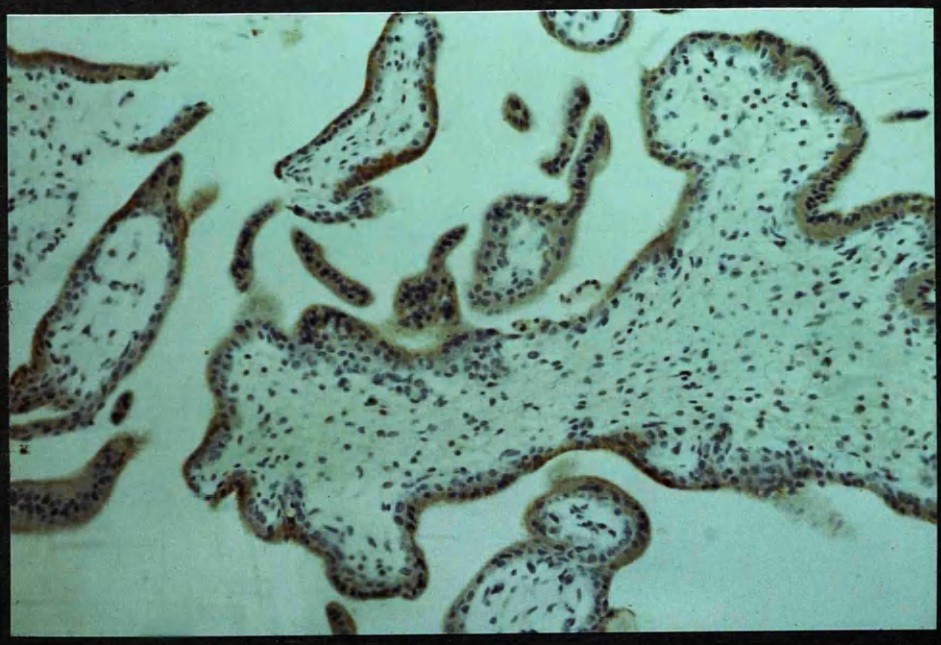
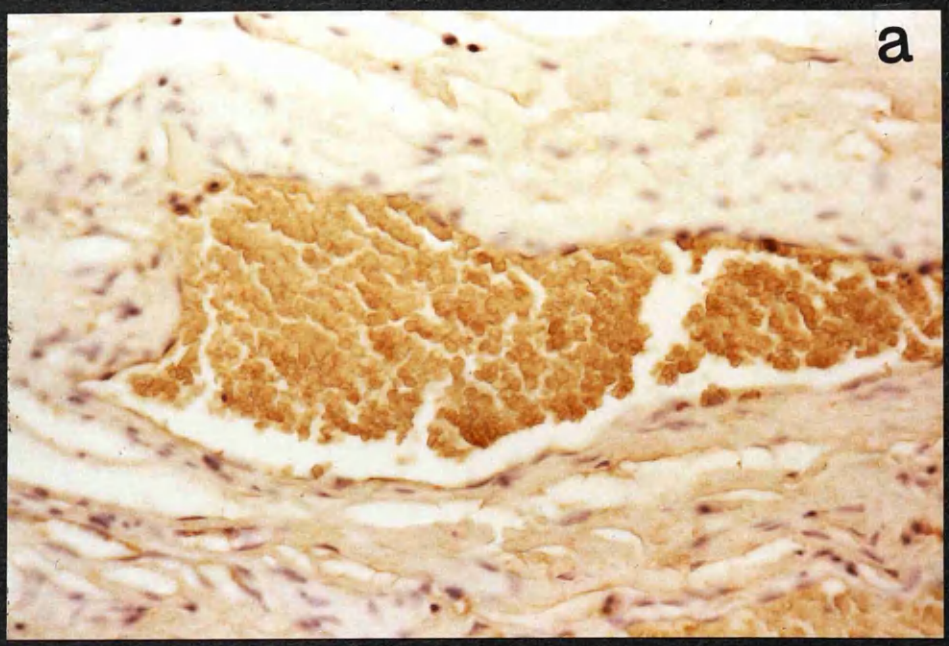


FIG. 6.10a SMP on red blood cell membranes, within a small blood vessel.
PAP method. x188.

FIG. 6.10b Control section, incubated with normal rabbit serum. The red blood cells are negative, showing that the brown labelling in (a) is not endogenous peroxidase.
PAP method. x188.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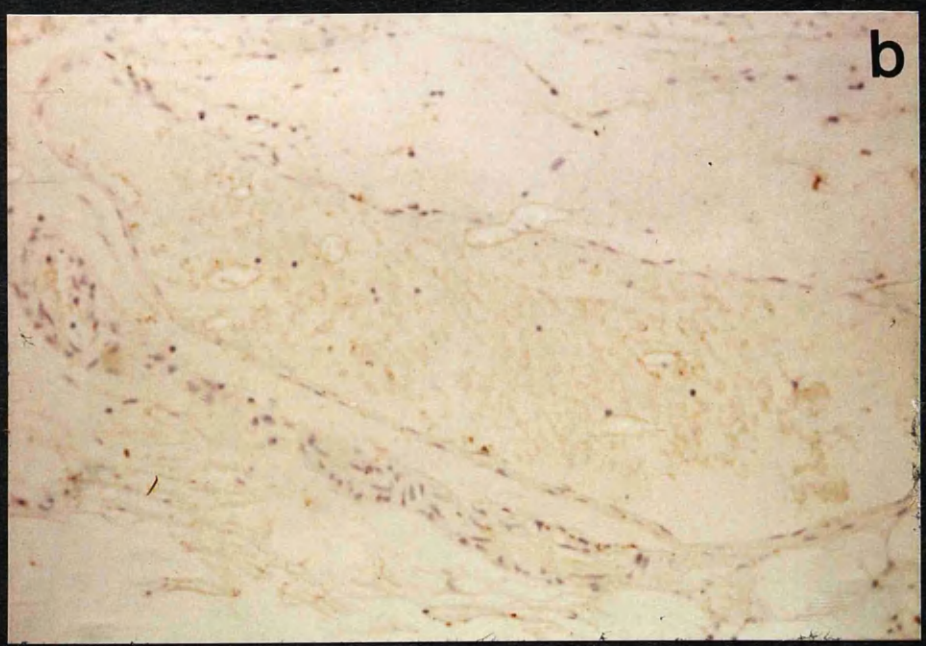
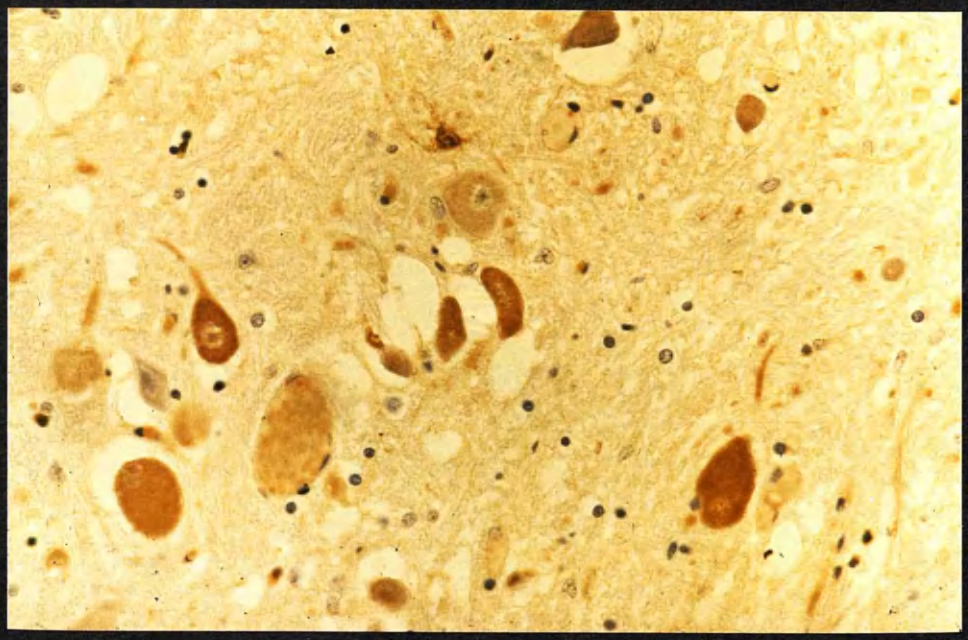

b

FIG. 6.11 SMP in neurones of brain.
PAP method. x288.

FIG. 6.12 Granular labelling of SMP in epithelium of choroid plexus.
PAP method. x470.



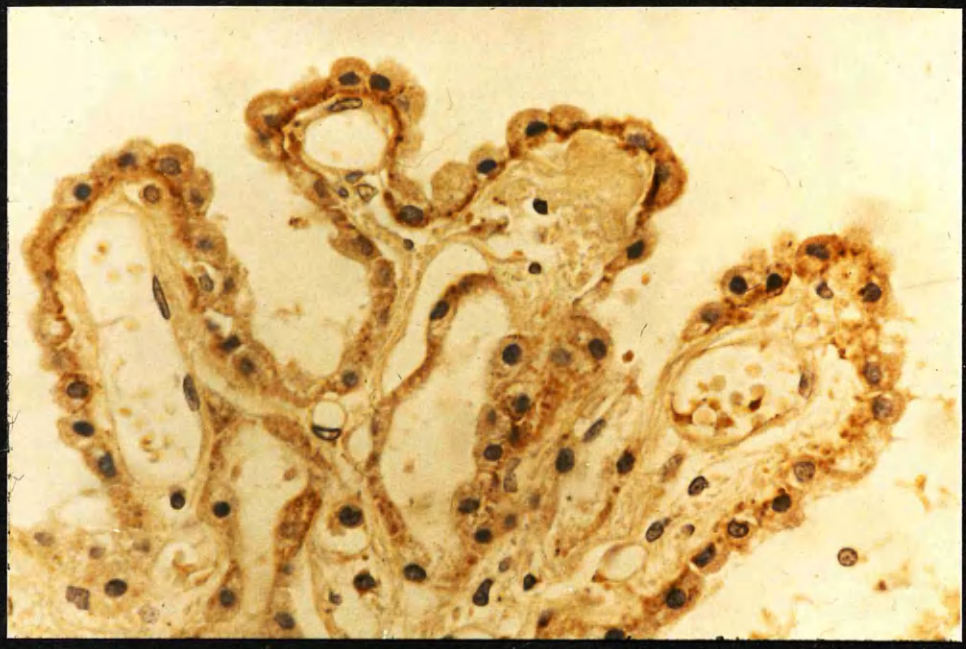
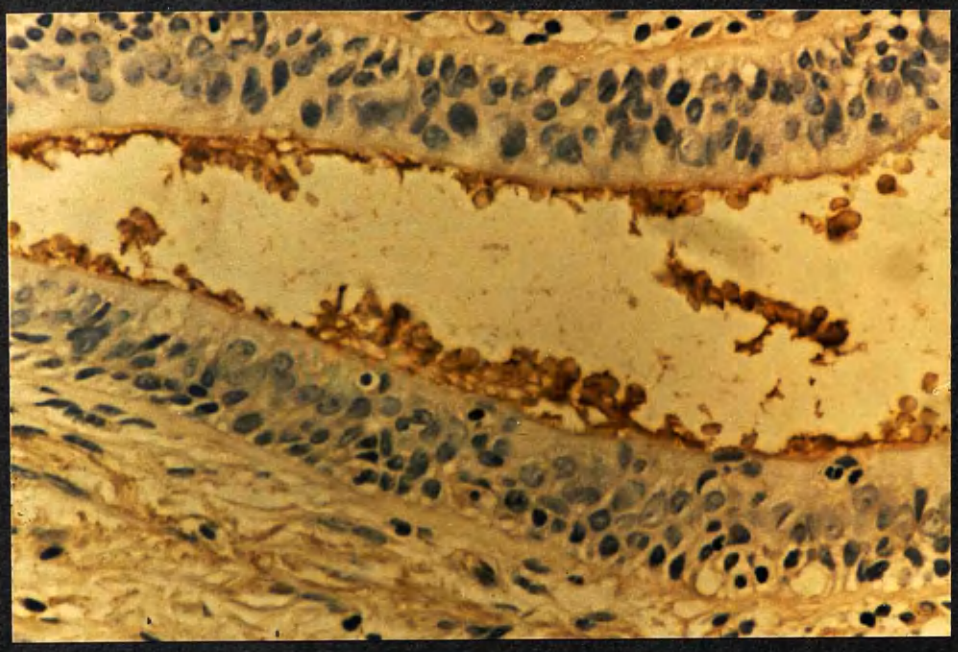
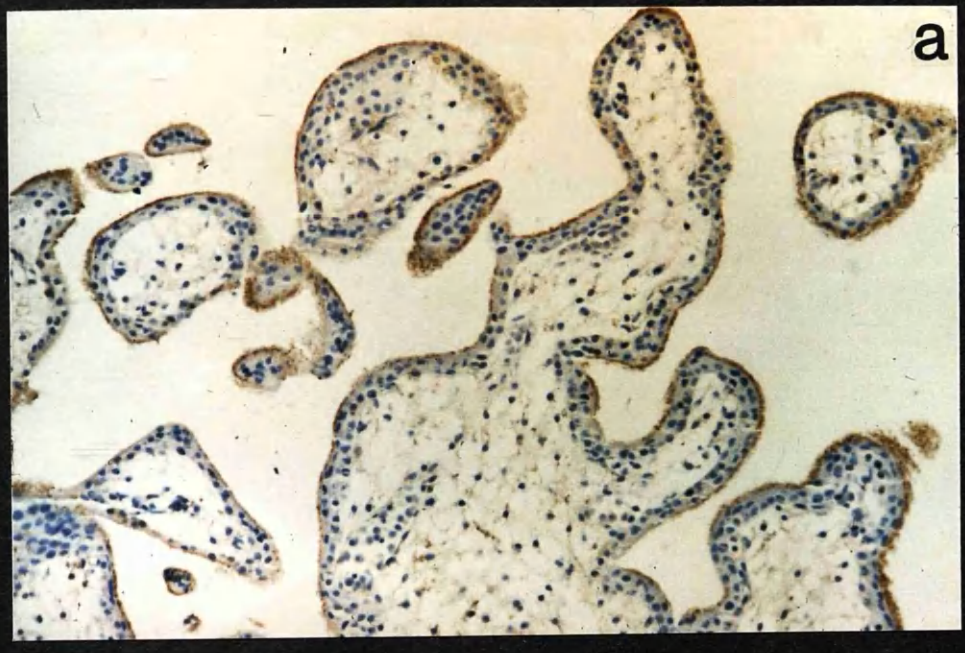


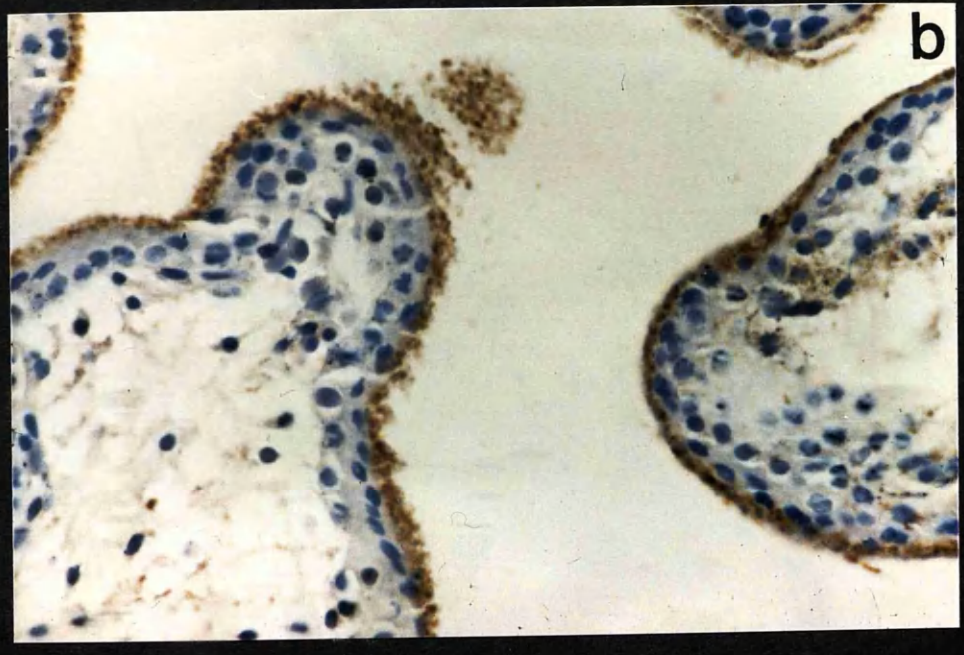
FIG. 6.13 SMP on surface of cilia, bronchial respiratory epithelium and on the membranes of red blood cells in the lumen.
PAP method. x470.

FIG. 6.14 a) SMP on surface of syncytiotrophoblast of chorionic villi.
PAP method. x188.

b) High power of a).
PAP method. x470.




a


b

DISCUSSION

The results confirm that cathepsin D is widely distributed in human tissues. Its presence in synovial cells (Poole et al, 1972; Matthews et al, 1981) has not been confirmed but in the kidney it was found in the same site, namely in the renal tubular epithelium, as was reported previously in the rat (Baricos & Shah, 1984). Cathepsin D appears to be found mainly in cells of the mononuclear phagocyte system. Like lysozyme (muramidase) (Mason & Taylor, 1975) it seems more prominent in reactive histiocytes, as in lymph node and spleen, than in resting phagocytes in liver, brain and placenta. Its presence in epithelium, for example of bronchi and bowel, shows that it does not indicate phagocytic activity, but that it may play a role in more general metabolic activity.

Cathepsin D is generally regarded as a lysosomal enzyme (Barrett, 1980). Its distribution in the body would be consistent with this, as would its granular appearance in cells, although the possibility of its being within other subcellular structures, for example in the gastric mucosa, cannot be excluded.

The present study has clearly established that the distribution of cathepsin D is different from that of lysozyme (Mason & Taylor, 1975; Klockars & Reitamo, 1975) and, according to published accounts, from that of α_1 proteinase inhibitor (antitrypsin) (Isaacson et al, 1981; Tahara et al, 1984), although it seems to be similar to that of cathepsin B (Crocker, Burnett & Jones, 1984; Howie, Burnett & Crocker, 1985), a biochemically unrelated enzyme in the cysteine proteinase category.

The SMP results did not yield convincing evidence of SMP in most cases studied. One problem in interpretation of the results is the effect of adjacent red blood cells, which were usually strongly positive. This appeared to explain the staining on the surface of the

bronchial epithelium and could explain that on the surface of the syncytiotrophoblast, which is normally in contact with blood. The labelling of choroid plexus is less easy to explain in this way and could be genuine, although further cases attempted were negative. Other ciliated epithelia of fallopian tube and vas deferens were negative. Similarly, the staining of neurones is not convincing and is under further study. The staining in the bowel could be genuine or could be due to diffusion of red blood cell products or gastric juice from higher up the bowel. Further study of the colonic mucosa would be worthwhile. It is not clear whether staining of the plasma cells is genuine, although plasma cells in malignant lymphomas were sometimes strongly positive (see Chapter 10).

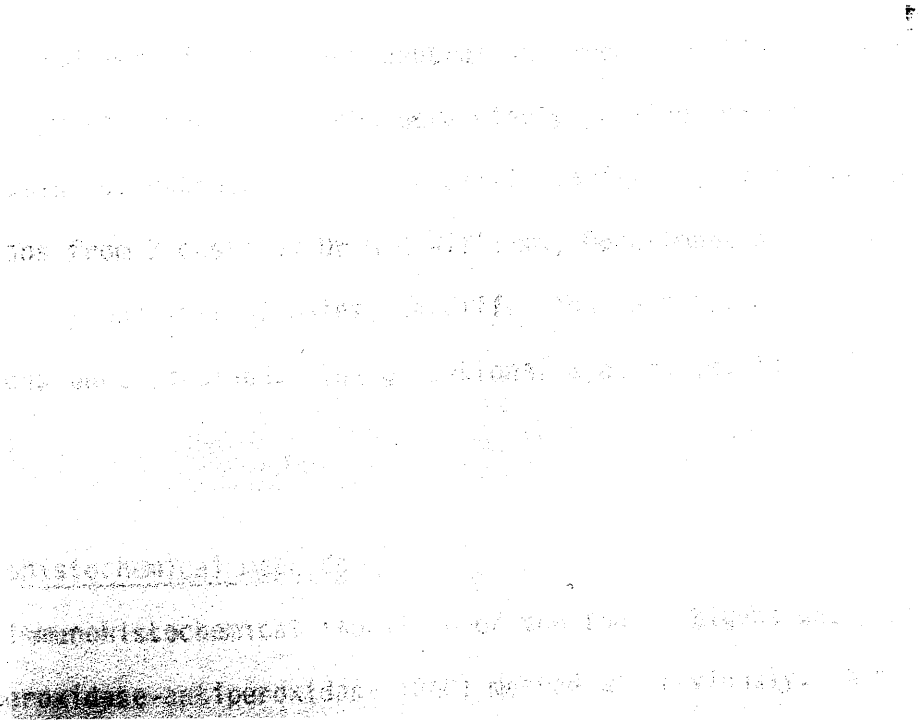
The wide distribution of cathepsin D and to a lesser extent, SMP is in strong contrast to that of the other aspartic proteinases, pepsin, gastricsin and renin. Why this is so is not clear. Because of the structural similarity between the aspartic proteinases a common evolutionary origin has been suggested. It could be that cathepsin D is the least highly evolved and that its distribution reflects an origin in unicellular organisms (Barrett, 1980b).

CHAPTER 7

THE LOCALISATION OF ASPARTIC PROTEINASES IN THE DEVELOPING STOMACH

INTRODUCTION

It will be shown that about 5% of gastric carcinomas contain pepsinogen; by contrast, the corresponding values for progastricsin, SMP and cathepsin D are 30%, 54% and 100% respectively (Chapter 8). By analogy with other oncofetal antigens, such as carcinoembryonic antigen, alpha fetoprotein (Purtilo & Yunis, 1971) and more recently, CL-187 (Bleday et al, 1985), the aspartic proteinases found in the neoplasms might be expressed by the corresponding normal fetal tissues. It is also possible that the enzymes produced most frequently by gastric carcinomas appear earliest embryologically and it was thus considered of interest to determine the time course in which the zymogens appear in the developing fetal stomach.



MATERIALS AND METHODS

Antisera

Polyclonal rabbit antisera to pepsin, gastricsin, cathepsin D and slow moving protease were used as described in detail previously.

Tissues

Stomachs were collected from fetuses of known gestational ages as assessed by crown-to-foot and crown-to-rump measurements. Whenever possible, the stomachs were sectioned longitudinally to include the body and antrum. The slices were paraffin embedded and 4 μm sections were cut, one being stained with haematoxylin and eosin for preliminary study. Only those cases which showed well preserved tissue and well orientated sections were used for immunohistochemical investigations. Twenty-three cases were collected from post mortem material which had been fixed and stored in neutral buffered formalin at the Leeds Maternity Hospital. Additional stomachs were collected from autopsies, conducted within 24 hours of death in the General Infirmary and St James's Hospitals, Leeds (8 cases) and Aberdeen Maternity Hospital (12 cases) and were fixed in 10% neutral buffered formalin. Paraffin blocks from 8 further fetal stomachs were kindly provided by Dr J W Keeling, Department of Pathology, John Radcliffe Infirmary, Oxford and blank sections from 2 cases by Dr G G Williams, Department of Pathology, University Hospital of Wales, Cardiff. Thus a total of 53 fetal stomachs were examined. The gestational ages ranged from 10 to 38 weeks.

Immunohistochemical methods

Immunohistochemical labelling of the four antigens was performed by the peroxidase-antiperoxidase (PAP) method as previously. Antisera to pepsin and gastricsin were diluted 1 in 200 and those to cathepsin D and SMP, 1 in 100 in 0.05 M TRIS - buffered saline, pH7.6. Sections were

overlain for 24 hours at $4^{0}C$ with dilute antisera. With every batch of slides, a positive control section was included, this being normal human spleen for cathepsin D and normal human adult gastric mucosa for the other enzymes. Swine anti-rabbit immunoglobulin (1 in 25) and rabbit peroxidase-antiperoxidase complexes (1 in 50) (Dakopatts A/S, Denmark) were used as secondary antibodies. Sections were developed in 3,3' diaminobenzidine (Sigma Chemical Co, Poole, Dorset) and counterstained with haematoxylin. In selected cases, antisera were pre-incubated with the appropriate antigen for 1 hour at room temperature before application to the sections.

RESULTS

There was no evidence of aspartic proteinases in the fetal stomach until 12 weeks, when SMP was identified in some cells of the surface epithelium and progastricsin in a few cells at the base of developing glands. At 14-16 weeks SMP was the more prevalent enzyme of the two and appeared in occasional cells in the glands. At 17-18 weeks pepsinogen and cathepsin D began to appear. Pepsinogen was present in the basal part of the surface epithelium cells and in the cytoplasm of occasional cells at the base of the glands, the pattern being similar to that of progastricsin. Cathepsin D was first seen in cells at the base of the glands, where there was diffuse cytoplasmic labelling, sometimes with more intensely reactive cytoplasmic granules. By this stage SMP was labelled strongly in surface epithelium and, in some cases, in the glands (Fig 7.1).

After 17 weeks all four aspartic proteinases were identified in most cases (Figs 7.2-7.4 and Table 7.1). From 17-24 weeks pepsinogen and progastricsin were less strongly labelled in surface epithelium than before, while positive cells in the gastric glands became more numerous. Progastricsin was present in and pepsinogen absent not only from the gastric antrum, but also from several cases of gastric body, although both were equally present in the body from 34 weeks onwards. SMP was identified in almost all cases until term, although the intensity of staining appeared to diminish somewhat after 22 weeks. Cathepsin D was also present in most cases and its pattern and distribution did not significantly change. Pre-incubation of antisera to pepsin and gastricsin with their respective antigens resulted in complete abolition of staining.

TABLE 7.1
ASPARTIC PROTEINASES IN THE EARLY DEVELOPING STOMACH

		Number of positive cases (equivocal cases in brackets)			
Gestation (weeks)	Total	Pepsinogen	Progastricsin	Cathepsin D	SMP
≤10	2	0	0	0	0
12	2	0	1	0	2
14-16	8	1	1(2)	0	4(3)
17-18	8	6	6	4(1)	7(1)
19-20	7	2(1)	5(1)	4	3

Note: after 20 weeks all four aspartic proteinases were present in most cases studied

FIG. 7.1 Slow moving protease in glands and surface epithelium in fetal gastric mucosa at 18 weeks gestation. (PAP method x 215).

FIG. 7.2 Progastricsin in the deeper parts of the glands in fetal gastric body at 17 weeks gestation. (PAP method, x 330).

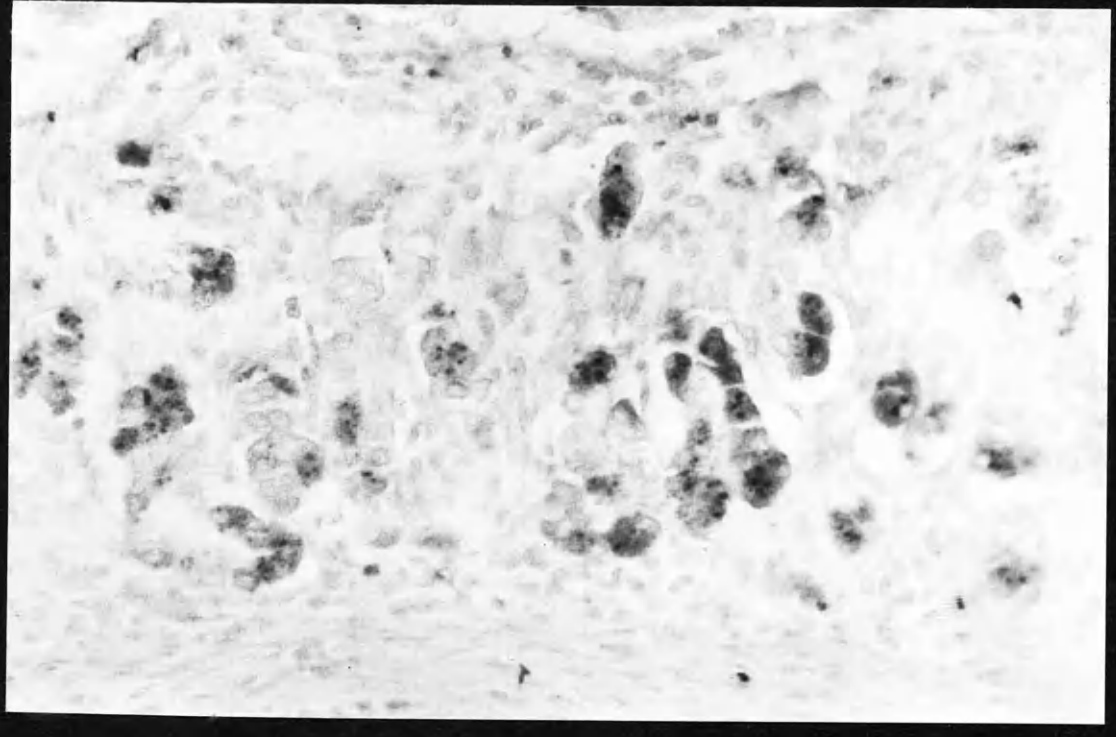
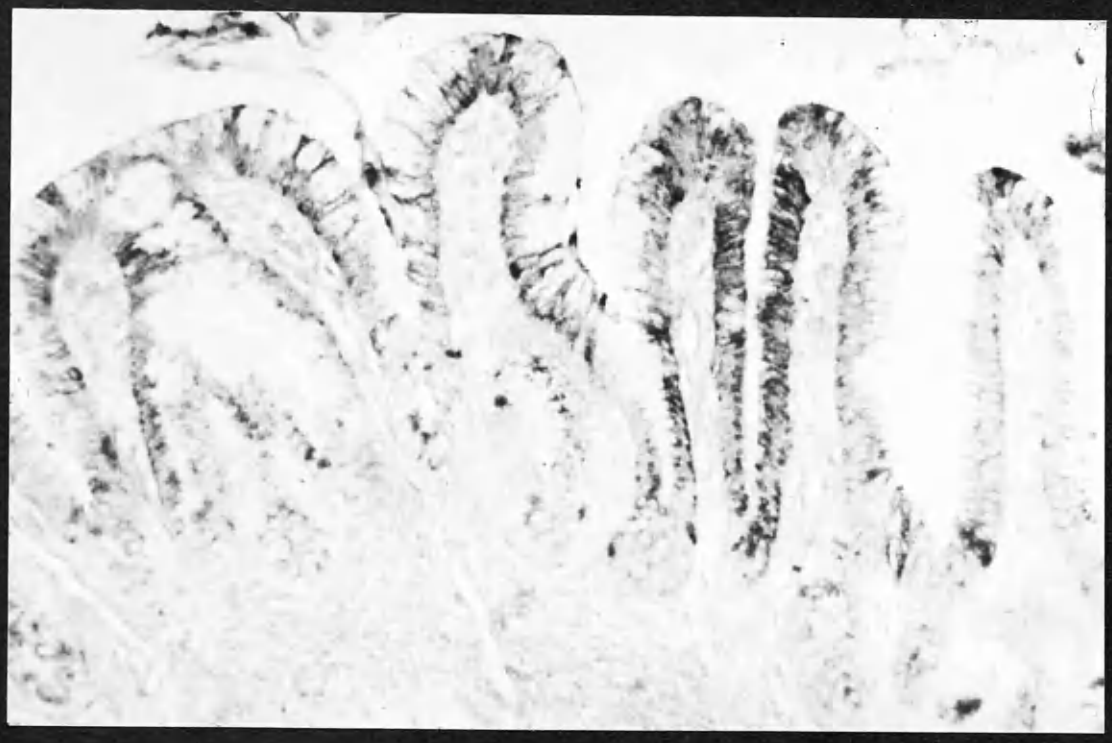
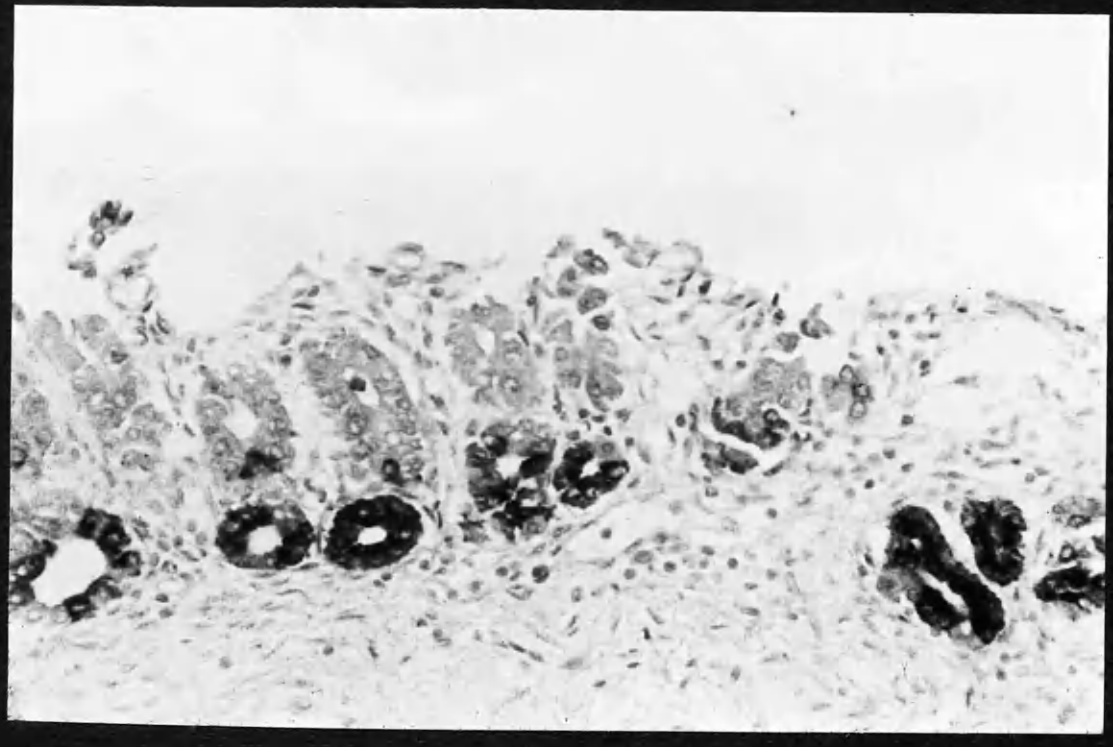


FIG. 7.3 Cathepsin D in cells of the glands in fetal gastric mucosa at 34 weeks gestation.
(PAP method x 215).

FIG. 7.4 Pepsinogen in the deeper parts of the glands in fetal gastric mucosa at 35 weeks gestation.
(PAP method, x 330).





DISCUSSION

It has long been known that, from as early as 16 weeks of gestation (Keene & Hewer, 1924; Keene & Hewer, 1929) the fetal gastric juice of various species, including humans, has acid proteinase activity, presumed to be due to pepsin (Langendorff, 1879). In previous investigations, immunoelectrophoresis of fetal gastric mucosal extracts suggested that progastricsin appeared during the third month of gestation and pepsinogen by the fourth month, although in histological sections both zymogens could only be detected by immunofluorescence after 5 months.

The present results indicate that SMP and progastricsin both appear in the developing stomach at 12 weeks, soon after the glands are formed (6 - 9 weeks) and just before the parietal cells become discernible (12 - 15 weeks) (Salenius, 1962) with the production of hydrochloric acid (16 weeks) (Deren, 1971). Progastricsin is, however, only occasionally present until 17-18 weeks, around the time the chief cells differentiate (15 - 23 weeks) (Salenius, 1962), after which all four aspartic proteinases are normally detectable.

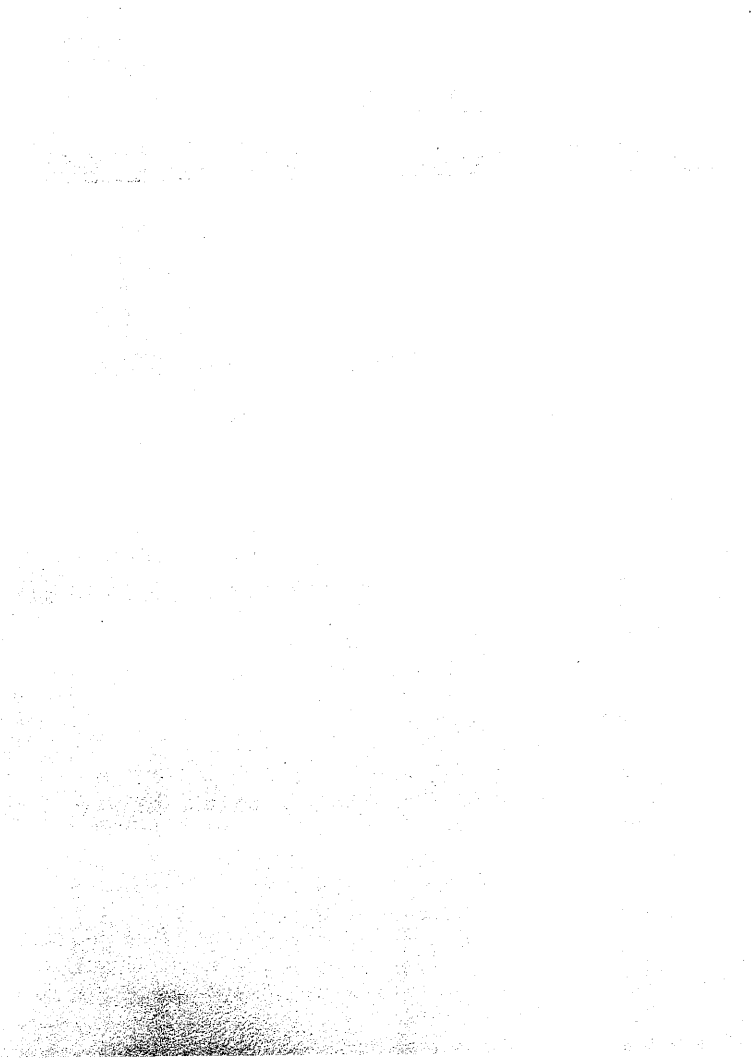
The amniotic fluid also appears to contain zymogens; immunoelectrophoresis yields progastricsin at 32 weeks and pepsinogen at 11 weeks, although the possibility that these are of maternal origin cannot be excluded (Liebman & Samloff, 1978). SMP, however, was noted by immunoelectrophoresis of fetal gastric mucosa from approximately 8 weeks of gestation onwards (Hirsch-Marie et al, 1976). In a separate study, SMP was proposed as main acid proteinase in the human stomach at birth (Foltmann & Axelsen, 1980). Clearly, there is some inconsistency in the findings reported in these earlier investigations and much of this may be explained by the different techniques and conditions, such as exposure to acid pH, employed in the various studies. The present

investigation was carried out to resolve these discrepancies and, by including cathepsin D, to consider all the aspartic proteinases now known to be present in the human stomach (Kay et al, 1987). The results support the earlier suggestions of Hirsch-Marie et al, 1976.

In animals, the gastric aspartic proteinases differ from those in the human. The stomachs of fetal piglets and calves express not pepsin or gastricsin but the milk clotting aspartic proteinase, chymosin (rennin) (Foltmann & Axelsen, 1980). Unlike the other enzymes, chymosin cannot digest immunoglobulins. The neonate can thus develop immunity by ingestion and absorption of antibodies from maternal colostum until chymosin production is replaced by that of pepsin approximately 4 weeks after birth (Foltmann, 1981). A similar protection mechanism for antibodies cannot exist in the human stomach as four enzymes, all of which can digest immunoglobulins, are present in gastric mucosa long before birth and postnatal immunity is maintained by placental transfer of antibodies.

The aspartic proteinases, including chymosin, all show a remarkable homology in structure and probably evolved from a common precursor (Foltmann, 1981). It has been suggested that cathepsin D might be the primitive intracellular enzyme of unicellular organisms (Barrett, 1980b). Cathepsin D appears to be a ubiquitous enzyme, and has been detected in almost all normal and neoplastic tissues so far studied (Reid et al, 1986). On the other hand pepsinogen, progastricsin and SMP, are mainly produced by the gastric mucosa and by neoplasms of the stomach. The present study suggests that SMP is ontogenetically more primitive than progastricsin and pepsinogen. The distribution of staining for progastricsin, which was more widespread and intense than that of pepsinogen, tends to suggest that progastricsin may develop before pepsinogen, although the number of cases studied over the critical gestational age range (12 - 18 weeks) is too small to allow a

definite conclusion. The descending order of frequency with which SMP, progastricsin and pepsinogen occur in the epithelium of gastric carcinomas is also consistent with the hypothesis that this reflects their order of appearance in the evolution of the human gastric mucosa.



CHAPTER 8

ASPARTIC PROTEINASES IN PRIMARY CARCINOMA OF STOMACH

INTRODUCTION

The possibility that gastric carcinomas may produce aspartic proteinases appears to have been somewhat neglected until recently, possibly because biochemical analysis of tumour extracts revealed no trace of acid proteolytic activity (Greenstein & Stewart, 1942; Dixon & Webb, 1964). Proteolytic activity, optimal at pH around 3.5 but almost absent at 2.0, was demonstrated in extracts of 6 gastric carcinomas (Taylor, 1960a). It is not clear whether this was due to cathepsin D, which is inactive at pH 2.0, or to gastricsin which is still active at this pH or to a mixture of the two enzymes. Similar proteolytic activity, maximal at pH 3.3 was found in extracts of adenocarcinomas from all parts of the intestine (Taylor, 1960b), which would also fit with either cathepsin D or gastricsin. Later, two enzymes were detected in gastric carcinomas and on the basis of chromatographic and electrophoretic properties were considered to be cathepsins D & E (Etherington & Taylor, 1972).

Kalinovsky & Parshin (1975) demonstrated uptake of tyrosine-I-C^{14} and serine-3-C^{14} into homogenates of gastric cancer and detected small amounts of radioactive pepsinogen in the tumour tissue. On electrophoresis of extracts of gastric carcinoma from 18 patients, Kalinovsky, Melnikov & Seits (1977) noted 3 fast-moving anode fractions, the positions of which were similar to those of pepsinogen and pepsin from normal gastric mucosa. These fractions proved to have proteolytic activity for gelatin at low pH. Similarly situated inactive pepsinogen bands were found in the metastases in para-aortic, omental and supraclavicular lymph nodes from these patients.

In an interesting study, Hirsch-Marie et al (1976) demonstrated by standard and bidimensional immunoelectrophoresis that pepsinogen and progastricsin appeared to be either absent or present in markedly smaller amounts in acetone extracts of 5 gastric carcinomas than in

normal mucosa. On the other hand, slow moving protease (SMP) was present in all 5 cases, usually in almost as great amounts as in normal mucosa.

Immunofluorescence studies confirmed the presence of progastricsin in one well differentiated carcinoma but pepsinogen could not be located in any of the tumours. SMP, on the other hand, was strongly labelled in dedifferentiated mucosal glands and in some isolated neoplastic cells.

The present study was undertaken in order to determine whether aspartic proteinases could be demonstrated immunohistochemically in gastric carcinoma. As preliminary experiments indicated that all the zymogens were well preserved in tissues fixed in 10% formol saline, material from the surgical files, fixed in this way and paraffin embedded, was used. This permitted retrospective examination of tissue which would otherwise take years to accumulate.

MATERIALS AND METHODS

Antisera

Antisera to pig pepsin and pepsinogen, human pepsin and gastricsin, cathepsin D, SMP and lysozyme were used as described in Chapter 2. All dilutions were in 0.05M Tris - HCL buffered saline (TBS) pH 7.6 except anti-gastricsin, which was diluted in 20% normal human serum in TBS. Anti-pig pepsin was used at 1:200, anti-lysozyme at 1:1000 and all others at 1:100.

Tissues

Blocks of gastric carcinoma removed by gastrectomy were selected from the routine surgical histology files of the Pathology Departments, Glasgow Royal Infirmary and the General Infirmary at Leeds. All tissues had been fixed in 10% formol saline and paraffin embedded. Altogether, 125 cases from Glasgow Royal Infirmary were examined for the presence of pepsinogen and 114 for progastricsin. Cases examined from the General Infirmary at Leeds included 19 which were studied for cathepsin D, 28 for SMP and a further 62 carcinomas with metastatic lymph node deposits in the gastrectomy specimen which were examined for progastricsin.

In all cases 4-6 μm were cut and labelled by the peroxidase anti-peroxidase (PAP) method as described in Chapter 3. Sections were counterstained with haematoxylin or methyl green.

Controls

Wherever possible blocks were chosen to include non-neoplastic gastric mucosa adjacent to the carcinoma. In addition, a section of normal gastric body mucosa served as a control for pepsinogen, progastricsin and SMP while a section of normal spleen was used as positive control for cathepsin D.

Sections of carcinomas were also incubated with antisera,

preincubated with the corresponding enzymes or zymogens, as in Chapter 3. The supplies of pure cathepsin D and SMP were too small to allow absorption experiments to be performed on the carcinomas, but experiments on selected strongly positive cases have been described in Chapters 3 and 10.

Selected carcinomas were also incubated with normal rabbit serum or with preimmune serum from the rabbit in which the antiserum had been raised.

Classification of tumours

Gastric carcinomas which contained either pepsinogen or progastricsin were classified 1) according to the degree of differentiation and 2) into diffuse and intestinal type by the system of Laurēn (1965). The pepsinogen positive cases were classified by Dr. J. Jass, Histopathology Department, Central Middlesex Hospital, London and the progastricsin cases independently by Dr. M.F. Dixon, Department of Pathology, University of Leeds and by the author.

Case details

The case notes of the pepsinogen positive cases were reviewed to determine a) the site of origin of the carcinomas in the stomach and b) the duration of survival.

The duration of survival of patients with gastric carcinoma in the General Infirmary at Leeds (LGI) was obtained by courtesy of Mr. D. Ward, Department of Surgery, LGI. The survival times of the patients with progastricsin-containing carcinomas was compared with those with progastricsin-negative carcinomas. Statistical analysis was performed by computer with a BMDP software package analysis (LOGRANK) program (BMDP Life Tables and Survival Functions, BMDP Statistical Software, Copyright 1983 Regents of California) (Peto et al, 1977). Analysis was performed in collaboration with the Department of Medical Statistics, University of Leeds.

RESULTS

Pepsinogen

Seven (5.6%) of the 125 gastric carcinomas examined labelled with both anti-pig pepsin and anti-pig pepsinogen. In all cases staining was focal, being present in some tumour acini and not others and often in only some cells of an acinus (Figs. 8.1 - 8.3). In areas of tumours where there was no acinar formation, staining was also focal, although often present in large numbers of cells (Fig. 8.4). All 7 cases showed the same pattern of labelling with both anti-pepsin and anti-pepsinogen. The reaction product was generally intense and often granular and was present in the cytoplasm of the malignant epithelial cells; none of the stromal cells were positive.

In most cases there was non-neoplastic gastric mucosa adjacent to the tumour. Mucosa of body type was always positive, even when there was severe inflammation, but areas of intestinal metaplasia were negative.

Two further cases gave equivocal results with only weak, diffuse staining in tumour cells. There was no evidence of pepsinogen in any of the other carcinomas, although the gastric body mucosa was always positive.

Controls

In all 7 cases labelling of tumour cells was abolished by prior incubation of antipepsin with commercial pig pepsin or of antipepsinogen with pure pepsinogen. As in the normal stomach (Chapter 3) the enzyme was found to be more efficient than the zymogen in blocking staining. In all cases sections incubated with normal rabbit serum in place of antiserum were negative, both in the carcinoma and non-neoplastic stomach.

Classification of pepsinogen-containing gastric carcinomas

a) by degree of differentiation

Five of the 7 cases were moderately differentiated adenocarcinomas and 2 were poorly differentiated carcinomas.

b) by the Laurén classification

Three of the carcinomas were of the so-called intestinal type and 2 of diffuse type. The other 2 were difficult to classify.

Case details

The case notes of only 4 of the 7 patients with pepsinogen-containing gastric carcinomas could be traced.

a) Site of tumour

One of the tumours was an ulcer-cancer of the antrum and 1 arose around the lower oesophagus, but the site of origin of the others could not be found in the case records.

b) Survival

One patient died 3 months after gastrectomy, but the outcome in the other cases was not known.

Progastricsin

Thirty-three (29%) of the 114 gastric carcinomas from Glasgow Royal Infirmary and 26 (42%) of the 62 cases from the General Infirmary at Leeds showed evidence of progastricsin in the cytoplasm of the malignant cells (Figs. 8.5 and 8.6). In addition, the latter series included 4 cases in which there was only faint diffuse labelling of the tumour cells; the result in these cases was considered equivocal. In 4 cases from Glasgow Royal Infirmary, the malignant cells close to the normal mucosa were labelled for progastricsin, but those further away were negative. This was considered to be an effect of diffusion and these cases were therefore considered negative.

Like pepsinogen, progastricsin was usually focally distributed within each tumour (Fig. 8.7) and most malignant cells were either

negative or strongly positive. The reaction product was often granular (Fig. 8.8). Sometimes there appeared to be cloning, with most cells in some parts of a given tumour being positive while other areas were negative (Fig. 8.9). In some cases the number of positive tumour cells lessened with the depth of invasion into the gastric wall, but in most cases malignant cells throughout the stomach wall and extraserosal fat were positive.

Controls

In all sections, any non-neoplastic mucosa of gastric body or antrum or duodenum gave strongly positive labelling of progastricsin in the cells, even when the carcinoma was progastricsin negative.

Labelling of tumour cells was blocked in all cases by prior incubation of antigastricsin with gastricsin or progastricsin. The 33 positively identified cases were divided into two groups for absorption experiments with gastricsin derived from two different human sites, i.e. stomach and seminal fluid. In the first group of 16 the labelling by antigastricsin was completely abolished by prior incubation with gastric gastricsin (10 µg/ml). In the second group, which included the 17 most strongly positive carcinomas, absorption experiments were performed with seminal progastricsin or gastricsin at 1 µg/ml. Seminal gastricsin was effective in abolishing labelling by the antiserum to the gastric enzyme whereas in only 2 cases did seminal progastricsin block labelling completely. In the other 15 cases labelling was either not reduced or only partly diminished on incubation with the seminal zymogen.

Twelve of the gastricsin-positive cases of gastric carcinoma labelled with preimmune serum were all negative.

All sections incubated with normal rabbit serum or with preimmune serum showed no evidence of labelling in either the carcinomas or the normal gastric body of antral mucosa.

Classification of progastricsin-containing carcinomas

a) by degree of differentiation

The 33 progastricsin-containing gastric carcinomas from GRI were of all degrees of differentiation. Three were papillary, 12 moderately differentiated, 11 poorly differentiated with some acinar formation and 7 poorly differentiated without acinar formation (Fig. 8.9). There thus appeared to be no predilection for any one histological type.

b) by the Laurēn classification

The 33 gastricsin positive cases, classified according to the Laurēn classification, were grouped as follows:

Diffuse: 13 (including 4 with some intestinal areas)

Intestinal: 16 (including 2 with some diffuse areas)

Mixed: 4

Comparison between pepsinogen- and progastricsin-containing carcinomas

Four of the pepsinogen-containing carcinomas were strongly positive for progastricsin, 1 was weakly positive and 2 others were negative. The other progastricsin-containing carcinomas did not contain pepsinogen.

Case details

Whether or not the type of surgical operation was taken into account, there was no significant difference in survival time between a group of patients with progastricsin-containing gastric carcinomas and that in which the tumours were progastricsin-negative.

Cathepsin D

In all 19 cases there was granular labelling of the cytoplasm of cells in the gastric carcinomas. In some tumours, labelling was predominantly in epithelial cells (Fig. 8.10), in some, in the non-neoplastic stromal cells (Fig. 8.11), while in others both types of cell were labelled (Fig. 8.12). The stromal cells were mononuclear and may

have been macrophages but detailed study was not undertaken to establish this with certainty. Tumours of all degree of differentiation contained cathepsin D (Fig. 8.13).

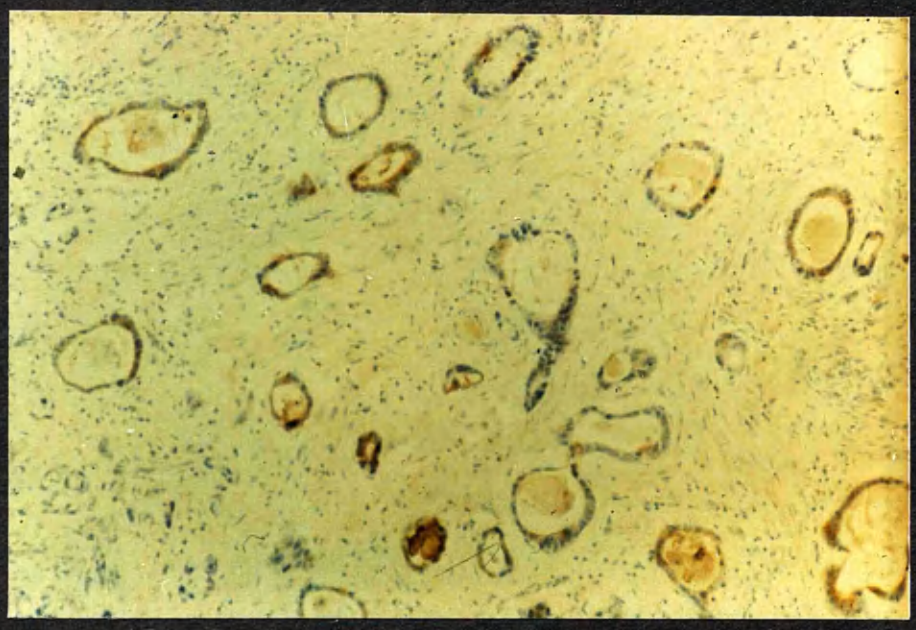
Lysozyme, on the other hand, was present mainly in stromal cells and was seen in fewer neoplastic epithelial cells than cathepsin D.

Slow moving protease

Of 28 cases examined, 21 showed labelling of tumour cells for SMP. The number of positive cells in each case varied from few to many and staining was usually strong (Figs. 8.14 and 8.15). As was observed with progastricsin, both moderately and poorly differentiated carcinomas were positive and staining was focal. Stromal cells were negative, apart from red blood cells, which were usually strongly positive and polymorphs which, in some of the cases where they were present, were weakly reactive. In 4 SMP positive cases where there was tumour in 7 out of 12 lymph nodes, 6 of the deposits were also SMP positive.

FIG. 8.1 Pepsinogen in moderately differentiated gastric adenocarcinoma. The secretions within some of the acini are positive.
Antipig pepsin, PAP method. x54.

FIG. 8.2 Pepsinogen in moderately differentiated gastric adenocarcinoma. Some acini are strongly positive but many others are negative.
Antipig pepsin, PAP method. x115.



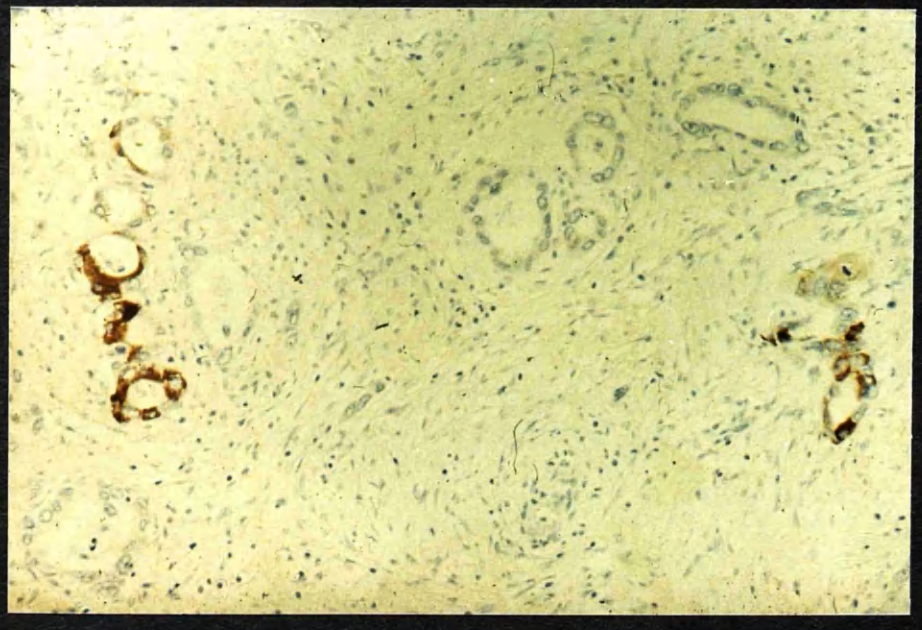
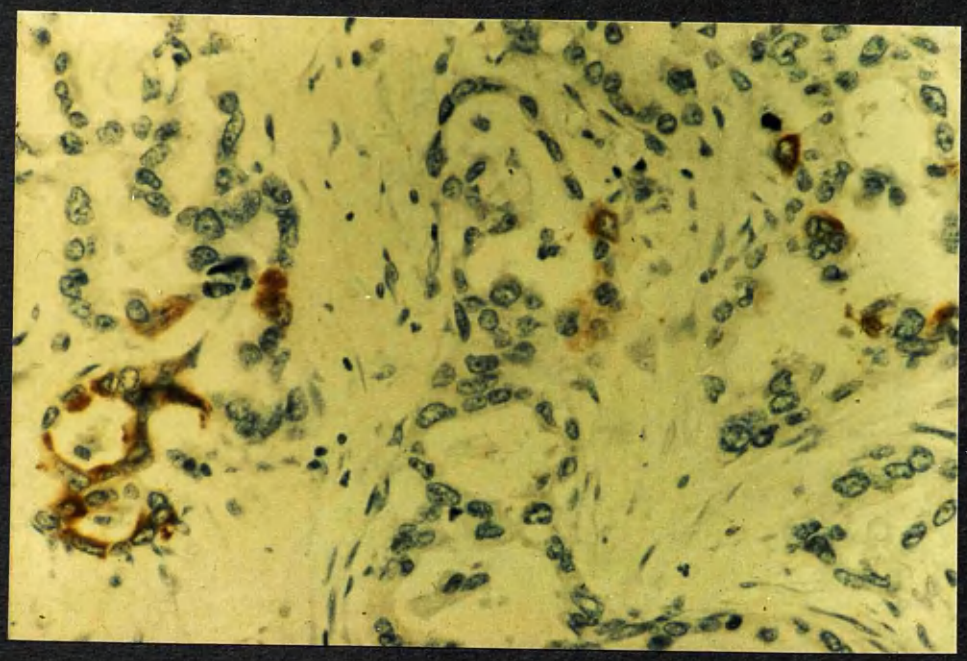


FIG. 8.3 Pepsinogen in moderately differentiated gastric adenocarcinoma. Labelling is notably focal, even within individual tumour acini.
Antipig pepsin, PAP method. x284.

FIG. 8.4 Pepsinogen in poorly differentiated gastric adenocarcinoma.
Antipig pepsin, PAP method. x284.



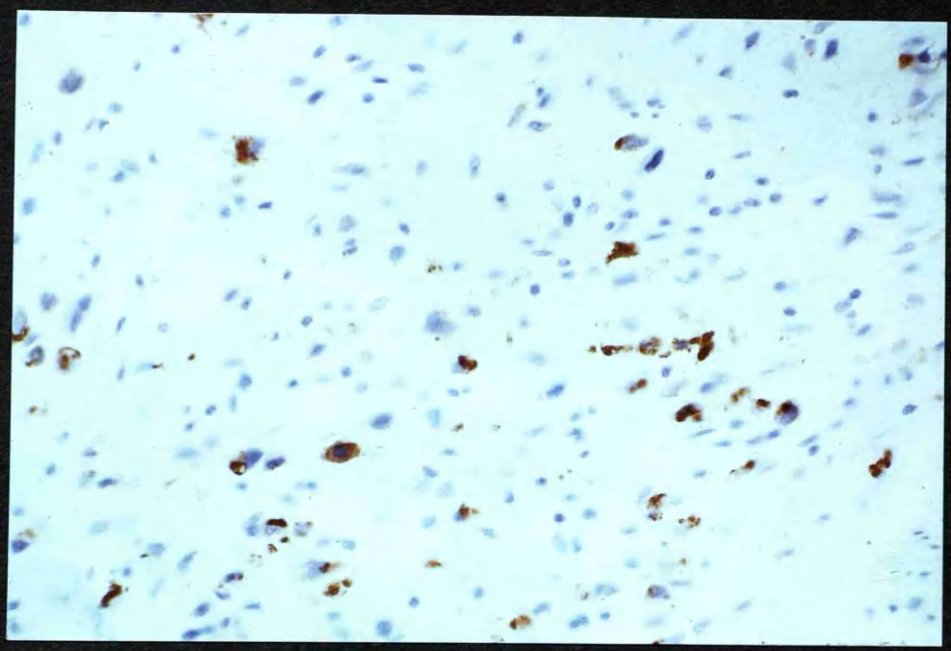
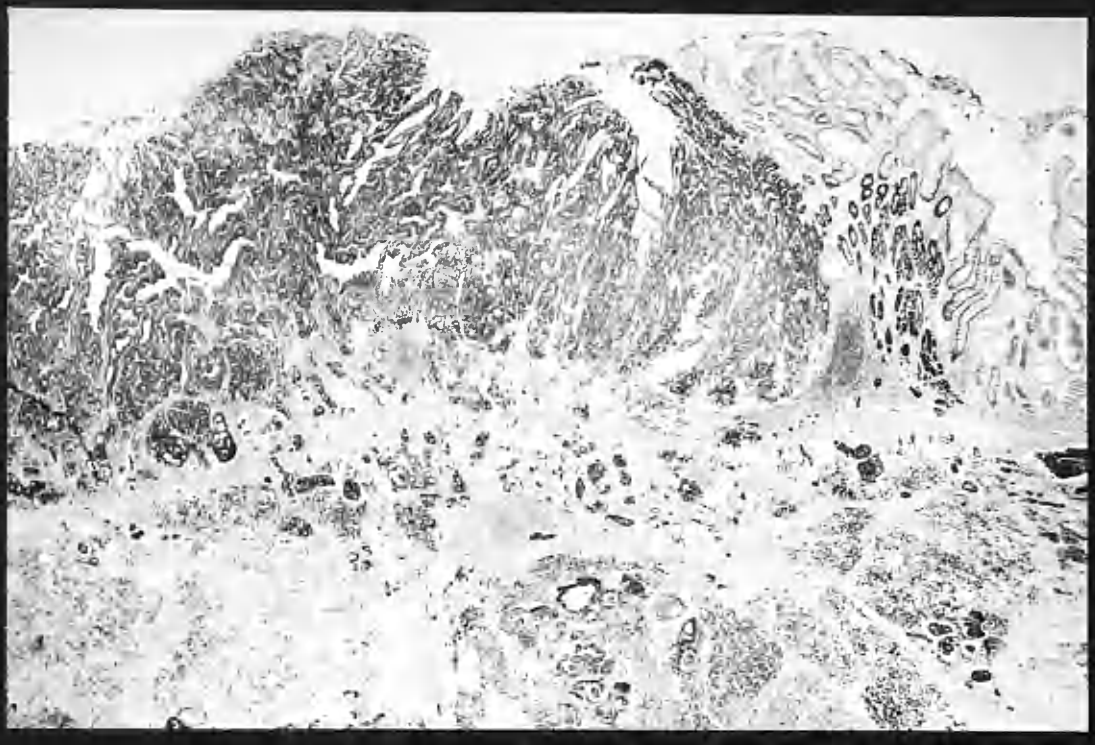


FIG. 8.5 Progastricsin in gastric carcinoma at origin in mucosa and in tumour cells invading through submucosa and muscle.
Antigastricsin, PAP method. x30.

FIG. 8.6 Progastricsin in gastric adenocarcinomas. In this case the tumour in lymphatic channels (L) is not expressing progastricsin while the cells elsewhere are.
Antigastricsin, PAP method. x72.



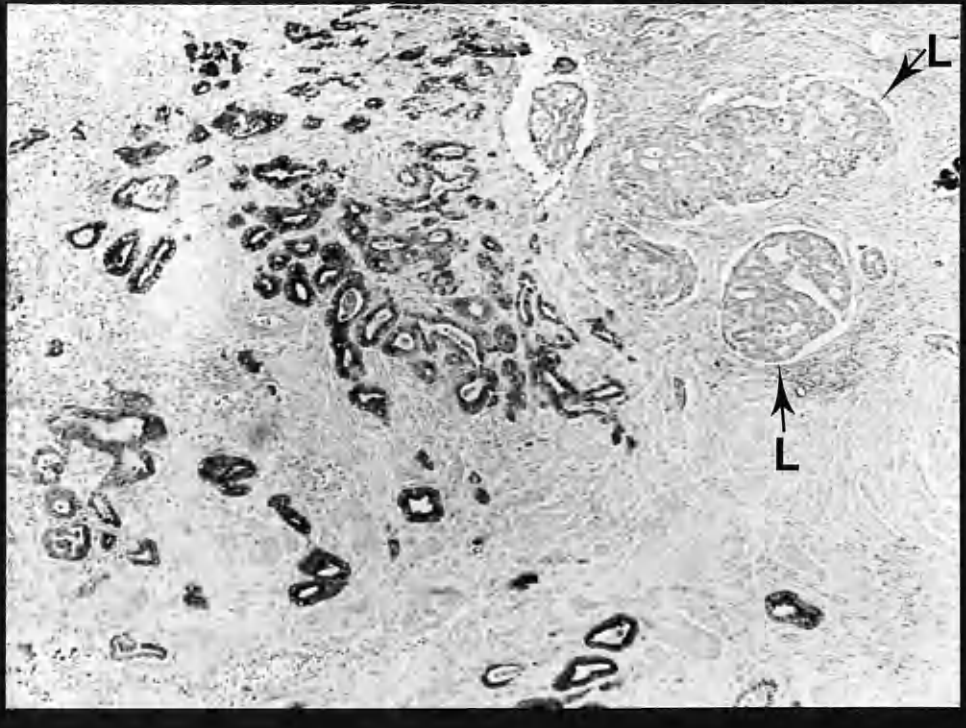
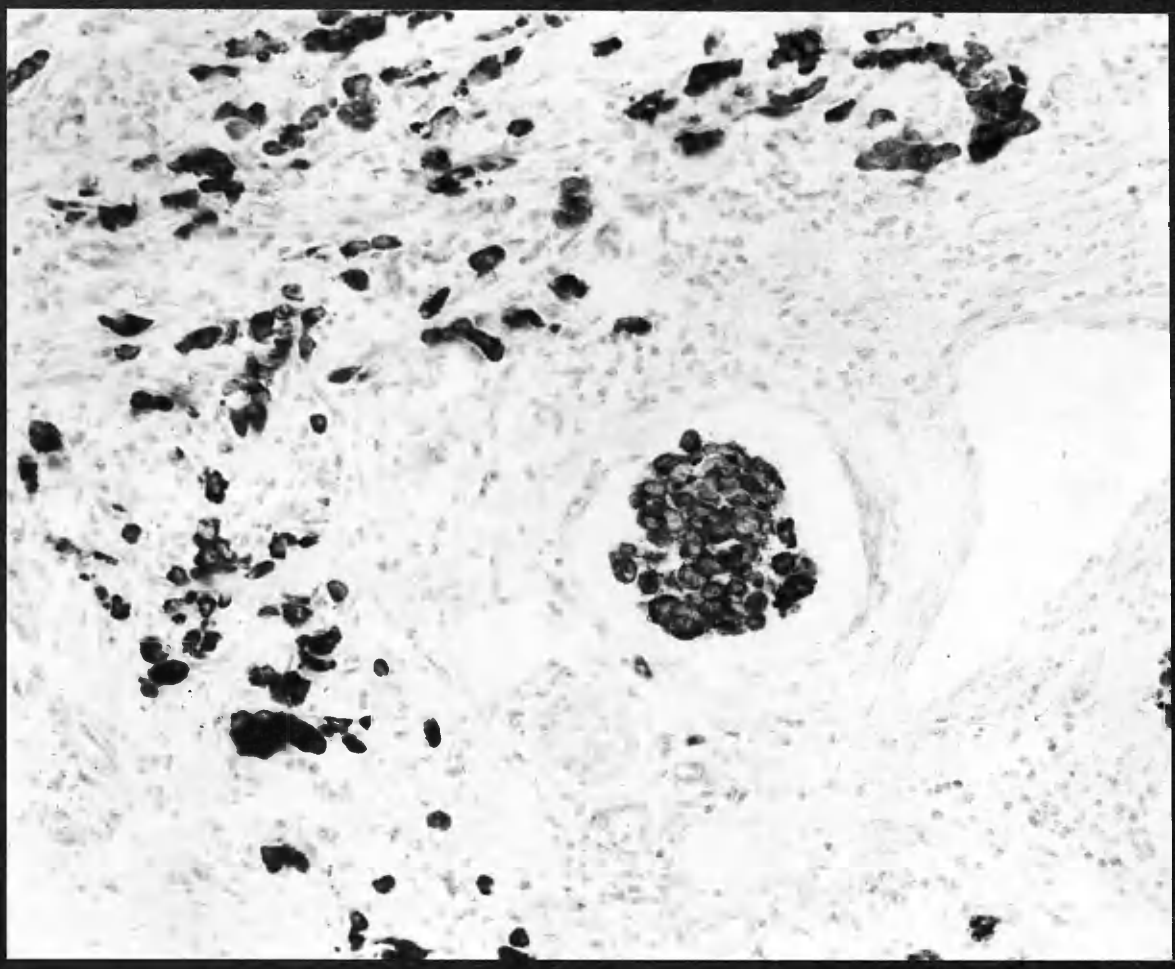

L
L

FIG. 8.7 Progastricsin in moderately differentiated gastric adenocarcinoma invading the muscularis propria of the stomach. Note that the staining is focal.
Antigastricsin, PAP method. x112.

FIG. 8.8 Progastricsin focally labelled in gastric adenocarcinoma. In some cells the staining is clearly granular.
Antigastricsin, PAP method. x348.

FIG. 8.9 Progastricsin in poorly differentiated gastric carcinoma, including cells in a lymphatic channel. Antigastricsin, PAP method. x470.

FIG. 8.10 Cathepsin D in gastric adenocarcinoma. There is focal labelling, often granular, of neoplastic epithelium and of cells in the stroma.
Anticathepsin D, PAP method. x340.



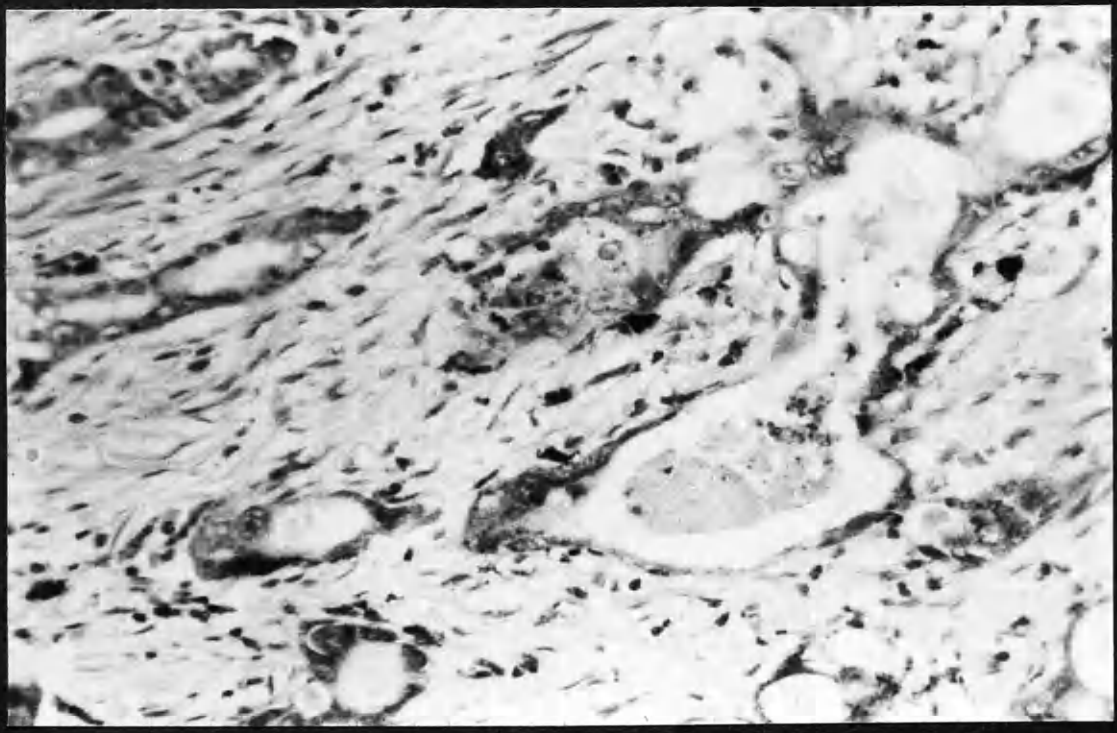
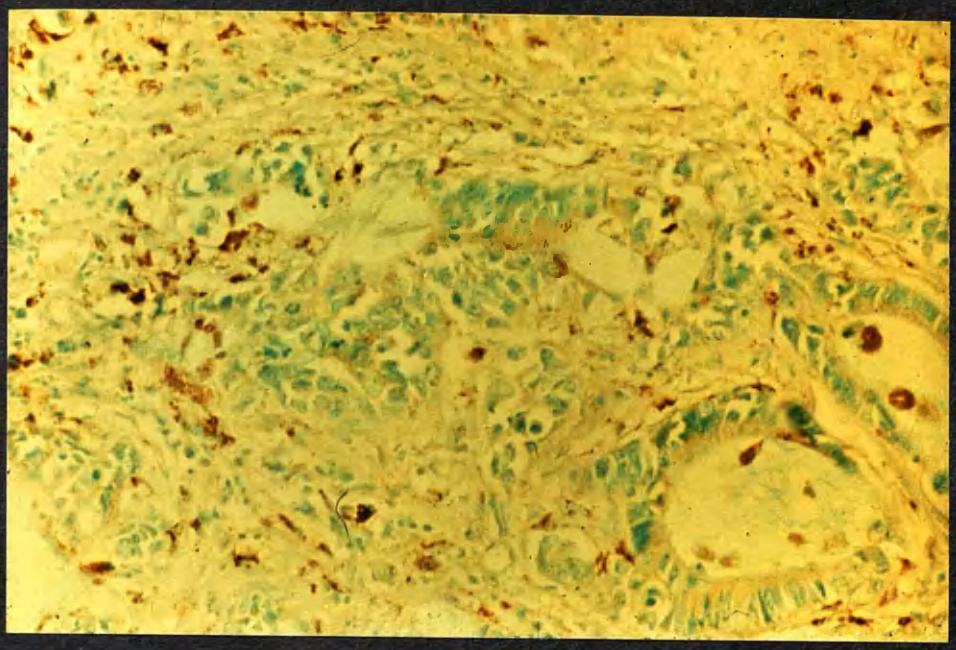
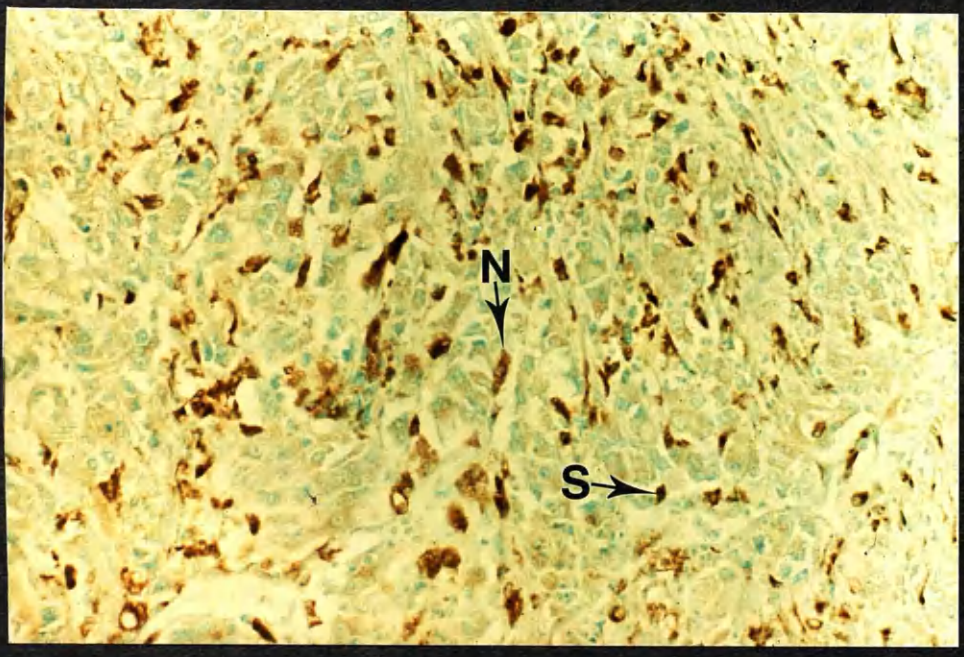


FIG. 8.11 Cathepsin D in gastric carcinoma, in this case mainly in the stromal cells rather than in neoplastic epithelium, which is mostly negative.
Anticathepsin D, PAP method. x340.

FIG. 8.12 Cathepsin D in gastric adenocarcinoma showing focal labelling of neoplastic epithelial cells (N) and of some stromal cells (S).
PAP method. x340.

FIG. 8.13 Cathepsin D in poorly differentiated gastric carcinoma, mainly in neoplastic epithelial cells.
Anticathepsin D, PAP method. x510.

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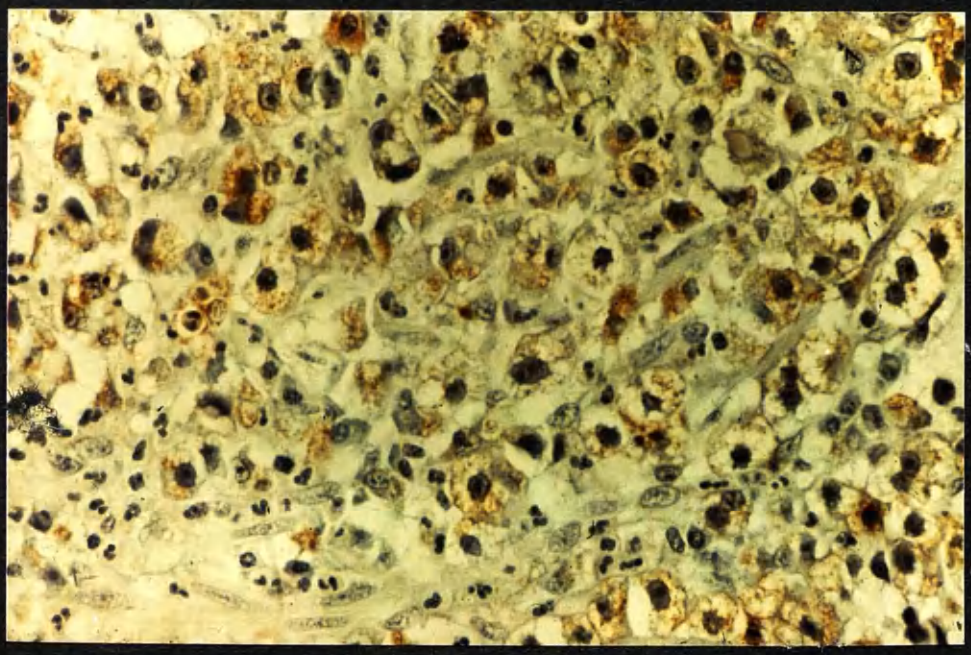
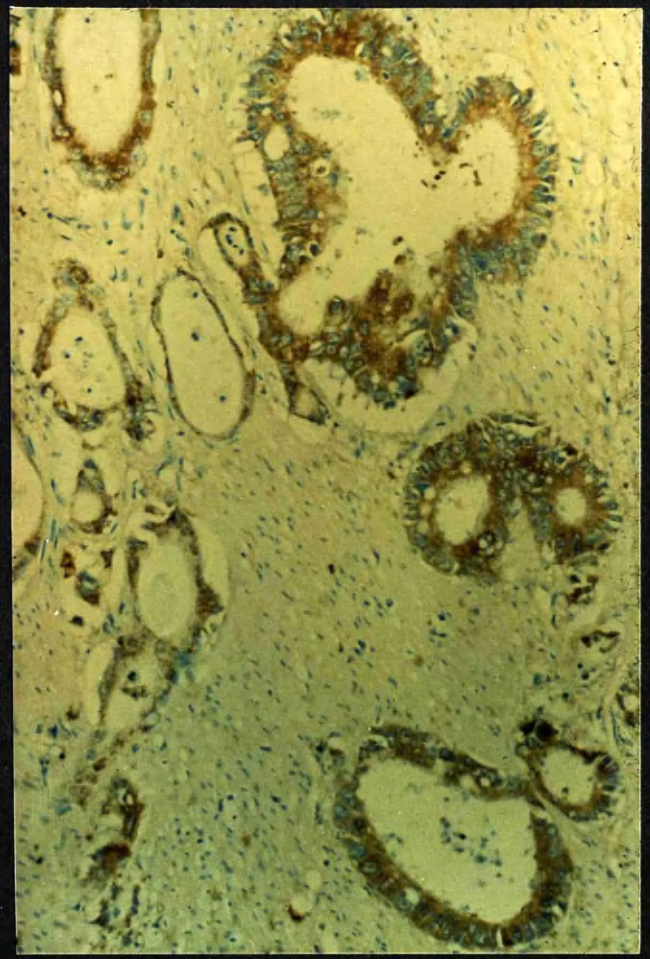
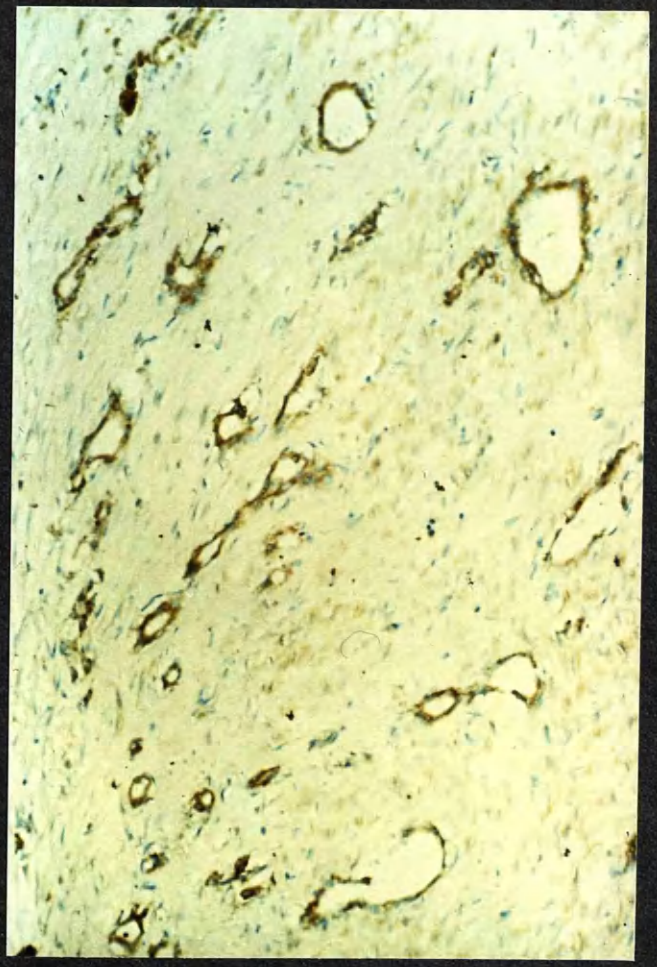


FIG. 8.14 SMP in gastric adenocarcinomas invading subserosal stomach.
Anti SMP, PAP method. x74.

FIG. 8.15 SMP in gastric adenocarcinomas invading submucosa.
Anti SMP, PAP method. x340.





DISCUSSION

The results show that gastric carcinomas contain aspartic proteinases. Presumably, pepsinogen and progastricsin are present in the tumour cells as zymogens, as in normal stomach, although this is difficult to prove by immunohistochemical labelling with antisera that react with both zymogen and active enzyme (Chapter 2). The possibility of selective uptake by diffusion has been considered in Chapter 4 and was taken into account in assessment of the immune labelling. Presumably the malignant cells which arise in the stomach retain the capacity to synthesize the zymogens of the cells from which they arise. It is not yet clear whether cathepsin D and SMP are also produced as zymogens or as active enzymes, although a precursor of cathepsin D has been postulated (Puizdar & Turk, 1981).

There are two possible reasons why biochemical studies have failed to demonstrate acid proteinase activity in gastric carcinomas. The first is that the zymogens are not present throughout the tissue but are only in the malignant cells and even then focally, so that if the tissue were homogenized they might well be diluted below the limit of detection by enzyme assay. The second is that the zymogens might not be normal. It is well known that hormones produced by tumours may differ structurally and functionally from those produced normally (Robbins, Cotram & Kumar, 1984). It is possible that at least some malignant cells produced abnormal molecular forms of pepsinogen or progastricsin, some of which may be inactive. Such a possibility has previously been suggested by detection of inactive forms of pepsinogen in gastric carcinoma (Kalinovsky, Melnikov & Seits, 1977).

Pepsinogen appears to be a much less common product of gastric carcinoma than is progastricsin. Eighty-five per cent of carcinomas of

the stomach arise in the antrum, where progastricsin but not pepsinogen is normally produced and the predominance of progastricsin in carcinomas could reflect this. It would be of interest to know whether the pepsinogen-containing tumours in the present study arose in the gastric body but the case records were insufficiently complete to allow this to be determined. Moreover, this might be difficult to prove retrospectively as the precise origin of any given carcinoma is likely to be obscure by the time the tumour is large enough to be clinically apparent. As pepsinogen-producing cells in the normal gastric body also produce progastricsin, it might be expected that this would also apply to carcinomas. Of three carcinomas which were strongly positive for pepsinogen, one was only weakly positive for progastricsin and two were negative. Possible explanations for this include deletion of the progastricsin gene or translocation to a region where it is no longer transcribed.

A second possible explanation for the higher prevalence of progastricsin than pepsinogen in gastric carcinomas is that the former might be the more primitive zymogen embryologically and might be more likely to occur as an oncofetal antigen. There is evidence from comparative biochemical studies in animals that this is the case (Foltmann & Axelsen, 1980; Foltmann, 1981; Foltmann et al, 1981) and this is supported by the observation that progastricsin appears earlier in gestation in the developing human stomach (Chapter 7). These two possible explanations for the higher prevalence of progastricsin than pepsinogen are not, of course, mutually incompatible.

The focal distribution of pepsinogen and progastricsin is analogous to that noted for many other tumour products, for example the aspartic proteinase renin in malignant tumours of kidney (Lindop & Fleming, 1984) and prealbumin in carcinoids (Millar et al, 1984).

While production by some cells of small amounts of product, below the limit of detection by the PAP method, might result in a focal pattern, the results of quantitative immunohistochemical studies suggest that there would be variation in the intensity of the staining reaction (Millar & Williams, 1982). In the present study this was not observed; most cells were either negative or strongly positive. It seems most likely, therefore, that the focality is because zymogen production is either switched on or not. Zymogen production could be switched on at a number of levels, but is most likely to happen before gene transcription (Alberts et al, 1983). The absence of progastricsin from some pepsinogen-containing carcinomas suggests that the two genes may become uncoupled in neoplastic cells.

Whether these cells secrete zymogen into the blood is not known, but there was evidence of secretion into the lumen of some tumour acini.

The prognosis of gastric carcinoma does not appear to be related to the presence or absence of progastricsin in the tumour cells. The numbers were too few and case records too incomplete to permit a similar correlation with the presence or absence of pepsinogen. Interestingly, parietal cell differentiation in gastric carcinoma has been said to be associated with a favourable outcome in the few cases described (Capella et al, 1984; Gaffney, 1987).

Although cathepsin D was found in all gastric carcinomas studied, its expression seems not to be a specific property of the neoplastic epithelial cells, as it was also found in the stromal cells. Its presence may therefore reflect some non-specific functional attribute of the cells, such as high metabolic activity, related to requirement for lysosomal enzymes. This has not yet been investigated experimentally as cathepsin D has not previously been localised within tumours.

Since the results of the studies described in this chapter were published (Reid, Thompson & Kay, 1983; Reid & Kay, 1983; Reid, Valler & Kay, 1985; Reid, Valler & Kay, 1986), they have been confirmed by Stemmermann, Samloff & Hayashi (1985), who in a series of 64 gastric carcinomas found pepsinogen in 3.1% and progastricsin in 29.7% of cases. In the intestinal types pepsinogen was found in 4.5% and progastricsin in 36.3%, whereas in the diffuse types pepsinogen was found in none and progastricsin in 15%.

Busby-Earle, Williams & Piris (1986) also found both zymogens in both intestinal and diffuse types of carcinoma, but their antiserum to pepsinogen labelled a higher proportion of carcinomas (75%) than their antiprogastricsin (22%). The reason for the discrepancy is not clear but differences in antibody avidity and the possibility of diffusion could be part of the explanation.

It has been suggested that the presence of progastricsin indicates a higher degree of malignancy, as it correlates with the depth of invasion of the gastric wall and with the presence of lymph node metastases (Fiocca et al, 1987).

In the present study, SMP was found in a higher proportion of gastric carcinomas than were pepsinogen and progastricsin. This is in accord with the results of a recent study of 74 gastric carcinomas, in which SMP was found in 54%, compared with progastricsin in 31% and pepsinogen in 5% (Shiraishi et al, 1987). In the same study it was found that some cancer cells expressed both SMP and progastricsin, unlike normal gastric mucosal cells, none of which express both enzymes.

A low pepsinogen concentration ($\leq$20 ng/ml) in the serum was found in about a third of Japanese men with gastric cancer and in only 6.3% of controls and it has been suggested that it is a useful subclinical marker of increased risk (Nomura, Stemmermann and & Samloff, 1980). The

serum pepsinogen to gastricsin ratio was of greater predictive value, although it detected mainly advanced carcinoma and was of little value in detecting early gastric cancer (Stemmermann et al, 1987). Whether or not SMP has clinical significance remains to be determined.

CHAPTER 9

PROGASTRICSIN IN CARCINOMA OF PROSTATE

INTRODUCTION

The enzyme acid phosphatase is produced by normal prostatic acinar epithelium. When carcinoma develops in the prostate, the malignant cells also secrete this enzyme (Jöbsis et al, 1978), often in such large amounts that the serum level is raised. As demonstrated in Chapter 5, the normal prostatic epithelium also contains progastricsin and it might be expected that this zymogen would also be found in carcinomas arising in prostate. This possibility is supported by the demonstration (Chapter 8) that progastricsin is found not only in normal gastric mucosa but also in carcinomas of the stomach. A series of cases of prostatic carcinoma was therefore studied in order to determine whether progastricsin could be demonstrated in the malignant epithelial cells.

MATERIALS AND METHODS

Antisera

Rabbit antisera to human pepsin, gastricsin and prostatic acid phosphatase were used in the peroxidase-antiperoxidase method with controls as described in Chapter 5.

Tissues

Fifty-four cases of prostatic adenocarcinoma from the routine surgical histology files of the Pathology Department, Glasgow Royal Infirmary were studied. In 44 cases the prostate had been removed by transurethral resection and in the remaining 10, by suprapubic enucleation.

Nine of the carcinomas were further studied by labelling alternate serial sections with rabbit antisera to progastricsin and to prostatic acid phosphatase (Miles Laboratories Limited). Sections were examined with a comparison microscope and the distribution of the two enzymes was compared.

The specificity of the immune labelling was studied as before (Chapter 5), 12 cases of prostatic carcinoma being examined.

Twenty-four prostatic carcinomas were also labelled with rabbit anti-pig pepsin.

With all staining runs a section of normal gastric body mucosa was included as a positive control for progastricsin or pepsinogen. The sections of prostate being examined for the presence of acid phosphatase served as their own control.

RESULTS

Progastricsin was identified in the cytoplasm of the malignant cells in 21 (39%) of the 54 cases of prostatic carcinoma studied. As in gastric carcinomas, progastricsin was present focally, the number of positive cells varying from a few in some cases to many in others. Despite its focal distribution, labelling, where present, was always intense (Fig 9.1). All positive tumours included at least some areas with a microacinar pattern, typical of prostatic carcinoma (Fig 9.2). In 9 cases there were undifferentiated areas; in 4 of these cases progastricsin tended to be in the less well differentiated areas (Fig 9.3), but in the other 5 cases no such tendency was noted.

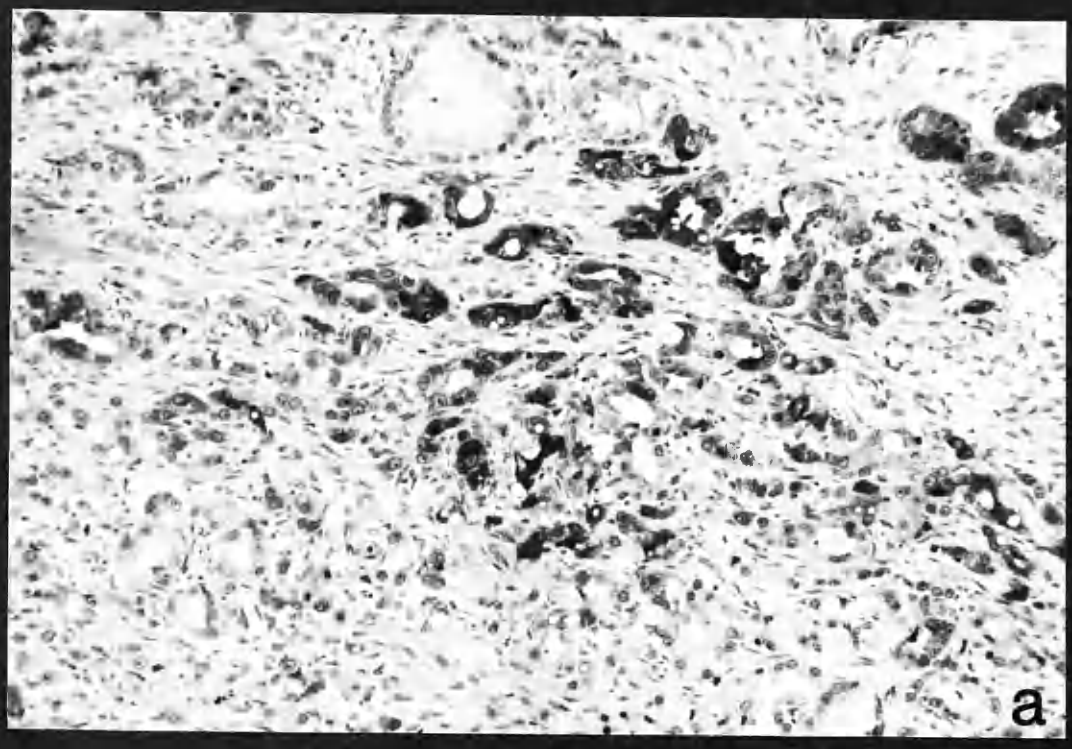
The 9 prostatic carcinomas in which progastricsin was most extensive were also labelled for acid phosphatase. In all 9 cases there was strong labelling for acid phosphatase in most of the tumour cells. Comparison microscopy of serial sections labelled alternately for progastricsin and acid phosphatase showed that most cells which contained progastricsin also contained acid phosphatase but that many cells contained acid phosphatase but not progastricsin.

FIG. 9.1a) Progastricsin in moderately differentiated adenocarcinoma of prostate. There is strong but focal labelling of neoplastic epithelium.

Labelled with antigastricsin, PAP method. x210.

b) High power of area of Fig. 9.1(a) showing granular labelling of the cytoplasm in some malignant cells.

Labelled with antigastricsin, PAP method. x540.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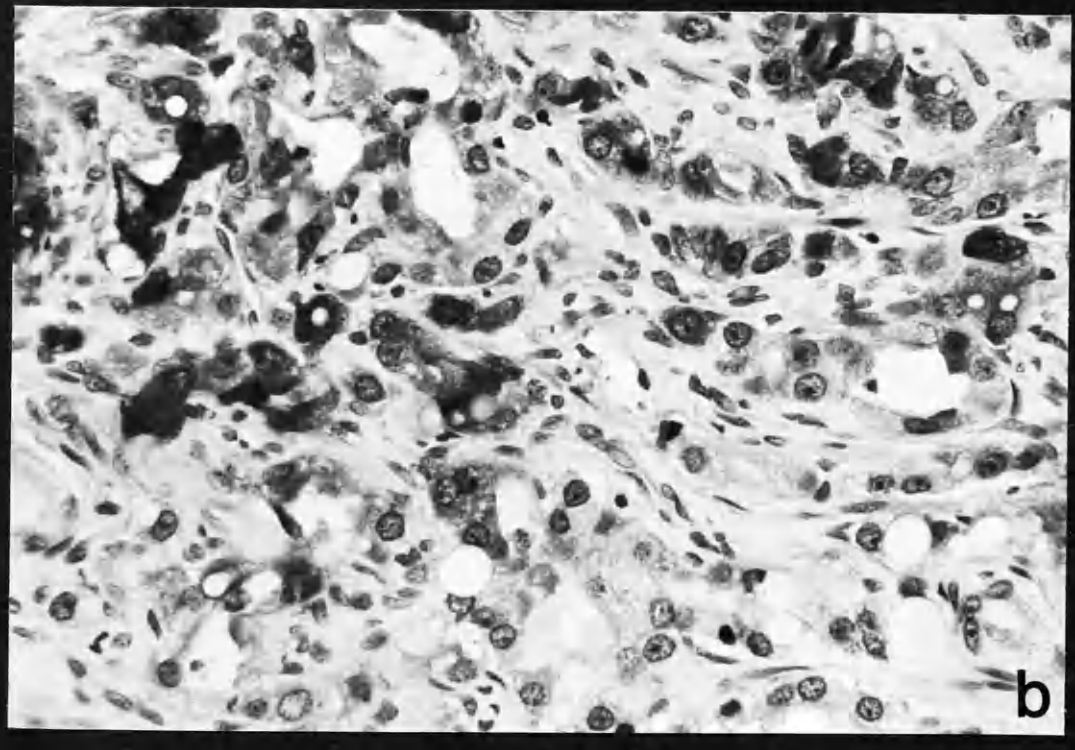
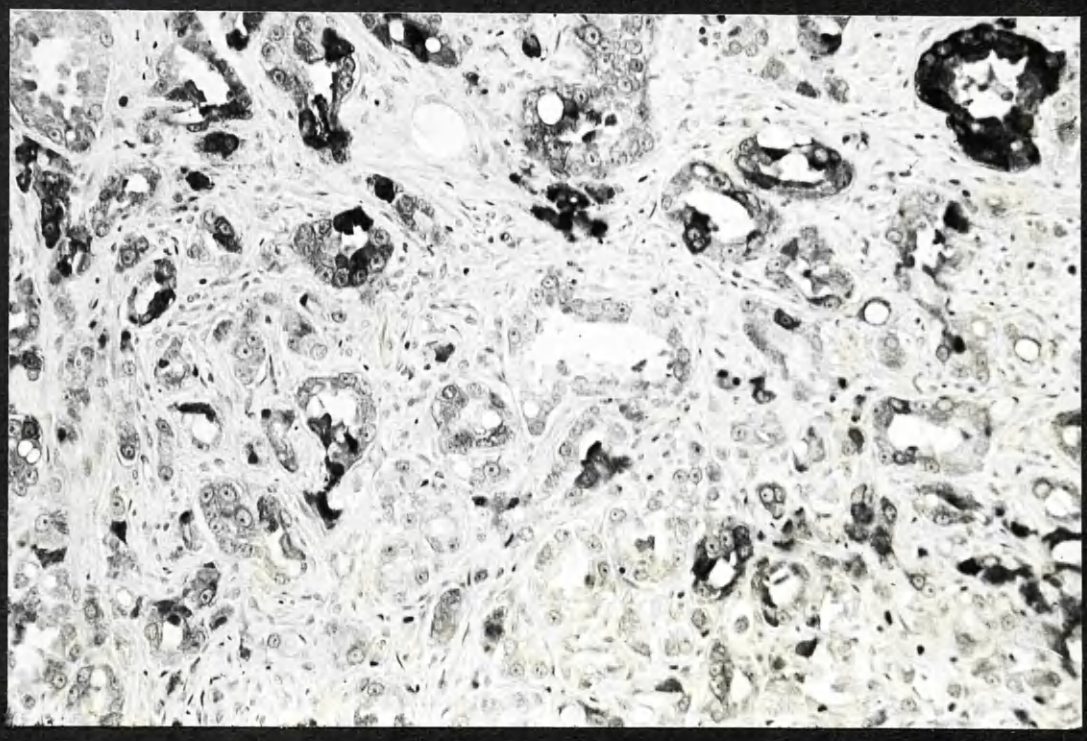

b

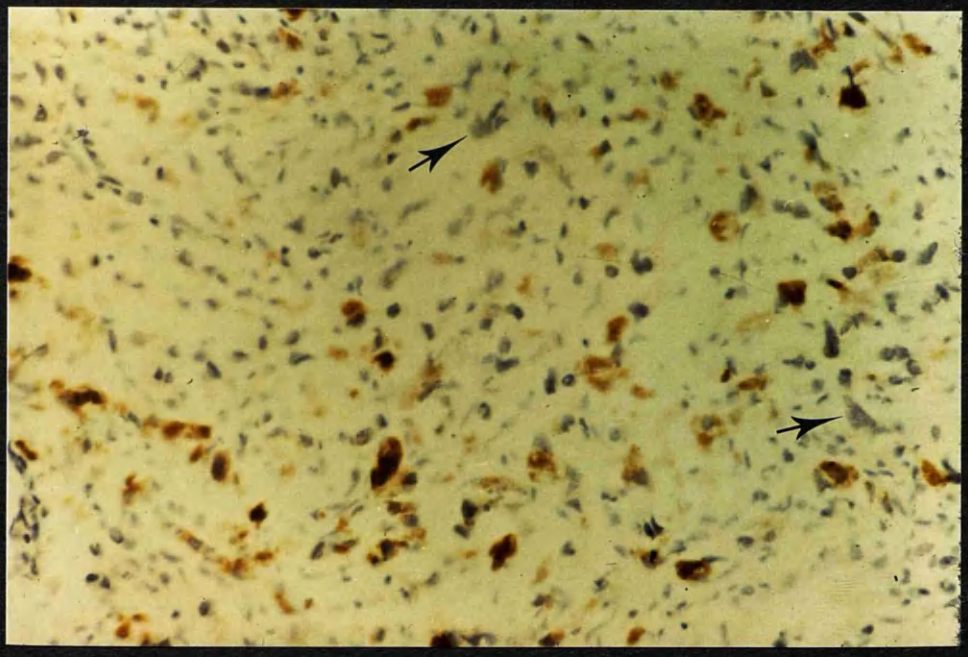
FIG. 9.2 Progastricsin focally distributed in moderately differentiated adenocarcinoma of prostate.

Labelled with antigastricsin, PAP method. x470.

FIG. 9.3 Progastricsin in poorly differentiated prostatic carcinoma. Some malignant cells (arrows) are negative.

Labelled with antigastricsin, PAP method. x210.





DISCUSSION

The results confirm the hypothesis that progastricsin production by the normal prostatic epithelium is reproduced in prostatic carcinomas. Similar arguments apply to the focality of labelling as in the case of gastric carcinoma (Chapter 8). The failure to detect progastricsin in 60% of prostatic tumours could be because the PAP technique was insufficiently sensitive to enable low intracellular levels to be demonstrated. However, it is more likely that these tumours do not produce the zymogen. This could be because they arise from cells which do not normally produce progastricsin. Recent work (Reese et al, 1987) suggests that progastricsin may be produced mainly in the central zone of the normal prostate and carcinomas arising peripherally might, on this basis, be postulated to be progastricsin negative. Studies have not yet been carried out to determine whether this is the case.

Although in principle progastricsin might be a useful marker of prostatic carcinoma, in practice this is for a number of reasons unlikely. Firstly, immune labelling of carcinomas for prostatic acid phosphatase is well established as being both specific and sensitive in indicating their prostatic origin (Jöbsis et al, 1978). Progastricsin labelling might complement but is unlikely to replace such a useful diagnostic test. Secondly, metastatic gastric carcinoma would have to be excluded. While raised serum levels of progastricsin might indicate production by a prostatic malignancy, the usefulness of this would be mitigated by the presence of raised or lowered levels due to gastritis (Samloff et al, 1982) as well as to gastric carcinoma. Further work has not so far been undertaken in this field.

At present the most likely practical application the results described in this chapter might have appears to be that if a deposit of metastatic adenocarcinoma contained progastricsin, a primary in prostate would have to be excluded before the presence of a gastric primary could be stated with confidence. This will be more fully discussed in Chapter 11.

CHAPTER 10

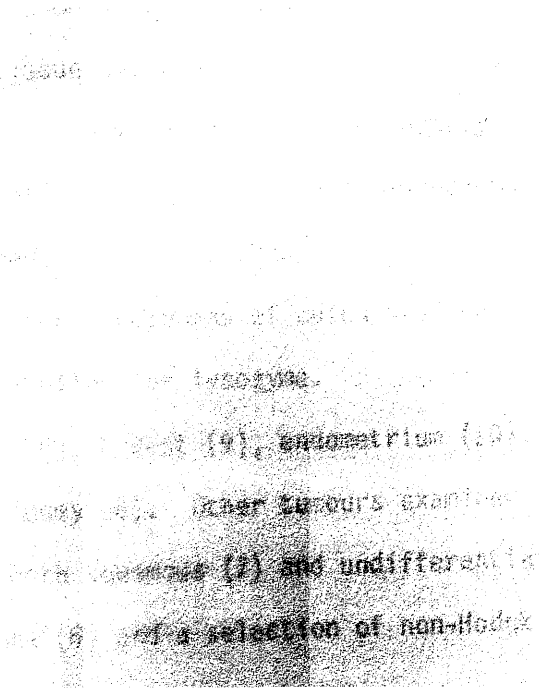
ASPARTIC PROTEINASES IN PRIMARY MALIGNANT TUMOURS OTHER THAN STOMACH AND PROSTATE

INTRODUCTION

It has been shown in Chapters 8 and 9 that pepsinogen and progastricsin occur in malignant tumours arising from the tissues in which the zymogens are produced normally. Renin is known to be present in malignant neoplasms of the kidney (Lindop & Fleming, 1984). It has not, however, been demonstrated that production of these enzymes or zymogens is confined to tumours of these sites. Cathepsin D and SMP, on the other hand, are normally widely distributed in the body and their presence might be anticipated in a variety of tumours.

In the present study, pepsinogen, progastricsin, cathepsin D and SMP were localised by the immunoperoxidase method in a variety of neoplasms.

The distribution of cathepsin D was compared with that of lysozyme.



MATERIALS AND METHODS

Antisera

Antisera to pepsin, gastricsin, cathepsin D and SMP were used in PAP method as described in Chapter 3.

Tissues

Adenocarcinomas from the following sites were studied for the presence of aspartic proteinases as follows, the number of cases being given in brackets:

Pepsinogen - colon (21), breast (18), endometrium (16), ovary (20), gall bladder (1), bile duct (2) and pancreas (14).

Progastricsin - colon (31), breast (18), endometrium (16), ovary (14), bile duct (2), pancreas (22). Serial sections of selected cases of pancreatic carcinoma were examined for progastricsin and pepsinogen.

Cathepsin D - colon (6), breast (6), ovary (6), pancreas (2), kidney (5). Other tumours examined were: carcinoma of bladder (8), squamous carcinoma of bronchus (8) and skin (5), various soft tissue sarcomas (5), Hodgkin's (5) and non-Hodgkin's (9) lymphoma. Some benign tumours, namely dermatofibroma (2) and breast fibroadenomas (5), were also examined.

Six of the carcinomas of colon studied for cathepsin D were also labelled for lysozyme.

SMP - colon (16), breast (9), endometrium (10), ovary (9), prostate (9), kidney (6). Other tumours examined were carcinoma of lung, both squamous (7) and undifferentiated (6), Hodgkin's lymphoma (6) and a selection of non-Hodgkin's lymphomas (9).

Absorption controls were performed on selected strongly positive cases. Antigastricsin was pre-incubated with gastricsin as previously described (Chapter 3). Anticathepsin D at 1 : 200 was pre-incubated for 1 hr at room temperature with a different preparation of cathepsin D (1 mg/ml), kindly supplied by Dr. A.J. Barratt, Strangeways Laboratory, Cambridge. The dilution of antiserum was higher than in the rest of the study to economise on antigen.

RESULTS

Pepsinogen

There was no evidence of pepsinogen in the carcinomas studied, except one case of pancreatic carcinoma, in which there was focal strong labelling of the tumour cells.

Progastricsin

Two cases of pancreatic carcinoma were considered to show definite evidence of progastricsin in the malignant cells (Fig. 10.1). One of these was the case referred to above, in which pepsinogen was detected in some of the progastricsin-containing cells. Two additional cases appeared to show staining; it was considered that this might be a result of diffusion from the overlying duodenal lumen, but similar sections labelled with antipepsin showed no sign of pepsinogen (Fig. 10.1a and b). One papillary tumour, probably of the ampulla of Vater, was also positive. One breast carcinoma showed strong reactivity in the cytoplasm of the malignant cells (Fig. 10.2). An absorption control in the latter case showed complete abolition of staining following pre-incubation of antigastricsin with gastricsin.

Cathepsin D

Carcinomas from colon, breast, ovary, kidney, bladder and pancreas all contained cathepsin D in neoplastic or stromal cells, or in both types, the predominance varying considerably from case to case (Fig. 10.3 and 10.4). Antilysozyme labelled the stromal cells of colonic carcinoma, although the neoplastic epithelial cells were negative.

Squamous carcinomas of bronchus showed labelling of only occasional tumour cells, although in some cases staining was widespread (Fig. 10.5). The connective tissue, however, showed strong labelling of macrophages as did the adjacent lung. Squamous carcinomas of skin

showed labelling of only occasional tumour cells and some stromal cells. The malignant connective tissue tumours were generally negative with labelling of only focal cells in one case. One of the two dermatofibromas showed a strongly positive reaction, apparently with histiocytes; the other was negative. Two of the breast fibroadenomas were positive with labelling of very occasional glands in each.

Malignant lymphomas

All lymphomas, except one case of non-Hodgkin's lymphoma, showed strong cytoplasmic reactivity, mainly in large cells scattered throughout the tumour. In one case of lymphocyte dominant Hodgkin's disease some of the cells in the granulomas seemed to contain cathepsin D, although the rest of the tumour tissue was negative (Figs. 10.6-10.8) .

Controls

At a dilution of 1/200, higher than that used for the rest of the study, the anticathepsin D still gave strong labelling in all the selected cases studied. The labelling of most cells was abolished, or in the most strongly positive cells, considerably reduced by pre-incubation of antiserum with cathepsin D.

SMP

There was equivocal staining in one case of colonic carcinoma and weak superficial staining in 5 others. Staining was cytoplasmic and not clearly granular. One carcinoma of ovary was positive and 3 others weakly so. Breast carcinomas were in 2 cases focally positive. Three renal carcinomas were strongly positive (Fig. 10.9), but it was noted that this was particularly prominent adjacent to areas of haemorrhage. The lung carcinomas were not convincingly positive. The Hodgkin's

lymphomas were negative, but in 2 cases there was intense labelling of plasma cells. The non-Hodgkin's lymphomas were likewise negative, but in 1 case there was strong labelling of some plasma cells.

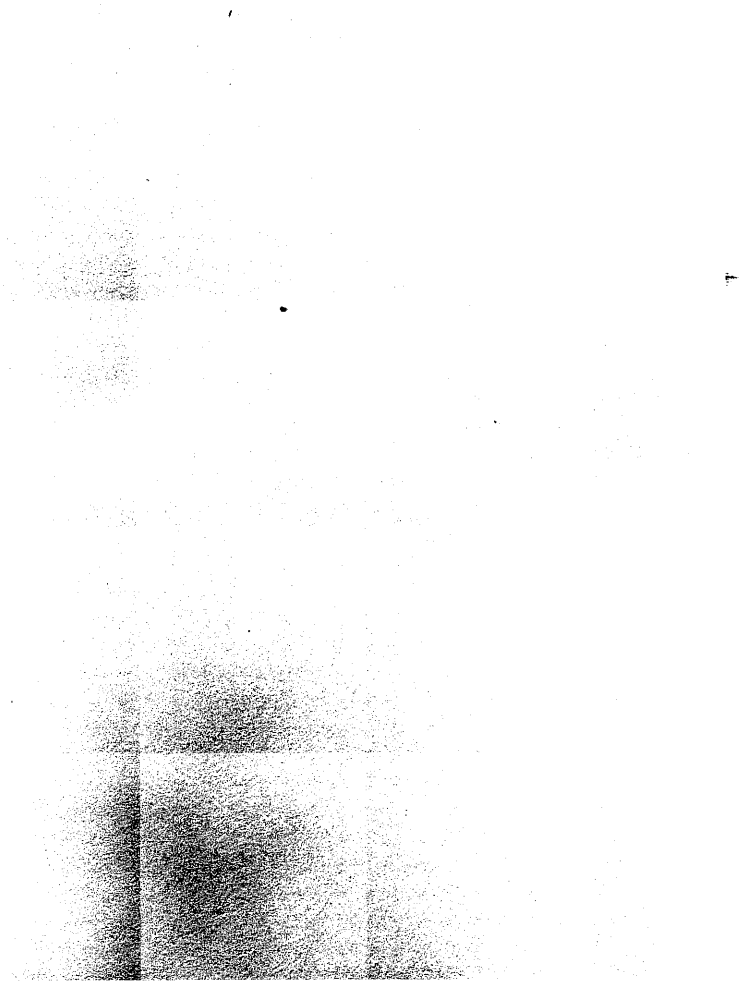
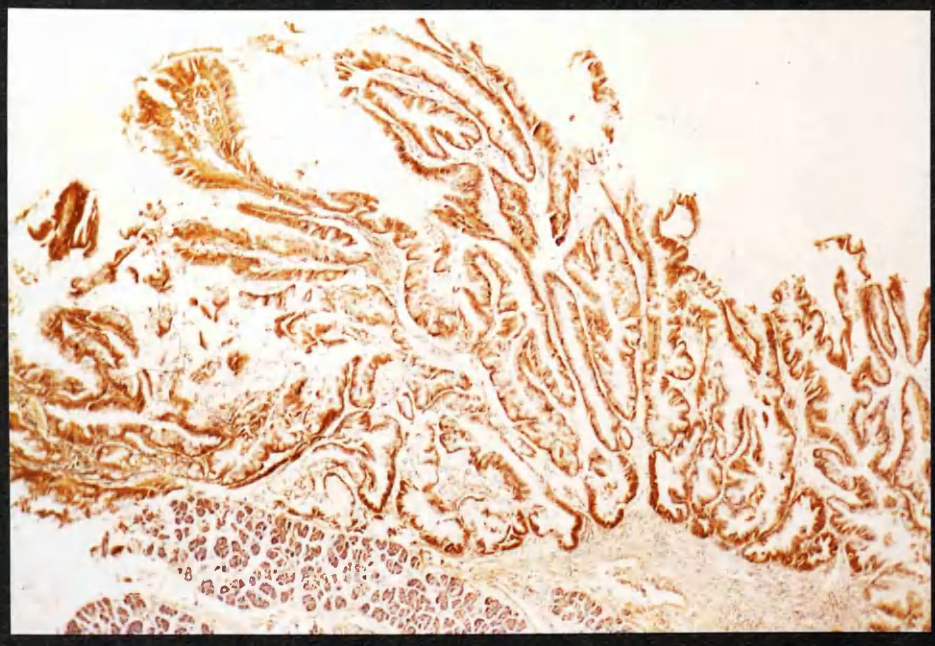


FIG. 10.1 Papillary carcinoma of pancreas projecting into duodenal lumen

a) Progastricsin in tumour cells.
Antigastricsin, PAP method. x42.

b) Absence of pepsinogen from similar field to a).
Antihuman pepsin, PAP method. x42.



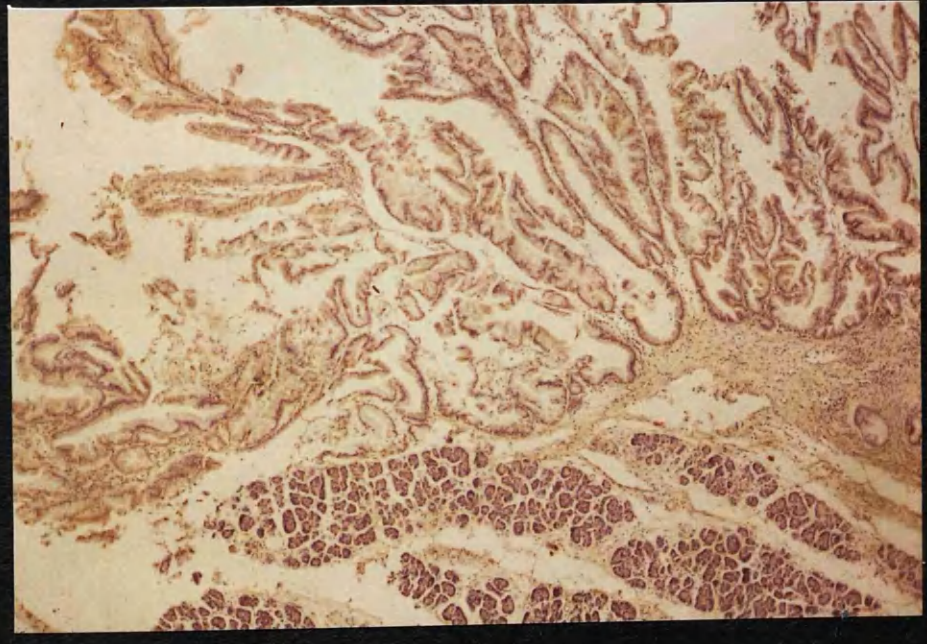
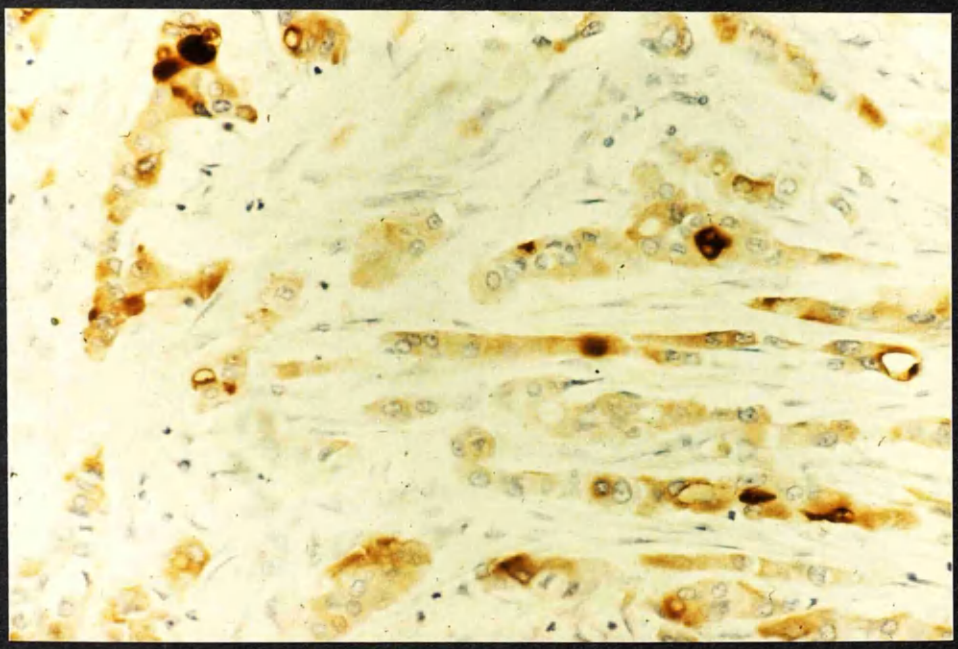
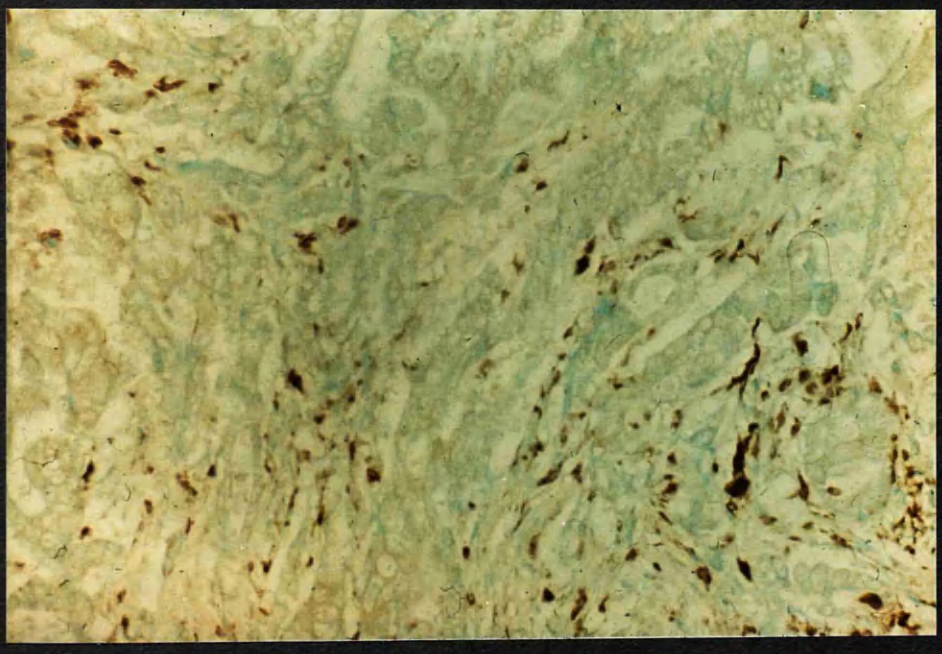


FIG. 10.2 Progastricsin in scirrhous carcinoma of breast.
Antigastricsin, PAP method. x420.

FIG. 10.3 Cathepsin D in adenocarcinoma of colon, in this case mainly in the stromal cells, most of the tumour acini being negative.
PAP method. x350.

FIG. 10.4 Cathepsin D in breast carcinoma. The enzyme is present both in the invasive carcinoma (C) and in macrophages (M) in the lumen of a normal breast duct.
PAP method. x155.





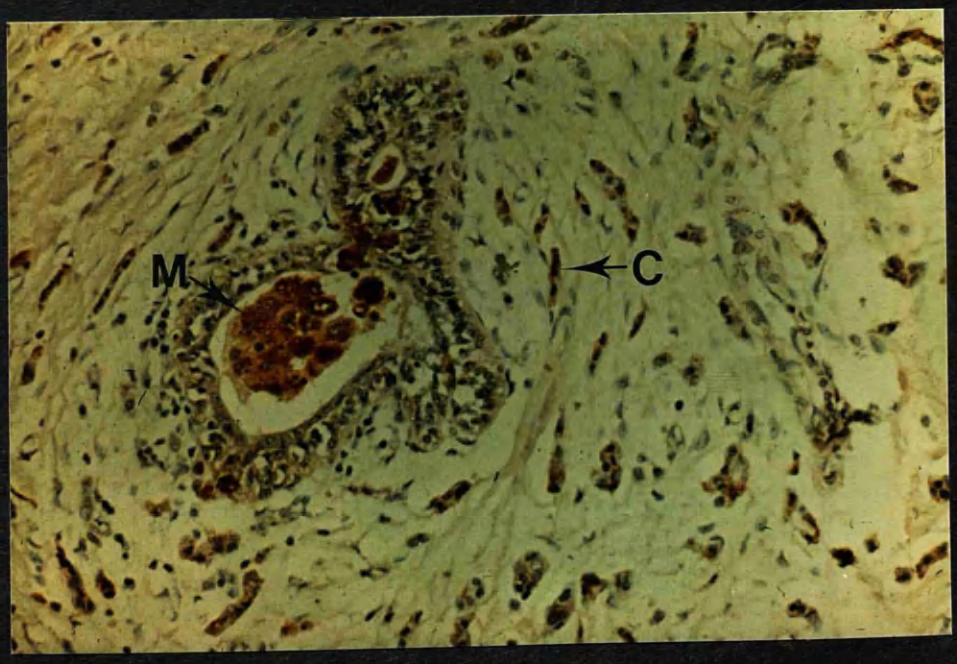
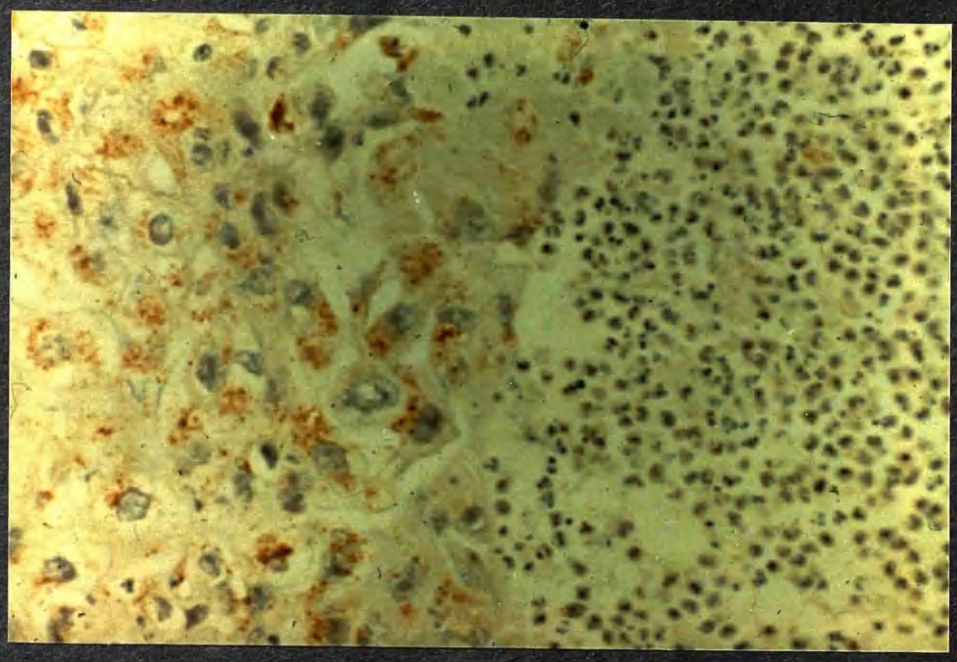
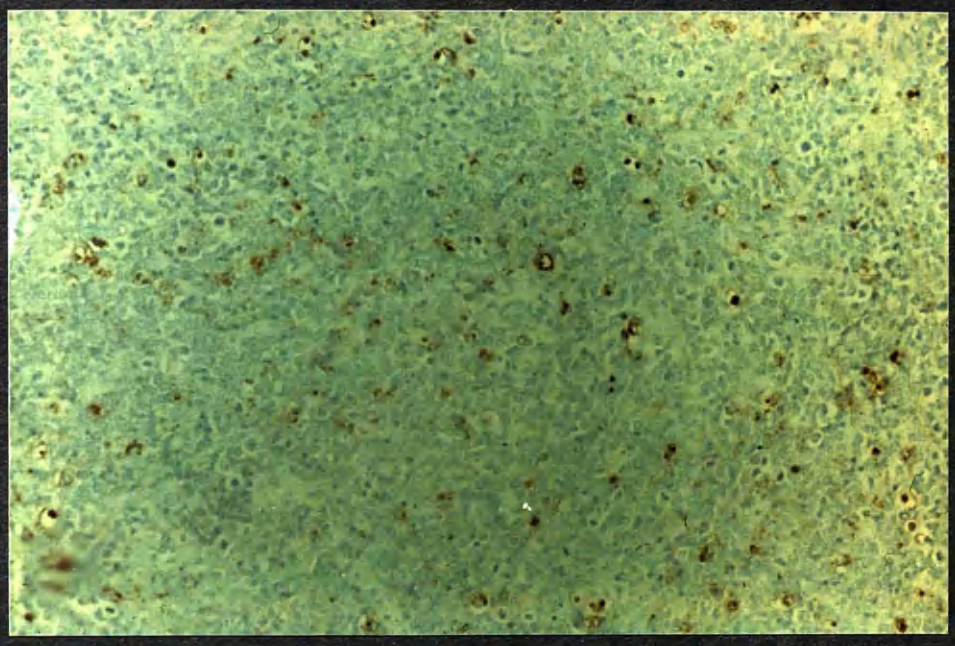

M
C

FIG. 10.5 Cathepsin D in squamous carcinoma of bronchus. There is granular labelling of the cytoplasm of the tumour cells (left), while the polymorphs (right) are unlabelled. PAP method. x508.

FIG. 10.6 Cathepsin D in malignant lymphoma (diffuse centroblastic). PAP method. x115.

FIG. 10.7 Cathepsin D in histiocytic cells of L & H Hodgkin's disease. PAP method. x115.





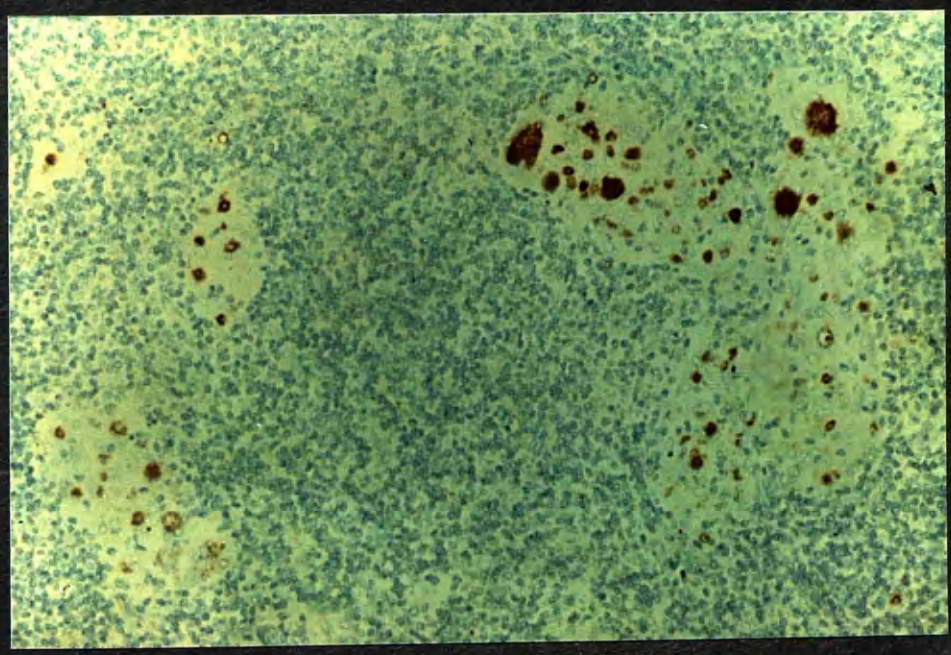
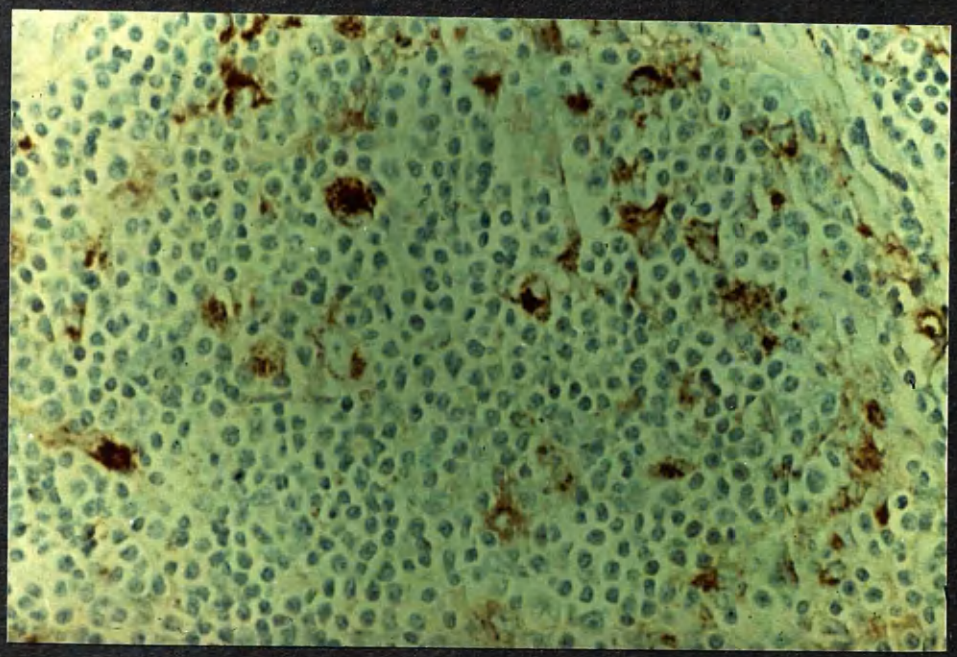
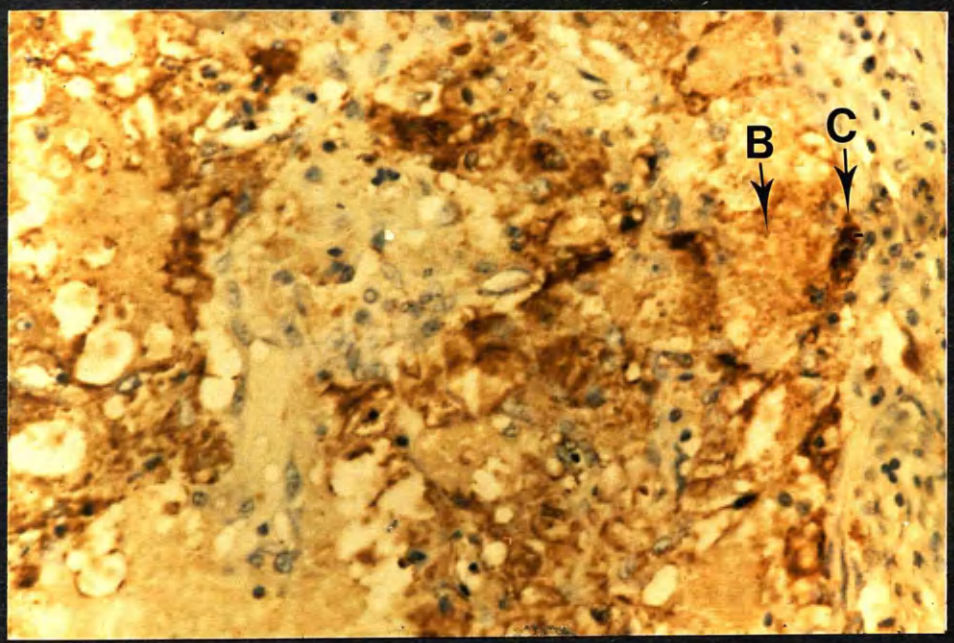


FIG. 10.8 Cathepsin D in nodular sclerosing Hodgkin's disease. The enzyme appears to be mainly in the cytoplasm of large cells.
PAP method. x324.

FIG. 10.9 SMP in carcinoma of kidney. Although there is labelling of tumour cells (C), there are large numbers of adjacent red blood cells (B) containing SMP. (Compare Fig. 6.13).
PAP method. x508.




B
C

DISCUSSION

Pepsinogen appears to be selectively present in carcinomas only of the stomach. Progastricsin, on the other hand, is produced by carcinomas of prostate (Chapter 9) and, less frequently, pancreas and sporadically, breast. Cathepsin D, however, appears to be present in most if not all tumours studied and may be a reflection of metabolic activity, of which lysosomal degradation of proteins is part. It has, therefore, no specificity as a tumour marker. Cathepsin D has been postulated to have a role in producing cachexia, possibly by enhancing proteolysis in muscle and other tissues (Greenbaum & Sutherland, 1983). This effect has been inhibited in animals by the aspartic proteinase inhibitor pepstatin, probably at the lysosomal level, although it is possible that increased production by the neoplasm itself could have a role.

Caution is required in interpretation of the SMP results. One problem is the presence of SMP in red blood cell membranes (Chapter 3). There was clear evidence of surface positivity in cells which were in contact with red blood cells (Fig. 6.13), and it is possible that diffusion could explain some of the positive results. Absorption controls have not been performed for SMP because a sufficiently large supply of pure enzyme was not available to us. This would not, however, overcome the possibility of diffusion either from red blood cells, or from intestinal contents into the surface of colonic carcinomas. Perfusion-fixation of tissues might help exclude the possibility of diffusion.

While there appears to be no published evidence of aspartic proteinases in other malignant tumours, there has been immunoelectrophoretic evidence of aspartic proteinases in benign tumours of the ovary (Hirsch-Marie et al, 1978). Pepsinogen (PGI-II in

the authors' terminology: see Chapter 1) was found in four cases, progastricsin (PGIII) in five and SMP in three, combinations being present in several cases. The terminology of the ovarian tumours is not clear but it appears that none of the carcinomas were positive. Immunohistological confirmation of this work would be worthwhile.

CHAPTER 11

DETECTION OF PROGASTRICSIN IN METASTATIC ADENOCARCINOMA

INTRODUCTION

It has been demonstrated previously (Chapter 8) that progastricsin occurs in the neoplastic epithelial cells of carcinomas of stomach and prostate, less commonly pancreas and only rarely in primary tumours of other sites. It thus appears to be a relatively specific marker for tumours of these tissues. However, any diagnostic value progastricsin might have in routine histopathology would be increased if it could be demonstrated that it was also present in metastases from primary tumours of these but not other sites. In this chapter two studies are described. The first was undertaken in order to determine whether progastricsin was present in the regional lymph nodes of gastrectomy specimens in which the primary carcinoma contained progastricsin; this study was undertaken initially on cases in Glasgow and later on cases in Leeds. The second study was conducted in order to establish whether progastricsin was present in metastatic adenocarcinomatous deposits and, if so, to determine whether the primary neoplasm was in stomach or some other site.

MATERIALS AND METHODS

Antiserum

Rabbit antigastric gastricsin was used in both parts of the study on metastatic carcinoma. In the first part, which deals with local lymph node metastases of primary gastric carcinoma, the antiserum was used at 1:100 in the PAP method as previously (Chapter 3), while in the second part, which concerns widespread metastases, it was used at 1:1000 in the immunogold-silver (IGSS) method. Normal gastric mucosa served as a positive control in all staining runs.

Immunogold-silver method (IGSS)

The method used was that published previously (Holgate et al, 1986). Titration experiments showed that the primary antiserum gave strong labelling of normal gastric body mucosa at all dilutions down to 1:1000.

Controls

Blocking controls were performed on selected cases as before (Chapter 3).

Cases studied

1. Local lymph node metastases of gastric carcinoma

In the study above (Chapter 8) the neoplastic epithelial cells of 33 cases of gastric carcinoma were found to contain progastricsin. In 12 of these cases the gastrectomy specimen included metastatic tumour in local lymph nodes, the total number of involved nodes being 36. Sections of these were labelled with antigastricsin by the PAP method. Thirty-five lymph node metastases from 14 cases of colonic carcinoma and 15 such deposits from 7 cases of breast carcinoma were also examined for progastricsin.

In a similar but separate study at Leeds General Infirmary, sections of local lymph node metastases of 26 progastricsin-containing gastric carcinomas (Chapter 8) were labelled for progastricsin, also by the PAP method.

2. Widespread lymph node and liver metastases from various primary carcinomas

Cases of metastatic adenocarcinoma in lymph nodes and liver were selected from the biopsy files of the Pathology Department, Leeds General Infirmary. The records of each case were examined and the following information was extracted:

a) the site of the primary carcinoma,

b) the site of the metastasis and

c) whether the metastasis was diagnosed before, after or at the same time as the primary tumour.

In addition, in all cases where the metastasis contained progastricsin and the primary tumour was non-gastric, at least one of any blocks available from the primary was labelled for progastricsin by the IGSS and PAP methods.

Statistical analysis

The Chi-square test with Yates' correction was used in analysis of the results.

RESULTS

1. Local lymph node metastases of gastric carcinomas

Twenty-eight of the 36 lymph nodes containing secondary gastric carcinoma labelled focally for progastricsin in the tumour cells (10 out of 12 cases) (Figs 11.1-11.3). The single metastasis from 1 breast carcinoma was strongly positive (1 out of 15 nodes) (Fig 11.4). None of the 35 lymph node secondaries from colonic carcinoma (14 cases) were positive.

The results in the Leeds cases were similar. In each of the 25 cases of progastricsin positive gastric carcinoma, at least 1 lymph node secondary was positive. Altogether 50 (76%) of 66 lymph nodes contained progastricsin positive carcinomas.

2. Widespread lymph node and liver metastases from primary carcinomas of various sites

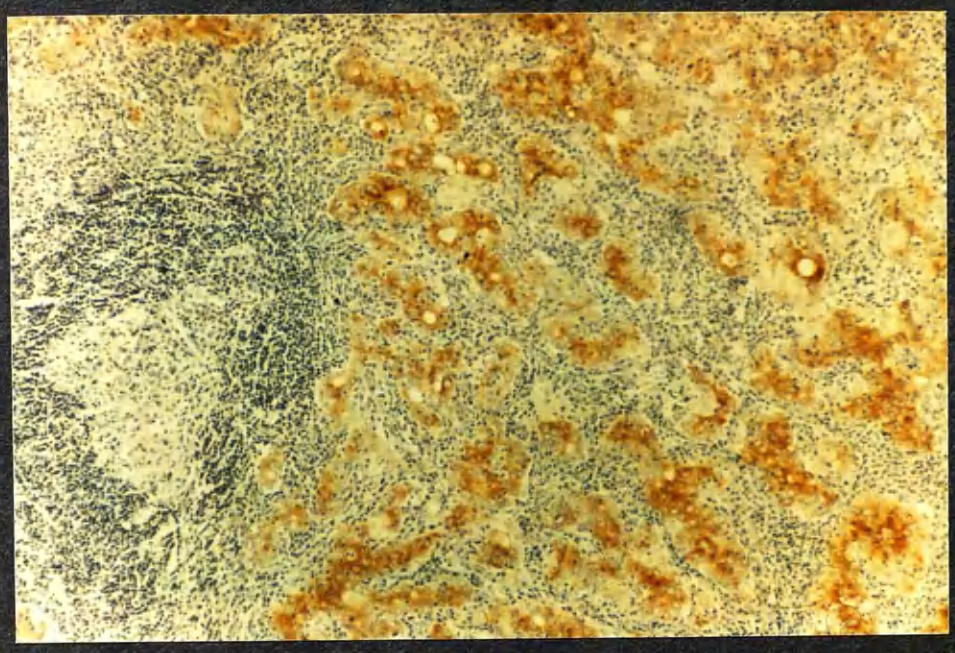
The results are summarised in Table 11.1. The results of statistical analysis are presented in Table 11.2. The figures show examples of progastricsin in metastatic carcinomas in lymph nodes from a primary in breast (Fig 11.4) and in liver from primaries in stomach (Fig 11.5) and breast (Fig 11.6).

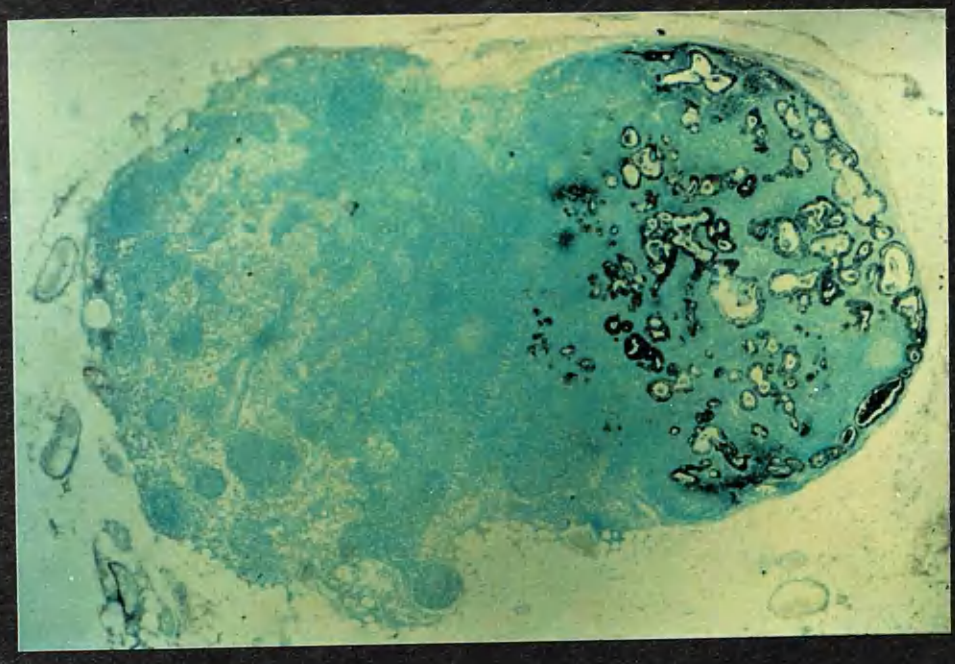
Blocks of the non-gastric primary tumours were available in the files in 4 cases in which there was a progastricsin-containing metastasis in lymph nodes and in 5 where there was one in liver. In the former group the primary was in breast (2), uterus (1) and lung (1) and in the latter group, breast (1), colorectum (2), and ampulla of Vater (1). In none of these primary carcinomas was progastricsin demonstrated, either by the IGSS or PAP method.

FIG. 11.1 Progastricsin in metastatic deposit of carcinoma in local lymph node in extraserosal fat of gastrectomy specimen.
Antigastricsin, PAP method. x250.

FIG. 11.2 Progastricsin in local lymph node metastasis of gastric carcinoma.
Antigastricsin, IGSS method. x47.

FIG. 11.3 High power of 11.2 showing progastricsin in tumour acini.
Antigastricsin, IGSS method. x288.





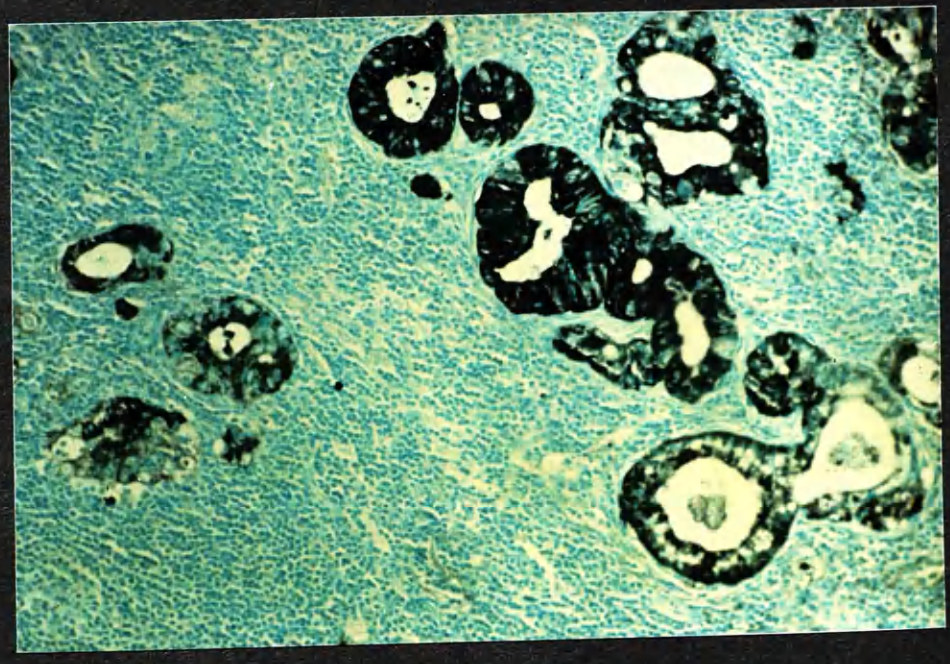
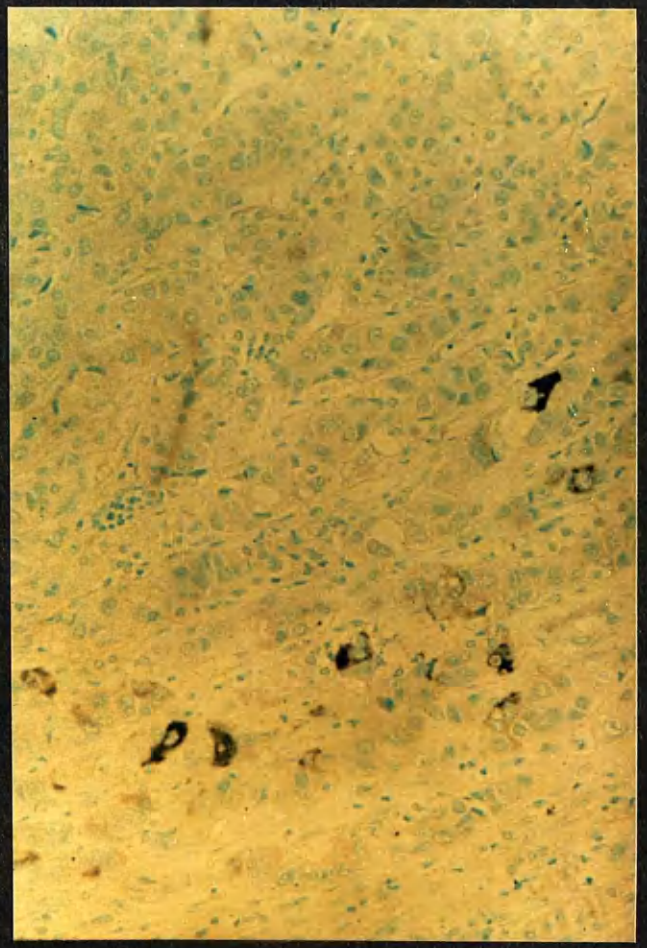


FIG. 11.4 Progastricsin in metastatic deposit of adenocarcinoma in axillary lymph node from primary in breast.
Antigastricsin, IGSS method. x288.

FIG. 11.5 Progastricsin in liver metastasis from primary gastric carcinoma. Histology is not available from the primary, which was not resected, but there was a large filling defect in the stomach on barium meal.
Antigastricsin, IGSS method. x190.

FIG. 11.6 Progastricsin in liver metastasis from primary carcinoma of breast. A small deposit in the adrenal was also positive in this case but the primary in breast was negative.
Antigastricsin, IGSS method. x288.



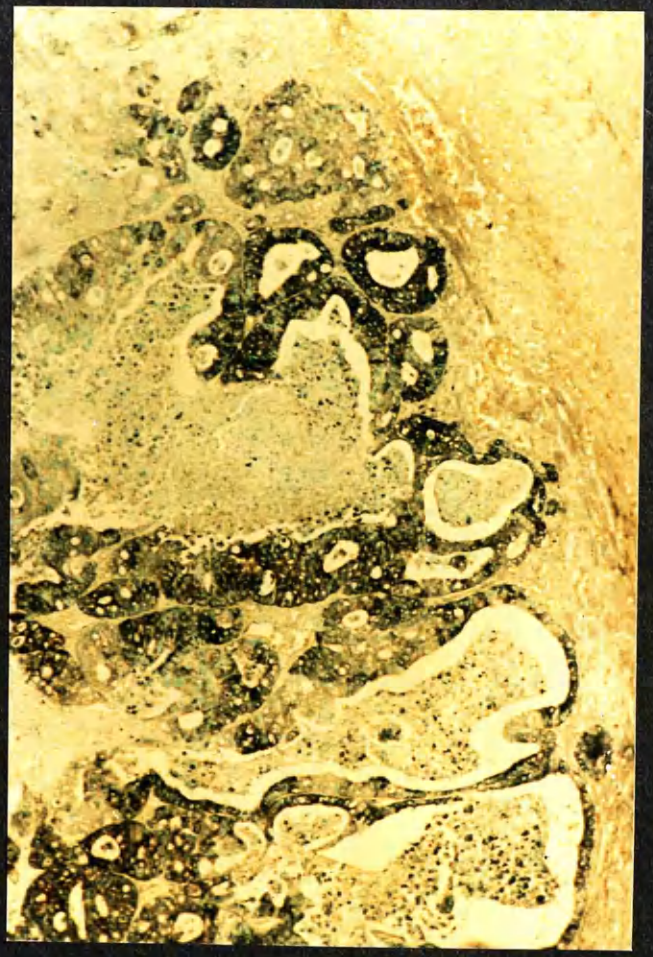


TABLE 11.1

NUMBER OF PROGASTRICSIN-CONTAINING METASTATIC ADENOCARCINOMAS IN VARIOUS SITES ACCORDING TO PRIMARY SITES (NUMBER OF CASES EXAMINED IN BRACKETS)

Primary site	Lymph Nodes						
	Neck	Axilla	Groin	Mediastinum	Abdomen	Liver	TOTAL
Stomach	2 (2)	-	0 (1)	-	5 (14)	5 (17)	12 (34)
Oesophagus	-	-	-	1 (1)	-	0 (3)	1 (4)
Pancreas	-	-	-	-	0 (1)	3 (16)	3 (17)
Colorectal	0 (1)	-	0 (1)	0 (1)	0 (2)	1 (28)	1 (33)
Prostate	1 (1)	-	-	1 (1)	-	-	2 (2)
Breast	0 (5)	2 (23)	-	0 (5)	-	2 (5)	4 (38)
Lung	1 (12)	-	-	1 (6)	-	0 (5)	2 (23)
Others	1 (3) (thyroid)	-	0 (1)	-	1 (3) (uterus)	1 (6) (ampulla of Vater)	3 (13)
TOTAL	5 (24)	2 (23)	0 (3)	3 (14)	6 (20)	12 (80)	28 (164)
Unknown	2 (8)	1 (1)	-	-	-	7 (45)	10 (54)
TOTAL (including unknown)	7 (32)	3 (24)	0 (3)	3 (14)	6 (20)	19(125)	38 (218)

TABLE 11.2

STATISTICAL ANALYSIS OF PROGASTRICSIN-CONTAINING METASTATIC CARCINOMAS

	Chi square (Yates' correction)	p
All lymph nodes : primary in stomach or prostate versus other sites	6.7836	0.0092
Abdominal lymph nodes : primary in stomach versus other sites	0.1020	0.7494
Liver : primary in stomach versus other sites	2.2277	0.1356
primary in stomach or pancreas versus other sites	1.4133	0.2345

DISCUSSION

It can be seen that progastricsin-containing metastases in lymph nodes are significantly more likely to come from a primary in stomach or prostate than in other sites. The results for metastases in abdominal lymph nodes only do not reach significance and metastases in liver do not significantly correlate with a primary in stomach or in stomach and pancreas together.

In a general hospital, 7-10% of patients with histologically confirmed metastatic tumour have no evidence of a primary malignancy (Stewart et al, 1979; Tattersall, 1987). This does not preclude therapy (Greco, Vaughn & Hainsworth, 1986), although it is important that treatable cancer be excluded. Investigation of the patient should, however, be minimised (Stewart et al, 1979; Altman & Cadman, 1986; Simon & Bartucci, 1986). Immunohistochemical study now allows diagnostically difficult malignancies to be classified into carcinoma, lymphoma or other type in 93% of cases (Gatter et al, 1985). Currently the site of a carcinoma cannot generally be further specified from histology or immunohistochemistry. It appears from the results of the present study that the demonstration of progastricsin in lymph nodes allows the presence of a primary in stomach and prostate to be suggested. Five out of 17 gastric metastases in liver were identified, but this was not statistically significant as metastases from each of a variety of primary sites were also sometimes positive. It may be noted that both cases of metastatic prostatic carcinoma in lymph nodes were progastricsin positive. In practice a primary in prostate could be diagnosed by clinical examination or by demonstration of acid phosphatase in the metastasis.

The presence of progastricsin in metastases from non-gastric primaries is also of interest. It has already been shown (Chapter 10) that progastricsin is not found in such primary sites, apart from

pancreas and one breast. In the present study, progastricsin could not be convincingly demonstrated in any of the primaries studied, even though it was present in occasional metastases of carcinomas from breast, lung, colorectum, thyroid, uterus and ampulla of Vater in addition to pancreas. The explanation could firstly be a cross-reaction of the antibody with some tissue component. Secondly, there could genuinely be progastricsin production by the tumour cells, presumably because its gene is switched on.

Finally, progastricsin was demonstrated in 10 out of 54 metastases from unknown primaries. No conclusions have been drawn from this observation, as the probability of the primary being undetected is likely to be influenced by its site, but it could well be that some or all of these primaries were in the stomach or, perhaps, prostate.

CHAPTER 12

FUTURE STUDIES

As a result of the work described in this thesis, four aspartic proteinases have been localised in normal tissues and in a variety of neoplasms. The relative specificity of pepsinogen and progastricsin and, possibly, SMP for carcinomas of stomach suggest a number of further lines of enquiry and some possible applications. If a paraffin section of a given metastatic adenocarcinoma is found to contain progastricsin, a gastric origin may be suggested with about 50% likelihood of being correct. This may be of value to the diagnostic histopathologist in the biopsy room. The possibility of a similar application for SMP is currently under investigation and it may be that a panel of antibodies would yield higher specificity and selectivity.

Tumour-specific antigens may have diagnostic uses in clinical practice. The serum levels of the aspartic proteinases have been studied, but do not appear to reflect the presence or amount of a tumour, as the effect of gastritis overwhelms the effect of zymogen production by tumour (see Chapter 3). Radiological localisation of tumour, however, may be possible. For example, radiolabelled monoclonal antibody to the oncogene protein $p62^{c\text{-}myc}$ has been used to localise lung tumours on scans (Chan et al, 1986). The protein is not on the cell membrane but in the nucleus and is assumed to be released by necrotic tumour cells. Such an application for antibodies to aspartic proteinases could be explored, despite the intracellular location of the zymogens. Similarly, a therapeutic role, by drug targetting with monoclonal antibodies to aspartic proteinases could be postulated (Embleton, 1987).

The biological role of aspartic proteinases is also under investigation. The function of SMP, or cathepsin E as it now appears to be, is completely unknown and requires further study.

Recently, aspartic proteinases have been found in a rather unexpected site, namely in retroviruses such as Human Immunodeficiency Virus (HIV), Type I (Kay et al, 1987). A retroviral proteinase, which processes the gag polyprotein into its separate core proteins, contains the sequence Asp-Thr-Gly, which is conserved in aspartic proteinases (Fig. 1.1). Study of a model of the pol proteinase of HIV I by pattern-recognition, structure prediction and molecular modelling techniques, suggests that the viral proteinase corresponds to a single aspartic proteinase domain, which may be active as a dimer (Pearl & Taylor, 1987). Furthermore, the proteinase activity of three retroviruses has been inhibited by pepstatin (Katch et al, 1987). Cathepsin D has previously been located in histological sections with biotin-labelled pepstatin (Matthews et al, 1981) and similar localisation of retroviruses might be conceivable. However, any future treatment of retrovirus infection such as AIDS with aspartic proteinase inhibitors would have to take into account the effect on normal cells in which aspartic proteinases have been demonstrated, as described in this thesis.

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