STUDIES ON THE GASTRIC PROTEASES IN THREE SOUTH AFRICAN SNAKE SPECIES

Thesis

Submitted in Fulfilment of the

Requirements for the Degree of

DOCTOR OF PHILOSOPHY

of Rhodes University

by

SIRION SHOLTO DOUGLAS ROBERTSON

June 1986

DEDICATION

This thesis is dedicated to the three people whom, indirectly, it most concerns:

Sally-Ann

Justin

Natalie.

'Mount Pleasant'
Stones Hill
Grahamstown.
30 June 1986.

From a certain point onward there is no longer any turning back. That is the point that must be reached.

Franz Kafka

1883 - 1924

ACKNOWLEDGEMENTS

In a general context, my greatest debt of gratitude is to my parents, Mr and Mrs D.D. Robertson, who gave me - amongst many other things - the three years leading to my first degree. This continued a pattern of circumstances from which I have benefitted greatly, and of which the present thesis is one ramification.

The earliest precursory parts of this work were carried out in the laboratory of Professor G.R. Delpierre, of the Department of Chemistry at the University of the Western Cape. I must thank him for arousing my interest in venom proteases, and for introducing me to the practical aspects of ion-exchange- and molecular exclusion-chromatography.

The project itself was commenced under the supervision of Drs M.J. Peak and F.T.. Robb, then of the Departments of Zoology and Microbiology at Rhodes University.

In the immediate context my greatest indebtedness is to my current supervisors, Professors J.F.E. Newman and D.A. Hendry, of the Department of Microbiology, Rhodes University. On the departure of Drs Peak and Robb they generously adopted the project when it might have been prematurely orphaned. In addition to providing a laboratory and extensive facilities, they introduced me to practical techniques in immunology (D.A.H.) and polyacrylamide slab gel electrophoresis and tryptic peptide mapping (J.F.E.N.), and gave me the benefit of their interest and enthusiasm at all times. They have also read the thesis in draft, and made helpful suggestions on presentation.

Dr D.R. Botes, head of the Molecular Biology Division, National Chemical Research Laboratories (C.S.I.R.), provided the facilities for composition analyses. Dr T. Haylett, also of the N.C.R.L., performed some of the analyses quoted in the text, and submitted my crude composition data to an independent error minimization programme.

Professor John Brand, of the Biochemistry Department at the University of Fort Hare, Alice, kindly made available his Department's amino acid analyser, and Mr James Gray, of the Department, supervised me in use of the machine.

Mr Eric Smith, a graduate student in the School of Pharmacy, Rhodes University, wrote an error minimization programme for the derivation of best-fit masses from the composition data.

The staff of the Rhodes Library, and in particular Miss Sue Arnott, has been most efficient in obtaining books, journals and reprints.

Hoechst Chemicals most generously donated two bottles of their inhalation anaesthetic Halothane.

Various people gave me general technical assistance. They include Messrs Ron Arnott, John Haslop, John Murray and Dr Santy Daya.

The Department of Nature and Environmental Conservation gave me permission to obtain and kill snakes for the project. Several snakes were supplied by the Port Elizabeth Snake Park. The majority of snakes used in the study were provided by Mr John Wood, of the South African Snake Farm, Kommetjie.

Funding has been generously provided by Rhodes University and by the

C.S.I.R. (1980).

In preparing the final report I have benefitted from critical comments by Professor G.R. Delpierre (University of the Western Cape, Belville,), and Professor A.P. Ryle (University of Edinburgh, Scotland). Professor Ryle has also brought to my attention some important references. I have had the advantage of a brief discussion with Dr R.J. Naude, (University of Port Elizabeth) on the treatment of amino acid analyses.

Finally, personal experience, first-hand reports and common sense indicate that the spouses of graduate students experience various stresses during academic gestation and the prolonged labour of parturition. Mine is no exception. In addition to providing the domestic tranquillity and continuity essential to productive cerebration, she has typed draft, fair copy and amended copy, checked spelling and advised on the formalities of layout.

ABSTRACT

The pepsinogens and pepsins of cobra, mole snake and puff adder have been studied. The pepsinogens of all three species fall into two distinct groups, here designated PI and PII. At least the latter group, in all cases, shows substantial microheterogeneity. Physicochemical studies suggest that the cobra and puff adder PII groups are more similar to each other than either is to the mole snake PII group.

Kinetic studies indicate that, in the cobra and mole snake, the PI and PII pepsins differ in their Arrhenius activation energies. Such difference is smaller, or absent, in the case of the puff adder PI and PII pepsins. These characteristics of the pepsins are assessed in the context of the differences between the oral secretions of the three species studied. The suggestion is advanced that the puff adder's strongly proteolytic venom has influenced certain properties of its gastric proteases.

TABLE OF CONTENTS

CHAPTER FIVE

CHAPTER SIX

CHAPTER SEVEN

APPENDIX

LIST OF TABLES

CHAPTER ONE

INTRODUCTION

CHAPTER ONE

INTRODUCTION

The earliest work on pepsins grew from studies on gastric digestion (Varro, 36 B.C.; de Reamur, 1752; Spallanzani, 1783, cited by Taylor, 1968). More recently, interest in pepsins has been promoted by their uniquely low pH optima, by a general concern with the active sites of catalytic proteins, and by the circumstance, common to all extracellular (vertebrate) proteolytic enzymes (Foltmann, 1981), that pepsin is the activation product of an inactive precursor or 'zymogen' - pepsinogen.

Medical interest in pepsin has been related in large measure to the regulation of its secretion (as pepsinogen) in the context of gastric and duodenal ulceration. Further, low pepsinogen levels in blood plasma have been found to correlate with the development of malignant gastric neoplasms, and such levels have been suggested as providing early indicators of malignancy (Nomura et al, 1980). Experimental animals for the study of pepsinogen secretion have included the frog (Inoue et al, 1985), dog (Magee and Naruse; 1983) and Man himself (Waldum and Burhol, 1980; Defize et al, 1984).

More generally, work on pepsinogens and pepsins has naturally drawn upon convenient sources of material. Porcine, bovine, chicken and monkey pepsins and their precursors are amongst the most extensively investigated and characterised.

Studies on the comparative biology of pepsins, motivated by interest in evolutionary relationships and/or adaptation, have been rather fewer. In this area reptiles in particular appear to offer considerable scope for further work. Blain and Campbell noted in 1942 that reptile digestive physiology had received little attention. This comment was echoed by Diefenbach in 1975, and the situation has not yet greatly altered. It is hoped that the present work will be seen as a small contribution to the subject.

In addition to the general appeal of contributing to an area not yet extensively researched, certain considerations apply particularly to members of the Order Ophidia,* and provide further incentive for an investigation of their gastric enzymes. At least three families within this Order have developed complex oral secretions - venoms - containing a variety of enzymes. Several of these enzymes have been shown to degrade components of animal tissues. Although there is interspecific - and indeed intraspecific - variation within several taxa (Murata et al, 1963; Jiménez-Porras, 1964), a number of genera in the family Viperidae include species possessing venoms particularly rich in proteases. These enzymes contribute to the haemorrhage and/or local necrosis commonly following bites by such snakes (Eagle, 1937; Okonogi et al, 1964; Visser and Chapman, 1978).

From these data it is a small step to the assumption that venom

---

* = Order Serpentes

proteases contribute significantly to the digestion of prey. This claim has frequently been made on the basis of indirect or reported evidence (Zeller, 1948; Minton and Minton, 1969). The only other conceivable function of snake venom is "to subdue or obtain their prey" (Schmidt, 1950). Since this function is ably served by venoms of the elapid snakes - generally lacking in proteolytic activity (Christensen, 1955; Robertson et al, 1969) - it seems difficult and unnecessary to seriously question a digestive role, at least amongst members of the Viperidae. Notwithstanding this, Thomas and Pough (1979) suggested that such an assumption did not rest on adequate experimental evidence. Working on venom of the rattlesnake (Crotalus atrox : Viperidae), they concluded that this venom does indeed contribute to prey digestion, especially at low temperature. From their own and other data they also advanced cogent arguments for believing that venom-aided digestion is a factor contributing to the geographic distribution of viperid snakes.

Recently Purves et al (1986) have isolated from puff adder venom a protease able to cleave one of the plasmin-degradation products of fibrin. These workers note, on the basis of preliminary data, that close relatives of the puff adder appear also to contain this protease, but that it does not occur in venom of the elapid Naja nigricollis. They suggest that the biological role of the enzyme includes tissue disruption.

Although it may be considered unnecessary to seek further evidence supporting a digestive role for viperid venom, it is nonetheless interesting to ask whether such venoms, playing a part in protein digestion, have exerted any detectable evolutionary influence on

other proteases of the digestive tract.

Presumably this possibility may apply to any of the alimentary canal proteases. Indeed, as some viperid venom enzymes resemble trypsin in substrate specificity and pH optimum (Delpierre, 1968a), examination of pancreatic proteases would provide a logical starting point for an investigation based on this rationale. Early attempts to analyse pancreatic proteases were unsatisfactory and discouraging however (Robertson and Paul, unpublished data), whereas preliminary assays of gastric proteases were more promising (Robertson and Reid, unpublished data; Robertson, 1979).

An investigation of snake alimentary proteases on these grounds is in some sense facilitated by the existence within the group of natural albeit imperfect "controls". Thus several snake families comprise members which are solid-toothed and non-venomous, while the family Elapidae includes many species which, though venomous, lack highly proteolytic venom enzymes (Mebs, 1970; Robertson et al, op. cit.). The family Viperidae includes species with the most actively proteolytic venoms. Attempts to compare alimentary proteases in members of these groups are complicated by their phyletic separation, but in view of the large number of genera in each of the groups, accumulating data may eventually reveal consistent patterns in support of the possible association suggested here. Such an adaptive association between venom proteases and those of the post-oral alimentary canal, in addition to further establishing the digestive role of venom, would provide another example of the complexity and subtlety of adaptive processes. "The job of the scientist is to

study, catalogue and relate the orderliness of nature ... " (Davies, 1983).

Inevitably studies on material derived from different groups have potential taxonomic interest. Some of the work in this study has been performed in the hope of indicating degree of relatedness between the three species, and in the interest of investigating the enzyme *per se*, rather than exploring the question of evolutionary adaptation of the gastric proteases to venom activity.

Assessment of the work should, I believe, take into account three considerations *inter alia*. Firstly, at no stage has it been intended as a treatise in biochemistry. Secondly, although the raw materials of the study - gastric mucosae of snakes - are not intrinsically in short supply, the ecological status of many feral animals, snakes included, imposes certain limits on the effective availability of research material. For this reason some of the data reported here do not have the definitive rigour rather easily attainable where material is available in large quantities. Thirdly, the main question posed - viz., the possibility of adaptation, by gastric proteases, to the presence of powerful oral proteases - cannot by its nature be finally answered in a single thesis of conventional size. The question as posed is nonetheless meaningful in the sense that such adaptation is conceivable in the general context of evolutionary theory. What is less clear is the question of the experimentally accessible parameters which might reflect such adaptation(s). This matter is discussed in the Conclusion.

The following species have been used:

1. Mole snake (*Pseudaspis cana*, Colubridae)

2. Cape Cobra (Naja nivea, Elapidae)
3. Puff adder (Bitis arietans, Viperidae)

(Classification is from Fitzsimons, 1962.)

These have been chosen to provide species which are, respectively, non-venomous (Fitzsimons, 1962); venomous with low venom protease activity (Robertson, et al, 1969), and venomous with high venom protease activity (Delpierre, 1968b).

CHAPTER TWO

GASTRIC PROTEASES : A BRIEF REVIEW

CHAPTER TWO

GASTRIC PROTEASES : A BRIEF REVIEW

2.1 Introduction

Gastric acid proteases are ubiquitous if not quite universal amongst vertebrates. They are absent in various groups of fish in which the stomach is rudimentary or absent. All members of the Subclass Holocephali lack a stomach, as do several genera within the families Cyprinidae and Cyprinodontidae (Barrington, 1957).

Pepsin was the first enzyme to be obtained in crystalline form, and the second, after Sumner's classic work on urease in 1926, to be shown to be a protein (Northrop, 1930).

The term "pepsin" was coined by T. Schwann in 1836, from a Greek root meaning "digestion"*. The same authority attributes the word "pepsinogen" to Foster in 1878.

General reviews have been published by Herriott (1962), Taylor (1968) and Foltmann (1981). In specific areas, nomenclature has been discussed by Etherington and Taylor (1967); secretion of the zymogens by Hirschowitz (1967); catalytic activity and the active site by Clement (1973), and

---

*Oxford English Dictionary, Vol VII, Part 1, Clarendon Press, 1905.

activation by Marciniszyn et al (1976b) and by Kay (1980). The evolution of the acidic proteases has been discussed by Foltmann and Pedersen (1977) and Tang (1979).

Complete sequence data for pig pepsin have been published by Sepulvida et al (1975), for chicken pepsinogen by Baudys and Kostka (1983) and for human pepsinogen A by Tang (1982 : not seen by the writer, quoted by Baudys and Kostka, op. cit.). Incomplete sequences have been reported for several pepsinogens, including those of Crab-Eating Monkey (Kageyama and Takahashi, 1980), rabbit (Kageyama and Takahashi, 1984), the major pepsinogen of Asiatic Black Bear (Kageyama et al, 1983b), rat pepsinogen I (Arai et al, 1984) and bovine pepsinogen (Harboe et al, 1974)

Crystallographic studies have been reported by Andreeva et al (1977) and by Andreeva and Gutschina (1979).

Methods of analysis of the activated enzymes have recently been summarised by Ryle (1984).

In addition to these there is a vast literature on the biochemistry of pepsinogens and pepsins from various species. Several of these papers are cited in the main part of this work.

2.2. Nomenclature

All vertebrate gastric proteases are classed within the group

3.4.23, as carboxyl (acid) proteases.

Foltmann and Pedersen (1977) note that these proteases generally have pH optima below 6. As a further generalisation, most of them have two aspartate groups at the active site, and are inhibited by pepstatin. They may be referred to as "aspartate proteases" (Foltmann and Pedersen, op. cit.).

The gastric proteases are subdivided as follows:

3.4.23.1 This category includes the major gastric protease of the pig, designated Pepsin A. Enzymes in this group are active against the substrates APDT and haemoglobin (Ryle, 1970).

3.4.23.2 In this division are placed pepsins designated Pepsin B. Parapepsin I is synonymous with Pepsin B (Ryle, op. cit.). Other names previously given this group are Gastric cathepsin, Non pepsin gastric proteinase and Foetal pepsinogen (Foltmann, 1981).

Enzymes in this group show high activity towards APDT, and little or no activity towards haemoglobin (Ryle, op. cit.).

3.4.23.3 Members of this group are designated Pepsin C. Gastricsin (Tang, 1970) is included in this category, as is Parapepsin II

(Foltmann, op cit., Ryle, op. cit.). Pepsin C has little or no activity against APDT (Ryle, op. cit., Tang, op. cit.), and is less susceptible to inhibition by pepstatin than are the other pepsins (Kay et al, 1983 : not seen by the writer, quoted by Ryle, 1984).

3.4.23.4 Proteases in this group are the Chymosins (= Rennin, Foltmann, op. cit.). They have enzymatic characteristics similar to those of Pepsin A, but greater milk-clotting activity.

All the gastric proteases are secreted as proenzymes. The designations of these zymogens are the same as for the active enzymes, but they are distinguished by the suffix -ogen, for pepsins, and the prefix pro- for chymosins (Dixon and Webb, 1979; Foltmann, op. cit.). For the precursor of gastricsin, Foltmann (op. cit.) and Kageyama and Takahashi (1985) use the term progastricsin. Tang (1979) refers to gastricsinogen.

Generally, workers have tended to identify pepsins and pepsinogens in terms of their chromatographic or electrophoretic behaviour. Thus Ryle and Porter (1959), using DEAE cellulose, isolated two minor pepsins ("parapepsins I and II") from pig pepsin A. These were later renamed pepsins B and C, and evidence was presented for the existence of a third minor pepsinogen,

which was named pepsinogen D (Ryle, 1965). This was subsequently purified, as pepsin D, and shown to be dephosphorylated pepsin A (Lee and Ryle, 1967).

This alphabetic system was followed by Bar-Eli and Merrett (1970) for dogfish pepsinogens.

Inevitably, with increasingly refined techniques for demonstrating heterogeneity, and the natural diversity of gastric proteases across the vertebrate spectrum, informal classification of the enzymes has itself diversified, and degrees of homology between them is in many cases unclear. As noted by Foltmann (1981) "we do not know how to classify the pepsinogens from dogfish and chicken relative to the individual zymogens from mammals ... ". (Baudys and Kostka, op. cit., tentatively assign chicken pepsinogen to the group EC 3.4.23.1, on the basis of sequence studies and kinetic characteristics.)

Etherington and Taylor (1967) recommended numbering human pepsins in order of decreasing mobility, at pH 5, on agar gel electrophoresis. They later showed (Etherington and Taylor, 1969) that their pepsin 5 is the same as the gastricsin of Tang et al (1959).

It has been shown that human pepsinogens are separable into two immunologically distinct groups. These groups are termed PGI and PGII. Group PGI includes the electrophoretically resolved zymogens Pg 1 to 5, which occur in gastric mucosa, blood and urine. Group PGII includes the electrophoretically

designated Pg 6 and 7, which occur in gastric mucosa, blood and seminal fluid (Samloff, 1969, 1971 : not seen by the writer, quoted by Taggart et al, 1979; Levine and Beer 1984).

Kageyama and co-workers (1976a and b; 1980; 1983b; 1984) designated pepsinogens by means of Roman numerals to indicate their order of elution from ion-exchange columns. The zymogens were further identified by alphabetic characters and Arabic numerals as polyacrylamide gel electrophoresis revealed microheterogeneity. These workers (Kageyama and Takahashi, 1983b) tentatively allocate all five pepsinogens isolated from Asiatic Black Bear to group 3.4.23.1, while noting that the activities of the Bear pepsins on APDT were 20% or less of that shown by pig pepsin A.

In this context it may be remarked that the characters used in grouping the proteases are themselves continuous variables, and the groups, as defined, must necessarily accommodate some internal diversity. The accumulation of detailed comparative data will presumably require that quantitative limits be set to the diagnostic characters in each group, and will probably result in an extension, or subdivision, of the existing categories.

2.3 Pepsinogens

2.3.1. Distribution of zymogen-secreting cells

The subject of gastric secretion has generated an extensive literature independent of studies on gastric proteases *per se*.

Inevitably, the study of gastric secretion has been motivated largely by interest in factors relating to the origin and control of peptic and duodenal ulceration in man, and our knowledge of the relevant processes derives largely from studies on man and higher mammals. In contrast, there is a paucity of data on gastric physiology in the lower vertebrates.

The human stomach is divisible into four main parts.

1. The cardiac portion is immediately adjacent to the gastro-oesophageal (cardiac) sphincter.
2. The fundus, or corpus, is an expanded area superior to the cardiac sphincter.
3. The body, or intermediate region, is the largest portion.
4. The pyloric region, or antrum, leads without clear demarcation from the body of the stomach to the pyloric sphincter.

(Hally *et al*, 1976)

The entire mucosal surface is lined with tubular glands which amongst them secrete mucus, hydrochloric acid and

pepsinogen.

The structure and functions of the glands vary in the different gastric regions. Thus the glands of the cardia secrete mainly mucus, the glands of the fundus (oxyntic glands) secrete mucus, acid and zymogens, while the glands of the pyloric region secrete the hormone gastrin, a small amount of zymogen, and mucus (Hally et al, op. cit.).

Pioneering studies by Linderstrøm-Lang and co-workers (1934 : not seen by the writer, quoted by Foltmann, 1981) showed that the zymogens are secreted mainly by the chief cells of the gastric glands. Later histochemical techniques (see below) have revealed that the mucus neck cells also secrete zymogen.

The application of immunoperoxidase and immunofluorescence techniques to gastric mucosal sections has demonstrated that chief cells of the oxyntic glands secrete a mixed group of pepsinogens, while chief cells of the antral glands secrete only those (Pg 6 and/or 7 - see Nomenclature) which react with antipepsinogen II antibody (Lechago et al, 1979). Foltmann (1981) notes that the antral zymogen is gastricsin-(ogen), and that it is also secreted by cells of the proximal duodenum. The pyloric zymogen recognised by Foltmann as gastricsin appears to be the same as "pepsinogen I", isolated by Seijffers et al (1963) from the human duodenum.

Data quoted by Foltmann (op. cit.) suggest that the distribution

of mucosal function in pig, cat and dog is at least similar to that observed in man. Amongst the submammalian vertebrates, however, cellular specialisation does not appear to have gone so far. Hirschowitz (1967) summarises several reports indicating that in birds, reptiles, amphibians and fish, the gastric tubular epithelium is composed of a single cell type, which secretes both enzyme and acid. Quoting Wright *et al* (1957), Hirschowitz (*op. cit.*) further notes that in tortoises, lizards and snakes, pepsinogen granules in the gastric secretory cells are present mainly in the fundus, and decrease progressively from the upper to the lower end of the stomach. The paper by Wright *et al*, cited by Hirschowitz, in fact deals only with lizards and a tortoise.

Recent studies have shown that in the Bullfrog, *Rana catesbeiana*, pepsinogen- and acid-secreting cells occur in the lower oesophagus, which is the main site of pepsinogen secretion (Inoue *et al*, 1985).

2.3.2. Regulation of synthesis and secretion

The process of synthesis and encapsulation of zymogen via the Golgi membranes belongs in the general sphere of cell physiology and will not be discussed here.

In view of the meagre data available on lower mammals and submammalian vertebrates - as noted above - widely applicable generalisations are difficult to make. Gastric

secretion appears to be controlled by both nervous and hormonal stimuli. In most if not all vertebrates feeding is periodic, and secretory activity is reduced or abolished completely between periods of digestion. Through acetylcholine, the vagus nerve stimulates gastric secretion. Secretion is also stimulated by the hormone gastrin (Prosser and Brown, 1961). Atropine, an anticholinergic agent, inhibits secretion, as do - at least in man - glucagon, calcitonin, growth hormone releasing inhibiting factor, and the prostaglandin PGE_2 (Hally et al, op. cit.). Secretin is reported by Hally et al (op. cit.) also to inhibit gastric secretion. Foltmann (op. cit.), quoting other workers not specifically cited, reports that secretin inhibits the secretion of acid and of gastrin, but that secretion of pepsinogen may be stimulated by secretin. Foltmann comments that "it is questionable whether this is a normal physiological effect in any of the species tested". Waldum et al (1979) quoting Berstad (1969 : not seen by the writer) note that secretin "is another strong pepsin stimulant". See also later comment on guinea pig chief cells in vitro (page 17).

It has been known for some time that severe hypoglycaemia is a powerful stimulus for gastric secretion in humans. This is a centrally-controlled mechanism mediated by the vagus nerve (Hirschowitz, op. cit.). More recently Webber and Morrissey (1979) have shown that, in the rat, different thresholds of blood glucose concentration exist for the secretion of pepsin

and of acid.

The local hormone histamine generally stimulates gastric acid and zymogen secretion in humans and a wide variety of mammals (Haggstrom and Hirschowitz, 1984) but as noted by Haggstrom and Hirschowitz (op. cit.), the detailed effects of histamine vary between several mammalian species. Thus in dogs and cats, histamine has a predominantly inhibitory effect on pepsinogen secretion.

It has been suggested (Hirschowitz, 1967) that secretion from the chief cells provides a stimulus for increased zymogen synthesis. Waldum et al (op. cit.) monitoring human PGI following infusion with pentagastrin* and secretin, obtained evidence in support of this. It has also been shown that serum PGI levels reflect the rate of secretion per se (Waldum et al, 1978 : not seen by the writer, quoted by Waldum et al, 1979).

Histamine or pentagastrin stimulation elicits a greater secretion of pepsinogen Pg 1 from smokers with gastric or duodenal ulcers, than from non-smokers with ulcers. Rates of secretion of the other pepsinogens were not elevated under the same conditions (Walker and Taylor, 1979). In addition to specifically implicating pepsin 1 as an aetiological

---

*Pentagastrin is a synthetic pentapeptide based on the terminal tetrapeptide of the hormone gastrin (Hally et al, op. cit.).

factor in smoking-induced ulceration, these findings suggest that secretion of the various gastric zymogens is differently controlled.

Periodic increases in basal pepsin and acid secretion in fasting dogs is well known, but attempts to relate these variations to nervous and endocrine control mechanisms have produced conflicting results (Magee and Naruse, 1983), and will not be discussed.

Raufman et al (1984), working on isolated guinea pig chief cells in vitro, found a significant increase in pepsinogen secretion when the cells were exposed to several secretagogues, including secretin and cholecystokinin. The former is believed to act via cyclic AMP, the latter by alteration in intracellular calcium levels (Raufman et al, op. cit.). These workers therefore suggest that c AMP and calcium represent two intracellular mediators of zymogen secretion. In this study the pepsinogen secreted was not specifically identified. It will be interesting to know whether differential secretion of zymogens, as mentioned above, may be effected by these two regulators.

2.3.3. Protein chemistry

a. Amino acid composition and primary structure

TABLE I shows the amino acid compositions of several pepsinogens. TABLE II indicates the origins of the listed

zymogens, their molecular masses, and sources of information.

TABLE I reveals that all the zymogens listed have high contents of the acidic amino acids glutamic and aspartic acid, and of the β hydroxyamino acids threonine and serine. Similarly, all contain relatively few of the basic amino acids lysine, arginine and histidine. Foltmann (op. cit.) notes that these general proportions of the acidic and basic amino acids are found in all the gastric proteases.

The masses of the listed zymogens are in the range 35 000 - 42 000 daltons, and most are grouped within the narrower range c 38 000 - 40 000.

It has been noted by Chiang et al (1967), of human gastricsin in comparison with human pepsin, that "the reversed ratios of aspartic to glutamic acid and of isoleucine to leucine are especially characteristic".

Such ratios are generally reflected in the pepsinogens designated C in TABLE I (Numbers 6, 8 and 10), though dogfish pepsinogen C (Merrett et al, 1969) does not seem to qualify, on these criteria, for such designation.

Referring to a doctoral dissertation by Kern (1953 : not seen by the writer), Herriott (1962) notes that reduction of all three disulphide bridges does not result in any increase in osmotic pressure, as would be the case if the bridges were interchain. The assumption of a single chain structure is supported by end-group analyses (Van Vunakis, 1956; Rajagopalan, 1966b).

TABLE I

PEPSINOGENS : AMINO ACID COMPOSITIONS

This Table shows the amino acid compositions, as residues (to the nearest integer) per mole, of several pepsinogens. Sources of these data are given in TABLE II

	SPECIES OF ORIGIN, AND DESIGNATION**													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
AMINO ACID	PIG PEPSINOGEN A	PIG PEPSINOGEN A	BOVINE (FUNDIC)	ASIATIC BEAR, PII-1	JAPANESE CRAB-EATING MONKEY PIII-3	JAPANESE CRAB-EATING MONKEY "C"	RABBIT PIII	JAPANESE MONKEY "C"	CHICKEN (3.4.23.1. ?)	QUAIL PEPSINOGEN C (?)	POLAR COD PEPSINOGEN "B"	RAINBOW TROUT Ia	DOGFISH PEPSINOGEN "A"	DOGFISH PEPSINOGEN "C"
Asp	46	44	40	40	38	30	45	27	39	34	51	38	44	44
Thr	27	26	27	21	27	25	29	25	26	32	26	27	23	27
Ser	46	46	50	39	44	39	45	35	41	42	47	30	43	51
Glu	29	28	32	40	34	42	29	38	25	47	27	44	39	41
Pro	18	19	15	22	23	21	17	17	18	10	26	17	19	22
Gly	36	35	35	31	36	35	32	32	31	41	39	27*	40	48
Ala	20	19	16	20	20	22	24	19	17	19	27	33*	18	18
Val	25	23	25	25	26	25	24	20	25	19	34	21	23	27
Cys	6	6	6	7	6	6	6	6	7	6	9	10	7	7
Met	4	4	4	7	4	5	6	5	9	8	6	10	7	6
Ile	27	25	32	22	27	18	25	13	23	20	24	19*	22	18
Leu	33	33	25	23	29	29	32	27	28	32	32	21*	28	24
Tyr	17	17	18	15	18	16	18	17	22	18	26	13	19	20
Phe	16	15	15	18	16	17	17	17	20	24	21	13	17	17
His	3	3	2	2	3	3	3	3	8	3	2	2	7	4
Lys	10	10	8	9	8	7	10	7	17	1	2	12	14	5
Arg	4	4	6	4	5	5	4	5	7	2	4	3	14	8
Trp	5	6	6	5	6	5	4	5	4	4	–	–	5	–

See Notes to TABLE I, overleaf.

Notes to TABLE I:

* Twining et al (1983) reverse the Ala-Gly residue numbers for pig pepsinogen (taken, by them, from Foltmann and Pedersen, 1977). It may be that their reported data for these residues in the Rainbow trout are similarly reversed in error.

** The designations are those used by the authors (See TABLE II). In the case of the pig pepsinogen, listed under columns 1 and 2, Pesinogen A is accepted as the official name. Where a query exists next to a designation (numbers 9 and 10) the authors have indicated that such designation is tentative.

TABLE II

SOURCES OF DATA AND MASSES OF PEPSINOGENS LISTED IN TABLE I

The numbers 1 to 14 refer to the zymogens listed in TABLE I. Full citation of sources is given under REFERENCES.

NO.	SOURCE AND DESIGNATION OF ZYMOGEN	MASS (DALTONS)	REFERENCE
1.	Pig pepsinogen A	38 944 (amino acid analysis)	Rajagopalan et al (1966)
2.	Pig pepsinogen A	39 630 (sequence)	Foltmann and Pedersen (1977)
3.	Bovine (fundic) pepsinogen	38 900 (amino acid analysis)	Chow and Kassell (1968)
4.	Asiatic Black Bear Pepsinogen PII-1	38 000 (SDS-PAGE)	Kageyama et al (1983b)
5.	Japanese Crab-Eating Monkey Pepsinogen III-3	40 000 (SDS-PAGE)	Kageyama and Takahashi (1980)
6.	Japanese Crab-Eating Monkey Pepsinogen C	38 000 (SDS-PAGE)	(As for 5, above)
7.	Rabbit Pepsinogen PIII	40 000 (SDS-PAGE)	Kageyama and Takahashi (1984)
8.	Japanese Monkey Pepsinogen C	35 000 (Gel filtration G150)	Kageyama and Takahashi (1976b)
9.	Chicken Pepsinogen 3.4.23.1. (?)	42 074 (sequence)	Baudys and Kostka 1983
10.	Quail Pepsinogen C (?)	40 000 (SDS-PAGE)	Esumi et al 1980
11.	Polar Cod Pepsinogen B	40 000 (SDS-PAGE)	Arunchalam and Haard (1985)
12.	Rainbow Trout Ia	(not reported)	Twining et al 1983
13.	Dogfish Pepsinogen A	41 500 (Sedimentation equilibrium)	Merrett, T.G. et al (1969)
14.	Dogfish Pepsinogen C	41 500 (Sedimentation equilibrium)	(As for 13, above)

b. Secondary and Tertiary structure

The low viscosity of native pepsinogen in relation to its mass suggests a shape which is neither rigid and rod-like nor randomly flexible. Some specific coiling is thus inferred (Herriott, op. cit.).

Andreeva et al (1977) obtained x-ray crystallographic data for pig pepsin. These data suggest a bi-lobed structure with a deep cleft some 12 Å wide between the lobes. Other evidence, including studies by Subramanian et al (1977) on pepstatin binding to an aspartate protease from Rhizopus sp., indicate that this cleft constitutes the binding site, and contains the residues Asp35 and Asp215, which are important in catalysis*.

The N-terminal segment of the zymogen, at neutral and alkaline pH, stabilises the molecule by electrostatic interaction between basic amino acids in the "tail" and negatively charged dicarboxylic acids in the enzyme moiety (Foltmann, op. cit.). Al-Janabi et al (1972), quoting Delpierre and Fruton (1965) and Tang (1971), note that pepsinogen does not react with diazo compounds or specific epoxide modifiers, both of which react with the active site aspartate groups of pepsin. They suggest therefore that the N-terminal chain of the zymogen may cover the catalytic residues of the enzyme.

In terms of the references cited by Al Janabi et al, this suggestion is difficult to evaluate. Thus Delpierre and Fruton (op. cit.) note

---

*The homologous active site residues in pig pepsin are Asp 32 and Asp 215 (Foltmann, op. cit.).

that pepsinogen is modified (at pH 5,1) by diphenyldiazomethane, although the rate of inactivation was found to be "somewhat slower than that of pepsin", and that such modification inhibits activation of the zymogen. These workers note that "the ability of DDM to react with pepsinogen so as to prevent its conversion to active pepsin suggests that, if one or more carboxyl groups are involved in the active site of the enzyme, they are accessible to chemical attack by DDM as well".

Rajagopalan et al (1966a) found that another diazo compound, diazoacetyl-DL-norleucine methyl ester, reacts with pepsin but not with pepsinogen, nor does it reduce the potential peptic activity of the zymogen.

In agreement with the assertion by Al Janabi et al, Tang (1971) reports that the epoxide 1,2-epoxy-3-(p-nitro-phenoxy) propane (EPNP) does not inactivate pepsinogen.

Later work by Marciniszyn et al (1976a), described below, indicates that pepstatin does not bind to pepsinogen at pH 5,6. This implies some protection of the active site by the "tail" segment of the zymogen, in support of the contention by Al Janabi et al (op. cit.) as noted earlier.

c. Activation of the zymogens

Herriott (1939) established that, in the range pH 4 - 4,6, activation of pepsinogen is autocatalytic, in the sense that pepsin catalyses its own production, and proceeds by second-order kinetics. He also noted that the activation rate was far greater below pH 4,0, and that it proceeded by mixed-order kinetics.

Pepsinogen held for long periods at alkaline pH, in circumstances producing irreversible inactivation of any pepsin which may be present, nonetheless quickly activates when the pH is brought to c 2. As noted by Al-Janabi et al (op. cit.), this implies that the zymogen must be able to activate by a method other than the autocatalytic (bimolecular) reaction observed by Herriott (op. cit.).

A unimolecular, first-order activation mechanism was proposed by Tang (1970 : not seen by the writer, quoted by Al-Janabi, op. cit.). Evidence for this was obtained by Bustin and Conway-Jacobs (1971), who bound pig pepsinogen to Sepharose , and showed that the rate of acid activation of the zymogen was independent of the Sepharose-pepsinogen ratio, thus implying that the activation rate was independent of the average distance between pepsinogen molecules.

Al-Janabi et al (op. cit.) showed that, in the pH range 1 - 3, semilogarithmic plots of remaining pepsinogen

concentration are a linear function of activation time. This indicates first-order kinetics, and hence intramolecular activation (Figure 1).

At pH 4 the same graphic co-ordinates as used in Figure 1 yielded a curve, suggesting mixed-order kinetics. The rate equation for the reaction is given by Al-Janabi *et al* (*op. cit.*) as

$$-\frac{d[Pgn]}{dt} = k_1[Pgn] + k_2[Pgn][Pep]$$

where $-(d[Pgn]/dt)$ = rate of disappearance of pepsinogen;

$[Pep]$ = pepsin concentration;

k_1 and k_2 = 1st- and 2nd-order rate constants.

On integration this equation fits the experimental pH 4 activation well (Figure 2). The initial concentration of pepsin was assumed to be negligibly small. The values of k_1 and k_2 were derived from the curve.

The influence of pH on the activation mechanism may be illustrated by plotting values of k_1 and k_2 against pH (Figure 3).

Al-Janabi *et al* (*op. cit.*) further suggest that unimolecular activation occurs at the same catalytic locus as that in the active enzyme. In addition to steric considerations, they point out that their observed k_1 pH rate profile resembles $\frac{k\ cat}{km}$ profiles for peptide hydrolysis by pepsin.

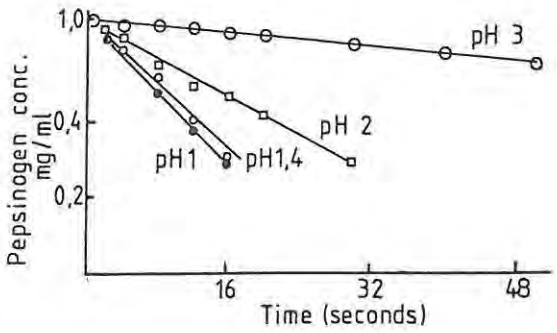

FIGURE 1

Semilogarithmic plots of pepsinogen activation, as indicated by remaining pepsinogen concentration, at various pH values. (From Al Janabi et al, 1972.)

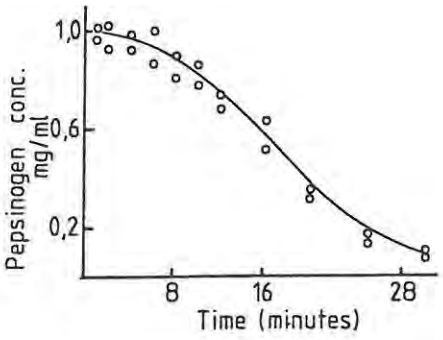

FIGURE 2

Pepsinogen activation, as indicated by remaining pepsinogen concentration, at pH 4. The line is calculated from the integrated form of Equation (1) (see text), assuming k_1 = 0,008 per min., and k_2 = 0,175 per (min.× mg per mℓ). (From Al Janabi et al, op. cit.)

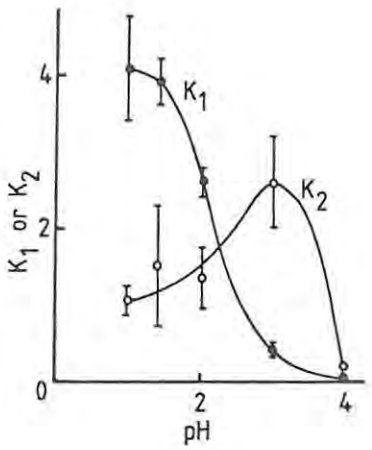

FIGURE 3

pH dependence of the first and second order rate constants k_1 and k_2. Units for k_1 = $(min)^{-1}$ and k_2 = $(min \times mg \text{ per } m\ell)^{-1}$. Vertical lines are root mean square deviations in the rate constant determinations. (From Al Janabi et al, op. cit.)

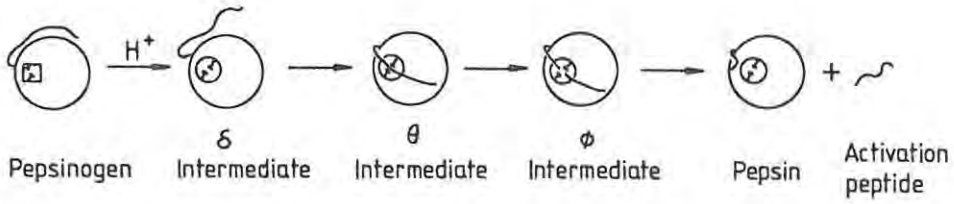

FIGURE 4

Proposed mechanism of pepsinogen activation, indicating intermediate forms. The square and the circle within the molecule (large circle) are respectively the undeveloped and developed active site, each containing two carboxyl groups. (From Marciniszyn et al, 1976.)

In a later publication by the same group (Tang's), Marciniszyn et al (1976b) showed that globin - a good pepsin substrate - acts as a competitive inhibitor to the intramolecular activation of the zymogen. This further implicates the active site of pepsin as that responsible for activation, and suggests the existence of an intermediate (intramolecular) enzyme-substrate complex, denoted intermediate δ (Figure 4). On the basis of these and other data, Marciniszyn et al propose the activation sequence shown in Figure 4. Other workers failed to show inhibition of activation in the presence of the similar pepsin substrate, haemoglobin, at nominally comparable pH. McPhie (1977) resolved the apparent contradiction by showing that Marciniszyn's group had not adequately controlled the pH of their enzyme/zymogen denaturation mixtures, thus obtaining falsely extended times for the activation process. With the denaturing pH more rigorously controlled, activation is revealed to be more rapid, and independent of the presence of haemoglobin at concentrations up to 20 mg/mℓ. "The original conclusion that pepsinogen is activated unimolecularly, without aid or hindrance from any other protein, still stands," (McPhie, 1977). Intermediate δ is thus not a necessary part of the activation sequence.

The suggested activation mechanism implies the release of a single activation segment. The sequences of pig pepsinogen and pepsin have been known for some time, and the activation segment is comprised of the first 44 residues of the N-terminus (Dykes and Kay, 1976). These workers noted, however, that isolation of the activation segment as a single peptide has never been achieved.

Hydrolysis of this segment was until then commonly ascribed to cleavage by the fully-activated enzyme.

Using pepstatin to inhibit the nascent enzyme, they succeeded in isolating the first 16 residues of the N-terminus, and an enzymatic moiety ("pseudopepsin") with composition intermediate between that of pepsinogen and pepsin. Dykes and Kay (op. cit.) thus proposed a multi-step activation sequence, in which the activation peptides are released sequentially. Results generally consistent with the findings and conclusions of Dykes and Kay (op. cit.) were obtained earlier for bovine pepsin by Harboe et al (1974). They found that "when activation was performed at pH 2 for the minimum time to achieve full conversion to pepsin ..... more than 7 peptides were formed". They also note that "It is remarkable that no investigation of the activation of gastric zymogens has yielded a single large activation peptide". These workers showed that the activation segment carrying the N-terminus of the zymogen contained 17 residues, and inhibited the milk-clotting action of the pepsin. This they suggest to be the "pepsin inhibitor" first mentioned by Herriott (1941 ; not seen by the writer, quoted by Herriott, 1955).

Later work on pig pepsinogen has shown the major inhibitory activity to be associated with the activation peptide comprising residues 1 to 16, with a k_I of 2,5 x 10^{-7}M at pH 5,5 (Dunn et al, 1978).

Kageyama and Takahashi (1982) reported the isolation of a single 47-residue N-terminal activation segment during activation of Japanese Monkey pepsinogen at pH 2, thus demonstrating the existence of a single-step activation mechanism. These workers subsequently (1983a) showed that, at pH 2, pig pepsinogen activates by single-step and double-step mechanisms simultaneously.

2.4. Pepsins

2.4.1. Molecular masses and isoelectric points

The substantive enzymes produced by activation of pepsinogens generally have masses in the range 32 000 to 38 000 daltons. As is implied by the loss, during activation, of predominantly basic amino acids, the pepsins are more acidic than their precursors. Herriott (1962) referring to other workers, notes that "the isoelectric point, if one exists for pepsin, must be below pH 1, for at this acidity the protein has not become cationic". Mahler and Cordes (1966) quote a pI for pepsin of *c* 1.0.

Later development of isoelectric focussing techniques on polyacrylamide gel has allowed more accurate determination of pI values. Righetti *et al* (1977) report pIs of 2,8 - 3,09 for pig pepsins, and pI 4,03 for "a major band" of chicken pepsin.

2.4.2. Substrate specificities

As summarised by Clement (1973), two types of synthetic substrates are commonly used for pepsin: aromatic dipeptides amd cationic substrates.

The former are based on the work of Baker (1951) and may be represented by N-acetyl-L-phenylalanyl-L-diiodotyrosine (APDT). This substrate, with pig pepsin, exhibits the highest catalytic rate constant (0,20 sec^{-1}) and the lowest K_m (0,075 x 10^{-3}M) of fifteen dipeptide substrates listed by Clement (op. cit.)

Investigation of k_{cat} and K_m data for several such substrates yields the information that phenylalanine residues are bound more strongly than tyrosine, indicating a hydrophobic binding site at the active centre, and that binding of the substrate involves both amino acid residues.

The cationic substrates have been developed by Fruton and co-workers (quoted by Baker, op. cit., and Clement, op. cit.). These substrates may be generalised as

A - X ⟋ Y - B,

where X and Y are amino acids which may be systematically varied; ⟋ denotes the bond cleaved; and A and B are protecting groups on the N- and C- termini respectively. These substrates offer certain advantages over aromatic dipeptides, in that they have much greater solubility below pH 4, and generally higher catalytic rate constants. The

greater solubility below pH 4 has enabled them to be used without the addition of organic solvents - a requirement complicating use of the aromatic dipeptide substrates (Fruton, 1977).

Kinetic data obtained using cationic substrates support those deriving from the aromatic dipeptide studies. Thus aromatic dipeptide residues are preferred in the X and Y positions, and particularly phenylalanine at X and either tyrosine, phenylalanine or tryptophan at Y*. The specificity involving residues immediately adjacent to the susceptible bond has been referred to as "primary specificity" (Fruton, op. cit.).

The properties of the protecting groups A and B also have considerable effect on the k_{cat}, and less effect on the K_m values for these substrates, indicating an extended binding site, and revealing the importance of binding subsites.

Other studies have made it clear that data obtained from synthetic dipeptide substrates are not fully applicable to the peptic hydrolysis of oligopeptides and proteins. Thus Sanger and Tuppy (1951 : not seen by the writer, quoted by Delpierre and Fruton, 1965) reported the cleavage of a Leu-Val bond in insulin, in addition to the anticipated cleavage of the Phe-Phe and Phe-Tyr bonds.

---

*The enzyme implicitly referred to here is Pepsin A (3.4.23.1). Pepsin C (= Gastricsin; 3.4.23.3) has a preference for tyrosine at position X, and is not active against APDT (Foltmann, 1981).

Fruton (1977) notes that, by studying the k cat and K_m characteristics of progressively longer synthetic (cationic) substrates, "it was inferred that the active site of pepsin can accommodate at least a heptapeptide". This corresponds to a length of about 25 Å (Sampath-Kumar and Fruton, 1974 : not seen by the writer, quoted by Fruton, op cit.).

Further information on binding subsites was presented by Powers et al (1977), who analysed reports on cleavage of naturally-occurring peptides by pig pepsin. The scheme (Figure 5) used by Powers et al (op. cit.) for designating binding sites in a protease with an extended binding site, is from Berger and Schechter (1970 : not seen by the writer, quoted by Foltmann, 1981).

Powers et al (op. cit.) amassed statistical evidence that the extended binding site is comprised of seven subsites, S_4 - S'_3. Bonds with high probability of cleavage (in positions P_1 - P'_1) are Phe-Val; Asp-Phe; Asp-Tyr; Phe-Tyr; Phe-His; Met-Met; Met-His; Trp-Glu; Phe-Ala; Cys-Tyr; Tyr-Met and Trp-Ile.

The amino acid at P_1 is the primary determinant of specificity, with cleavage probabilities (in descending order of susceptibility) favouring the (hydrophobic) amino acids Phe, Met, Leu and Trp. At subsites other than P_1 , most amino acids have no influence on the probability of cleavage at the P_1 - P'_1 locus. Certain amino acids, however, at subsites P_2 - P_4 and P'_1 - P'_3, increase or decrease the likelihood of cleavage at the P_1 - P'_1 site. (Powers et al, op. cit.)

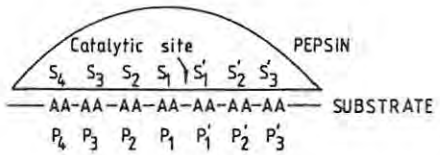

FIGURE 5

Diagrammatic representation of a polypeptide substrate (-AA-AA-AA-AA- ...) and extended binding site of pepsin. Subsites of the enzyme are designated S_4 ....S'_3. The corresponding residues in the substrate are P_4 ...P'_3. The bond cleaved is between P_1 and P'_1. (From Powers et al, 1977.)
(In the work quoted, the bond cleaved is given as that between P_1 and P_2. This appears to be a typographic error.)

In activation of pig pepsin, Kageyama and Takahashi (1983a) note that cleavage occurs between residues 44 and 45 (Leu-Ile) and/or residues 16 and 17 (Leu-Ile). Clearly these data are broadly consistent with the susceptibilities calculated by Powers et al (op. cit.).

Foltmann (1981), evaluating Powers' findings in the light of other work, notes that subsite preferences of pepsins from different sources may show interspecific variation, and that there may be co-operativity between the subsites within the extended binding site. In the light of this probable involvement of the entire binding site in a "unified" interaction with the substrate, Fruton (1977) has rejected the term "subsites", as implying independence of each other.

2.4.3 Mechanism of Action

"Enzymes, like midwives, ease the difficult passage of substances from one metastable situation to another." (Wolfenden, 1976)

Studies involving catalytic parameters, specific inhibitors and selective modification of amino acids in the pepsin molecule have contributed to an extensive, though incomplete, knowledge of the mechanism of peptic hydrolysis.

The work of Delpierre and Fruton (1966) and of Tang (1971) involving modification of carboxyl groups in pepsin, led to the later demonstration that aspartate residues at positions 32 and 215 in the chain are directly involved in catalysis. Herriott (several publications not seen by the writer : quoted by Delpierre and Fruton, op. cit.) showed that acetylation or iodination of tyrosine residues also

inactivates pepsin. Subsequent work, quoted by Foltmann (1981) implicates Tyr-75 specifically in the catalytic process.

The active site aspartate residues are located at the centre of the cleft accommodating the extended binding site (Tang, 1979). A serine residue at position 35 is hydrogen-bonded to Asp-32. This serine residue appears, with the catalytically important aspartate groups, to be strongly conserved among the carboxyl proteases (Tang, op. cit.).

Kinetic studies indicate that the pKa values of the carboxylic acid side chains of Asp-32 and Asp-215 are about 1 and 4,8 respectively (Clement, 1973).(Tang, 1979, quoting Clement, op. cit., reports the pKa of Asp-32 as about 2.) Foltmann (1981) reports a value of "about 1". Hartsuck and Tang (1972) calculated a pKa of 2,85 for this aspartate residue, but noted that this value depended on certain assumptions, and could be in error by at least 0,5 of a pH unit. As noted by Hartsuck and Tang (op. cit.) the normal pKa for an aspartyl side chain in a polypeptide is c 4. The unusually low pKa determined for Asp-32 , whatever its precise value, may be explained by a nearby carboxyl side chain providing a hydrogen bond to the Asp-32 carboxylate ion. In outlining a possible reaction mechanism, Foltmann (1981) shows the Asp-32 residue as stabilised by hydrogen bonding with Gly-217, Asp-215 and Ser-35 residues.

Several early mechanistic proposals for pepsin activity involve the participation of two carboxyls, one protonated and the other ionised, (Hartsuck and Tang, op. cit.). At the pH optimum of pepsin (c pH 2), the protonated moiety is Asp-215.

Tang (1979) notes that Asp-32 probably acts directly or indirectly by nucleophilic attack on the carbonyl carbon atom of the scissile bond.

Fruton (1977), summarising aspects of the mechanism of pepsin action, notes that, "Perhaps the most vexing questions about the mechanism of peptic action on synthetic substrates relate to the order of the release products, and whether a covalently bound acyl-enzyme or amino-enzyme is an intermediate in the catalytic process."

James et al (1977), working on the acid (carboxyl) protease penicillopepsin from the fungus Penicillium janthinellum - an enzyme showing considerable homology with the vertebrate gastric proteases - presented crystallographic evidence that the β-carboxyl group of Asp-32 "is too buried for this group to act as an attacking nucleophile directly". On this and other grounds they propose that an activated water molecule, hydrogen-bonded to Asp-32, attacks the (polarised) carbonyl carbon atom of the scissile peptide bond in the substrate. These workers note that by thus invoking a general base-catalysed mechanism, the consistent failure to trap covalently-bound acyl-enzyme or amino-enzyme intermediates is explained. They further note that the order of release of products may be assumed to be determined by the relative affinities of the acyl and amino groups for their respective binding sites.

The same reaction mechanism, embodying, in the transition state, a tetrahedral configuration of the carbonyl carbon of the scissile bond, provides an acceptable basis for explaining the transpeptidation reactions which the acid proteases are able to mediate at pH 4 and above (Fruton, et al, 1961; not seen by the writer, quoted by Clement, op. cit.).

Foltmann (1981) offers a tentative mechanistic model for the catalytic activity of pepsin. This scheme embodies the observations of several workers, including those of James et al (op. cit.), and "accommodates all published properties of the catalytic activities of aspartic proteinases" (Foltmann, op. cit.).

In confirmation of the earlier suggestion of James et al (op. cit.) Schmidt et al (1985 : seen in abstract) using carbon-13 NMR spectra of a pepsin-bound pepstatin analogue showed that water, rather than an enzyme nucleophile, adds to the peptide carbonyl to yield a tetrahedral diol in the catalysed reaction. In a subsequent paper by the same group, Holladay et al (1985 : seen in abstract) report further NMR-derived evidence for the direct participation of water, and conclude "pepsin apparently catalyses the hydrolysis of peptides by a general acid-base mechanism and not by a nucleophile mechanism".

2.4.4. pH-dependent denaturation

Pig pepsin may be stored in half-molar H_2SO_4 at 5°C " .... for weeks with only minor losses of peptic activity" (Herriott, 1962). In general, pepsins are quickly denatured under conditions of neutral or mildly alkaline pH (Northrop, 1930; Ryle, 1970; Kageyama and Takahashi, 1976b). Chicken pepsins and at least one pepsin from Japanese Quail are unusually stable at alkaline pH (Levchuck and Orekhovich, 1963; Bohak, 1969; Esumi et al, 1980).

Baudys and Kostka (1983), discussing the primary structure of chicken pepsinogen and pepsin, note that in this pepsin several basic residues

are scattered along the polypeptide chain. They point out that this distribution differs from that in group A pepsinogens, where the basic residues are concentrated in the N- and C-terminal regions, and in the region of the first disulphide bridge.* They suggest that this distribution may account for the greater alkaline stability of chicken pepsin, since the presence of basic residues is a requirement for stabilising the tertiary structure of the molecule by allowing the formation of ion pairs.

Differences in susceptibility to alkaline denaturation have been used as a means of distinguishing between pepsins within a single species (Kageyama and Takahashi, op. cit.; Walker and Taylor, 1979). The latter workers showed that human pepsin 5 (= gastricsin) is more alkali stable than other human pepsins, and also demonstrated that admixture of this pepsin with pepsin 3 affords the latter a degree of protection against alkaline denaturation.

Foltmann (1981) notes that small differences in primary structure profoundly influence pH-dependent conformation, hence "alkali stability is not a suitable criterion for classification of enzymes".

2.5. Evolution of Carboxyl Proteases

Sequence and x-ray crystallographic studies indicate considerable

---

*Notwithstanding this difference they suggest, as noted earlier, that chicken pepsin be classified as pepsin A.

similarity amongst the carboxyl proteases from widely diverse organisms. Penicillopepsin and (mammalian) gastric proteases exhibit similarities which may at first sight appear astonishing. The correspondence is most marked around catalytically important residues, and extends to the tertiary folding of the enzymes. Foltmann (1981) notes that "It seems most unlikely that this homology should arise by a convergent evolution", and suggests that these enzymes derive from a common ancestral stock embracing fungi and mammals.

The "superfamily" of carboxyl proteases includes not only the gastric (acid) proteases and penicillopepsins, all of which are extracellular, but also the intracellular (lysosomal) renin - an enzyme, produced in the kidney, which is released into the bloodstream and is involved in the regulation of blood pressure (Pickford and Lambie, 1976).

Since lysosomes, and lysosomal carboxyl proteases, occur in low (unicellular) organisms, Tang (1979) suggests that the extracellular gastric carboxyl proteases are derived from an evolutionary precursor which is represented in extant forms by cathepsin D. Shewale and Tang (1984) reported the complete sequence of porcine spleen cathepsin D, and showed that this enzyme exhibits 32,7% homology, in terms of shared residues, with renin and pepsin.

Sepulveda et al (1975) noted the close homology between two segments of the pepsin molecule : residues 30 - 42 and 213 - 225. Each of these segments carries one of the catalytic aspartate groups (Asp-32 and Asp-215). These workers thus proposed that pepsin may be the result of gene duplication and fusion.

Further evidence in support of this argument was provided by Andreeva et al (1977), who obtained crystallographic data indicating that two major domains exist in the molecule, representing N-terminal and C-terminal ends, and separated by a cleft accommodating the active site. "Symmetrically related molecules occupy the entrance into the cleft of each other." (Andreeva et al, op. cit.)

Andreeva and Gustchina (1979) later showed that each domain is itself divisible into two sections. Discussing these data, Tang (1979) notes that "the conformation among these four units is indeed very similar", and suggests that the ancestral gene coded for a protein of some 8 500 daltons, or 80 residues. Duplication and fusion would produce a polypeptide of about 160 residues, containing a two-fold symmetry. A second duplication, and mutation, would yield an enzyme containing two non-identical sub-units. Fusion of these genes would result in a single-chain protease, of 320 residues, and possessing the observed internal symmetries.

Among the vertebrates, gastric proteases appear to have diversified into three groups - pepsins, gastricsins (= pepsin C) and chymosin - early in mammalian evolution. Foltmann (1981), summarising earlier work, notes that pepsin from one mammalian species is structurally more similar to pepsins in other species than it is to gastricsin or chymosin of the same species. Further support for this was obtained by Arai et al (1984), working on the N-terminal sequence of rat pepsin. In terms of common residues, they show that the N-terminal 50 residues of their rat "pepsin I" is more similar to human gastricsin than to human or pig pepsin A, hence suggesting

that the rat pepsin should be classified as pepsin C. They further point out that the homology between this rat protease and human gastricsin (88%) is higher than that existing between human gastricsin and human pepsin (54%).

Kageyama and Takahashi (1985) reported the sequence of the NH_2-terminal 60 residues of Japanese Monkey pepsinogen C (= progastricsin). Comparing their data with those for pepsinogen A (human, monkey, rabbit, bear, cow and chicken), other progastricsins (human and cow) and prochymosin (cow), they were able to construct phylogenetic trees supporting the view that these three carboxyl proteases constitute natural groups which diverged at or prior to the time of radiation of the mammalian orders. Their analysis further suggests that progastricsin diverged first. These workers note that the mutation rate of pepsinogens A has been greater than those of the other two zymogens.

Figure 6 shows the general relationship between carboxyl proteases, as proposed by Tang (1979).

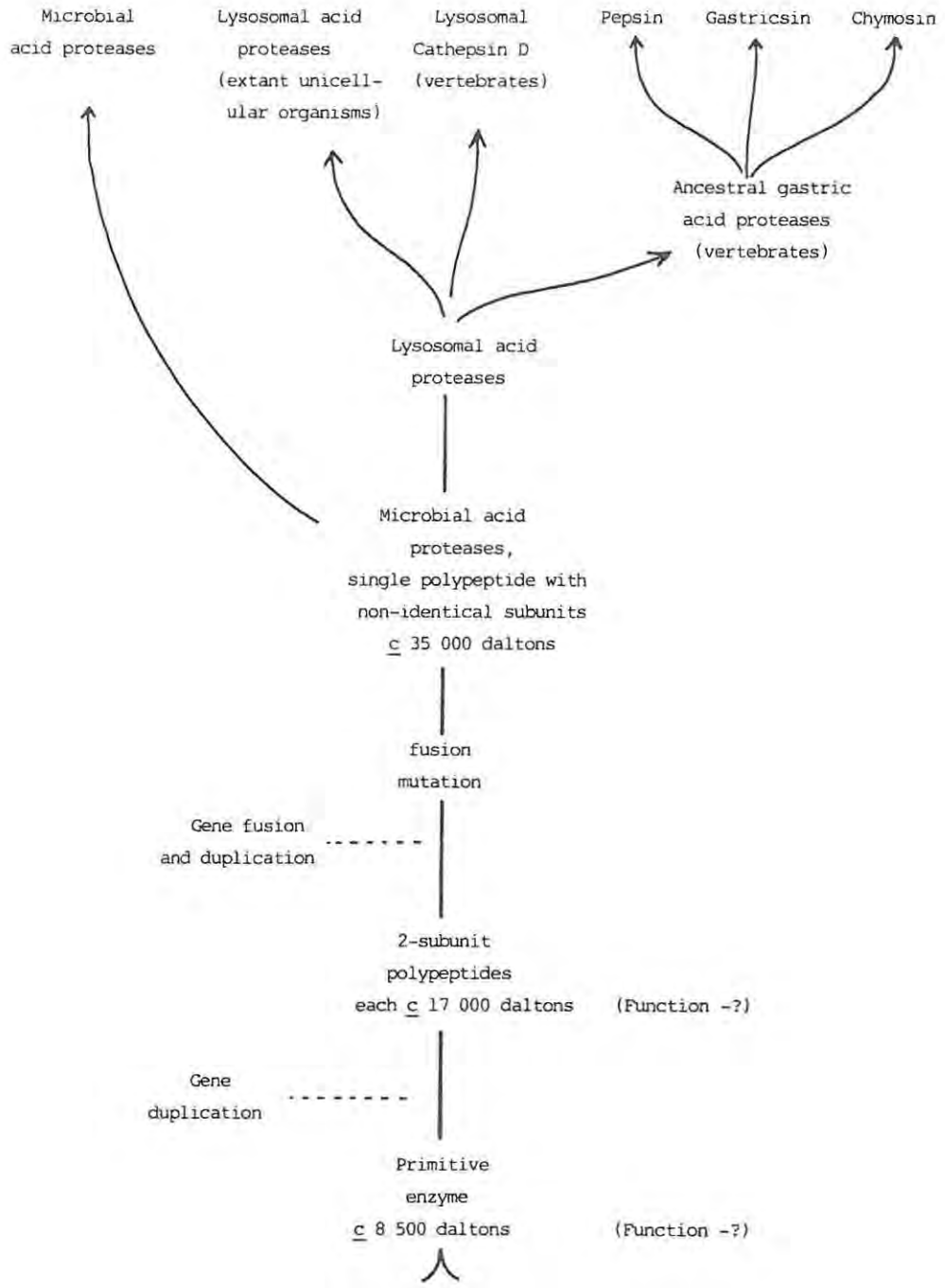

FIGURE 6

Proposed evolutionary relationships of the carboxyl proteases. (Redrawn, from Tang, 1979.)

2.6 Conclusions

A substantial body of knowledge exists on various aspects of the biochemistry of several carboxyl proteases.

In a comparative context, sufficient is known, from studies on sequence and higher levels of structure, to suggest broad evolutionary relationships among these enzymes. This basic framework of understanding will facilitate the systematic accumulation of detail, drawn from increasing numbers of taxa.

While sequence studies of the proteins - and ultimately of the relevant genetic material itself - will give increasingly detailed knowledge of phyletic antecedents of existing genomes, investigation of structure-function adaptations will involve the integration of these data with kinetic studies and other aspects of the biology of the whole organisms. In this area, much remains to be done.

CHAPTER THREE

MATERIALS AND METHODS

CHAPTER THREE

MATERIALS AND METHODS

3.1. Introduction

Extracts of animal tissues are usually, if not invariably, heterogeneous, and much biochemical technique derives from the need to separate the material(s) of interest from other components.

The methods used in this work are generally applicable to the purification of proteins, and estimation of such purity. Polyacrylamide "disc" gel electrophoresis in the presence of the anionic detergent sodium dodecyl sulphate has been adopted as a powerful, convenient and sensitive method for demonstrating heterogeneity in protein solutions. The technique offers the added benefit of yielding information on molecular masses when used in multi-lane slab systems with suitable marker proteins (Shapiro et al, 1967; Weiner et al, 1972).

Enzyme assays have been used as a means of estimating the extent of purification at each step, and to give an indication of specific activity at or near purity.

More generally, the analytical methods have been chosen so as to provide a basis for comparing the proenzymes and enzymes in the context of the rationale described in the introduction to this study.

A list of abbreviations is provided in the Appendix.

3.2. Materials

3.2.1. Snakes

In the early stages of the work snakes were purchased from the Port Elizabeth Snake Park. Several of these died during the holding period (see below), and many were found, on dissection, to be deficient in fat bodies and heavily infected with nematodes. For these reasons snakes for the main body of the work were obtained, with three exceptions, from the South African Snake Farm, Kommetjie. (See Footnotes, Table IV, page 95) In general their apparent state of health was far better. The three snakes not obtained from the Snake Farm were also apparently in good condition. All snakes were procured in the same season - Autumn - though not all in the same year. They were routinely held for about two weeks without food, but with free access to water, to ensure that all were in a post-digestive state. (But see Footnote to Table IV.)

3.2.2. Other materials

a. Enzymes

i. Pig pepsin: -ICN, 3 x crystallised, Cat. no. 100949, lot 6454.

ii. Pig pepsinogen: -ICN Technical grade, Cat. no. 100951, lot 3788.

-ICN 3 x crystallised, Cat no. 100952, lot 1187.

-Sigma 3 x crystallised, Cat. no. P 7250, Grade III, lot 71F-0165 and lot 29C-0155.

iii. Trypsin: Sigma Cat. No. T1005, lot 40F-8005, E.C. 3.4.21.4, Type XI, DPCC treated*.

b. Enzyme substrates

i. For routine pepsin assays the substrate was Bovine Haemoglobin Enzyme substrate powder, Miles Laboratories, Cat. No. 82-051, lot 41.

ii. For preliminary investigation of assay systems, Miles purified bovine haemoglobin was used, cat. No. 95-021, Batch 10A.

iii. NaOH-Urea-denatured haemoglobin was produced, from bovine haemoglobin (ii, above), as described by Delpierre (1968b).

iv. Human haemoglobin, also used in preliminary assays, was obtained by the method of Nyman (1959) from blood supplied by the Port Elizabeth Transfusion Service.

v. Globin was prepared from bovine haemoglobin (ii, above), as described by Marciniszyn et al (1976b).

vi. APDT: Sigma, Cat. No. A4501, lot 24C-2580.

c. Enzyme inhibitor

Pepstatin A; Sigma, Cat. No. P4265, lot 97C-0076.

d. Other proteins and Amino acids

i. BSA : Sigma, Cat. No. A4378, lot 128C-8000.

---

*Diphenyl carbamoyl chloride : chymotrypsin inhibitor.

ii. Molecular Mass marker Proteins:

- Pharmacia, Cat. No. LMW17-0446-01, lot 9010.
- Sigma kit No. MW-SDS-70L, lot 63F-6040.

iii. Amino acid standards for amino acid composition analysis : Beckman, Cat. No. 312220, lot 80711.

e. Other materials

Acrylamide: LKB 1820.101, Batch No. 9550120E.

Bis-acrylamide: Bio-Rad, Cat. No. 161-0200, lot 18945.

TEMED: Bethesda research Laboratories, Code 5524UB, lot 40214.

Freund's Incomplete Adjuvant: Miles Laboratories, Code 64-286, lot 11.

Freund's Complete Adjuvant: Miles Laboratories, Code 64-285-1. lot 54.

3,5-DIT: ICN, Cat. No. 101592, lot 9897.

$Na^{125}I$: Amersham International, Code IMS.30, batch 12B.

Molecular exclusion chromatography gels: Sephadex Pharmacia.

Ion exchange chromatography resins: Whatman DE52.

Routine Laboratory reagents were of ANALAR grade or equivalent.

3.3 Methods

3.3.1. Preparation of gastric mucosae

For sacrifice, snakes were introduced, two or three at a time, into a closed container with a volume of 37ℓ. To this was added c 11mℓ of the anaesthetic Halothane (2-Bromo-2-chloro-1,1,1 trifluoroethane) recommended by Gans and Elliott(1968) as a suitable anaesthetic for snakes. After apparent unconsciousness had supervened the animals were removed and decapitated. The oesophagus of each was plugged with cotton wool to prevent efflux of gastric secretions, and the body held vertically to drain blood into a collecting vessel. Blood from each specimen was collected separately, and 0,1 mℓ of a heparin preparation (25 000 units Pularin: Allen and Hanbury, Wadeville, Transvaal) was added to each sample to prevent coagulation. These samples were subsequently centrifuged, and the plasma individually lyophilised and stored in screw-top bottles at -10°C.

The body of each snake was opened with a longitudinal incision. The distal limit of the stomach is clearly demarcated by the pyloric constriction. Once the digestive tract has been opened the anterior limit of the stomach is found by the well-defined commencement of the gastric mucosa. The mucosa is easily removed by scraping with a scalpel. Each mucosa was treated separately. After rinsing in cold buffer the mucosae were blotted and weighed, then placed in beakers with sufficient buffer to give a 10% tissue : buffer homogenate. The buffer used was sodium phosphate, pH 7,8, 0,06 M.

After several methods of homogenisation had been investigated (Potter-Elvehjem hand-held homogenised; mortar-and-pestle; blender) the method chosen involved use of an Ultra-Turrax homogeniser (Janke and Kunkel KG, Staufen i, Breisgau). This disrupts tissue by shearing between concentric castellated metal rings, the inner one of which rotates at a variable speed. The method allows for some standardisation of the degree of tissue disruption - 15 minutes per mucosa was routinely used - and, by using a low speed, frothing is almost completely eliminated.

The homogenised samples were then individually stirred, with a magnetic stirrer, for 16 - 20 hours at 4°C. After extraction the samples were centrifuged for 30 minutes at c 3600G (c 6000 r.p.m., average radius 9 cm). A volume of c 0,2 mℓ was removed from each sample for activity and protein assays. (See 3.3. below.) Material for pepsin assay was ordinarily diluted 10-fold, while material for protein assay was diluted 2-fold.

The remainder of each supernate was lyophilised and stored in screw-top bottles at -10°C.

3.3.2. Assays for peptic activity

a. The method of Anson (1938) was used as the basis for the routine pepsin assay. Haemoglobin was dissolved in 0,040 M HCl to 2,1% (m/v) solution, giving a pH of c 1,8 (tested electrometrically). This solution was added, in 2,5 mℓ or 3,9 mℓ volumes, to standard 160 mm test tubes, and brought to 37°C (±0,2°C) in a waterbath controlled

with a Julabo V thermostat (Juchheim Labortechnik KG, D-7633 Seelbach, West Germany). Reaction was initiated by adding 0,1 mℓ of enzymatic material to each tube, and terminated by adding 4,0 mℓ of 10% (= 0,61 M) TCA. Blanks were prepared by incubating duplicate samples without enzyme, precipitating with TCA, then adding enzyme.

All samples were filtered through Whatman No. 41 filter paper, and the amount of TCA-soluble products was measured absorptiometrically at 280 nm in 1 cm quartz cells, using a Beckman 25 or Pye Unicam SP8-400 dual beam spectrophotometer. All samples were read against 5% TCA, and A_{280} values of blanks were subtracted from those of active samples.

Assays were routinely performed in duplicate, for at least two incubation times : commonly 2 and 4 or 5 and 10 minutes. Crude enzyme extracts were ordinarily diluted, with HCl/KCl buffer, pH 1,8, to give concentrations of $\underline{c}$ 0,2 - 0,5 mg protein/mℓ. Purified samples were diluted to 0,1 - 0,2 mg/mℓ. An hour at ambient temperature was routinely allowed for activation of pepsinogen prior to assay.

b. pH Optimum Assays

The above method was slightly modified for the determination of pH/Activity profiles. A stock solution of 2,1% haemoglobin was prepared in 0,01 M sodium citrate, yielding $\underline{c}$ pH 5. This solution was adjusted by dropwise addition of concentrated HCl ($\underline{c}$ 10 M). At the required pH values, as monitored electrometrically, samples were drawn off and added to assay tubes.

As an alternative, measured volumes of concentrated HCl were added to a series of small beakers, followed by 12,0 mℓ volumes of haemoglobin-citrate stock solution, yielding a set of pH values over the range cpH 1,4 to cpH 4,5. In all cases the pHs were checked before the solutions were pipetted into assay tubes. The volumes of concentrated HCl required were determined by reference to a previously prepared titration curve of quantitatively diluted HCl vs haemoglobin citrate stock solution. In this assay the buffer strength cannot be greatly increased above 0,01M, since this leads to precipitation of substrate.

The assay is complicated by the fact that the rate of non-enzymatic hydrolysis is pH-dependent (being greatest between pH 3,5 and pH 4). For this reason duplicate blanks were prepared at each pH, in addition to duplicate active samples, each of 3,0 mℓ volume.

There are certain shortcomings inherent in the use of haemoglobin as a substrate for pH optimum studies. These are mentioned in Section 6.7.1., p191 *et seq*.

The method used by Schlamowitz and Peterson (1959) to prepare haemoglobin yields a substrate exhibiting less variation thoughout the experimental pH range than undoubtedly exists in the substrate solutions prepared by the method adopted in the present study. Schlamowitz and Peterson (1959) dialysed 5% stock solution, which was then adjusted to pH 1,0 and allowed to stand at room temperature for 1 to 2 hours. Aliquot volumes were then adjusted to the required pH values and used immediately.

c. Assay of Inhibition by Pepstatin

The method adopted was essentially that of Kageyama and Takahashi (1976a).

i. Preparation of Inhibitor

Pepstatin has very low solubility in aqueous solution. Umezawa et al (1970) note that "Pepstatin ... is insoluble in water ...". Fortunately the water solubility is sufficient for kinetic studies, but care is needed to ensure complete solution and to guard against precipitation. Aoyagi et al (1971) used methanol to dissolve pepstatin for enzyme inhibition studies. Following the demonstration by Tang (1965) that certain aliphatic alcohols inhibit pepsin, Schlamowitz and Trujillo (1968) have questioned the practice of using methanol as a solvent for synthetic substrates. Presumably the same cautionary argument applies to the use of methanol as a solvent for pepstatin.

For the present work, a stock solution was prepared by adding 2,4mg of pepstatin to 615mℓ of water and stirring for 24 hours in the coldroom before use. Assuming complete solution, and a molecular mass of 685,9 daltons (Sigma Chemicals) this yields a solution of 5,67nmol/mℓ. About 100mℓ of this was centrifuged and the supernate, lacking any apparent insoluble material, was stored at 4°C and used over the three days during which the relevant assays were carried out. The use of so small a quantity of inhibitor inevitably leaves some doubt about its concentration, but the use of a single stock solution allows for greater comparability between assays.

The stock solution was diluted with distilled water to give six

solutions ranging in pepstatin concentration from 5,6 to zero nmol/mℓ. A sample of each dilution was mixed with an equal volume of pepsin solution (c 0,2 mg/mℓ). Final concentrations of pepstatin in the enzyme-inhibitor pre-incubation mixtures were 2,8; 2,3; 1,7; 1,1; 0,6 and 0,0 nmol/mℓ*. Pre-incubation was allowed to proceed for 30 minutes prior to commencing the assay.

ii. Enzyme solutions

In order to standardise enzyme concentration, enzyme solutions of known optical density were quantitatively diluted with 5mM acetic acid to bring their A_{280} values (1cm light-path) within the range 0,18 - 0,20 mg/mℓ (Figure 7). Assuming molecular masses of 33 000 daltons for the snake pepsins, 0,20 mg = 6,0 nmol. When added to the inhibitor solutions, this yielded a maximum pepstatin : pepsin molar ratio of 1 : 1,7. From reports by Umezawa et al (1970) and Marciniszyn et al (1976a) this ratio was expected to produce substantial inhibition of activity, while allowing use of stock inhibitor concentration low enough to minimise the danger of precipitation during the storage period.

Pilot assays produced erratic and unsatisfactory results. It was thought that this might be due to adsorption of the inhibitor on glass surfaces, and accordingly all glassware used in preparation of the stock solution, and in subsequent procedures, was pre-coated with Formvar (Merck). Thereafter assay data were more satisfactory.

---

*Values are corrected to one decimal place.

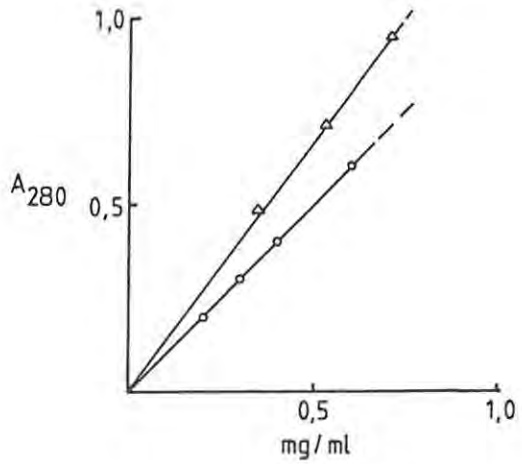

FIGURE 7

A_{280} values of pig pepsinogen (○—○) and pig pepsin (△—△) as a function of concentration. Pepsin in 5mM acetic acid; pepsinogen in sodium phosphate buffer, pH 7,8, 0,02M.
Corrected for water content: pepsin, 3,2% (calculated: see text) and pepsinogen, 4% (estimated: see Appendix 2).

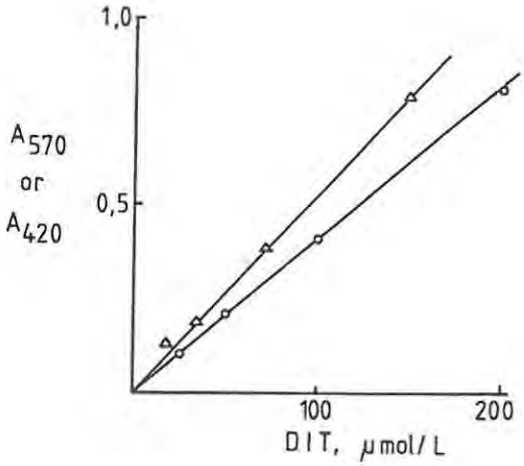

FIGURE 8

Standard curves for DIT concentration, as assayed by the trinitrobenzene sulfonic acid technique (Fields, 1972) (○—○: 420nm), and the ninhydrin technique (Moore and Stein, 1954) (△—△: 570nm).

d. Alkaline Stability Assay

Enzyme samples, 0,4mℓ in 0,02M acetate buffer, pH 5,2, were incubated at 37°C with two volumes of sodium phosphate buffer, 0,2M, at pH values in the range pH 7,0 - pH 7,5. Aliquots of 2 x 0,1mℓ were removed at incubation times of 2 to 40 minutes and assayed, in duplicate, against haemoglobin as described in 3.3.2. Incubation time for the assay was ordinarily 30 minutes. As controls, the stock enzyme, in acetate, was diluted with two volumes of the acetate buffer, pH 5,2, and assayed against haemoglobin for 10 and 20 minutes. In preliminary work, certain of the incubations were carried out in TRIS buffer at pH values up to 8,1.

e. Assay of Temperature-Activity Profiles

For assay of purified snake pepsins, six temperatures were used in the range 13,5°C - 43,2°C. For assay of crude mucosal extracts and of (commercial) pure pig pepsin, eight temperatures were used in the range 9,8°C - 45,8°C. In all cases, for temperature assays above 40°C, the enzymes, or extracts, were pre-incubated for 30 minutes at the assay temperatures prior to the actual assays. Assays were performed at pH 1,8.

Temperatures below ambient level were obtained by the use of an immersion cooling coil and a Julabo V thermostat (Juchheim Labortechnik KG, D-7633 Seelbach, West Germany) set to the required temperature. Actual water-bath temperatures were monitored with a laboratory alcohol thermometer before and during the assays. The temperatures remained constant over the assay periods. Substrate was pre-equilibrated with

the stabilised assay temperatures, and assays were performed in duplicate, as described in 3.3.2.a. Incubation time in all cases was one hour. Blank assays, in duplicate, were performed at each assay temperature.

f. Assay of Activity against the Synthetic Substrate APDT

i. Preparation of substrate

As with other synthetic peptide substrates, the aqueous solubility of APDT is low (Schlamowitz and Trujillo, 1968). Following the practice of Jackson et al (1965), the substrate (3,6gm) was dissolved in 0,1mℓ of warm, 0,1M NaOH, and 1,0mℓ of warm distilled water added. This was then added, drop-wise, to the required volume of (warm) HCl/KCl buffer, pH 1,8, with continuous stirring. In this way the stock solution was made to 0,155µmol/mℓ.

For assay, 0,9 mℓ volumes of substrate were brought to 37°C, after which 0,1 mℓ of enzyme solution, pre-equilibrated to 37°C, was added to each. Samples were incubated, in duplicate, for three assay times in the range 7 to 21 minutes. Reaction was terminated by adding 0,1 mℓ of 0,23M NaOH to each tube. Blank assays were performed by adding enzyme to the incubation mixture after the addition of NaOH.

Free amino termini generated during hydrolysis of the substrate were routinely asayed by the ninhydrin method of Moore and Stein (1954), as described by Plummer (1978). Ninhydrin/Hydrindantin solution in methyl cellosolve (ethylene glycol monomethyl ether), and acetate buffer, pH 5,5, was added to each assay tube in 1,0 mℓ volumes. (For details of reagent preparation and procedure, see Appendix 3.) the tubes were held in a

bath of boiling water for 15 minutes. During heating, each tube was closed with a lead shot weighted glass stopper to reduce loss of solution. After cooling, 1,5 mℓ of 50% ethanol was added to each tube, and after a further 10 minutes optical densities of the solutions were read in 1 cm cuvettes at 570 nm.

ii. Ninhydrin-Positive Substrate Blanks

To avoid spurious positive results in assaying column effluents for APDTase activity, it was found necessary to assay individual fractions for free amino groups in the absence of the synthetic substrate. Peptic hydrolysis of endogenous contaminant proteins or peptides in the effluent fractions leads to significant colour development in the Ninhydrin test. Such values were subtracted from the A_{570} results for the APDT-containing fractions.

In preliminary work, the trinitrobenzenesulfonic acid method (Fields, 1972) was used as an alternative assay for amino groups. Using both methods to assay a range of standard dilutions of 3,5-diiodotyrosine it was found that the Ninhydrin method gave A_{570} values approximately 130% of corresponding values obtained with the TNBS method when the latter were measured at the recommended wavelength of 420 nm (Fields, 1972).

The Ninhydrin/DIT standard curve (Figure 8) was used to quantitate APDTase activity.

3.3.3. Assays of Soluble Protein

a. Goa's MicroBiuret Method (Goa, 1953)

This method was used for routine determinations of protein in supernatant fractions of mucosal extracts and in solutions of lyophilised crude or purified material. Where necessary, protein solutions were quantitatively diluted to fall within the concentration range $\underline{c}$ 2 - 10 mg/mℓ. All assays were performed in duplicate. (See Appendix 4 for details of reagents and procedure.) Calibration curves in the range 0 - 10 mg/mℓ were prepared with solutions of commercial BSA. Concentration values were corrected for a moisture content of 3,8%, determined by heating two samples, each of $\underline{c}$ 0,4 g, for 20 hours at 102°C and determining the mean percentage mass loss (Estoe and Courts, 1963). (See Figure 9, page 62.)

The MicroBiuret method of Itzhaki and Gill (1964), not used in the present work, appears to offer a substantial increase in sensitivity (5 - 10-fold) over Goa's method. Similarly, Bradford (1976) has developed a method for the accurate measurement of microgram quantities of protein using Coomassie Brilliant Blue, involving measurement of absorption at 595 nm. In bringing this method to my attention, however, Ryle (pers. comm.) notes that it is insensitive for pig pepsin.

b. The Method of Lowry et al (1951)

Hartree's modification (1972) of this method was used for determining protein concentrations below 1 mg/mℓ. Test solutions were quantitatively diluted to give concentrations in the range $\underline{c}$ 20 - 120 µg/mℓ. Reagents and procedure are given in Appendix 5. In addition to giving an alkaline copper-based biuret reaction, the Folin-Lowry assay also

depends on a chromogenic reduction of phosphomolybdate by the amino acids tyrosine and tryptophan in the protein, or free in the solution (Plummer, 1978). The colour reaction therefore varies in intensity, at any given protein concentration, according to the prevalence of these residues in the test solution. For this reason calibration curves were prepared for both BSA and pig pepsinogen (Figure 10, Page 62).

c. Absorbance at 280 nm

As a rapid means of estimating protein concentrations in column effluents, etc., use was made of the empirically determined relationship between concentration and absorbance at 280 nm.

Calibration curves were prepared for commercial pig pepsinogen and commercial pig pepsin (Figure 7, page 56).

3.3.4. Assay of Carbohydrate

The method chosen was the phenol-sulphuric acid assay (Dubois et al, 1951; Hodge and Hofreiter, 1962). Samples of 0,5 mℓ of the test materials, each containing c 0,5 mg of protein, were added to a series of uniform, thick-walled 160 mm test tubes. To each was added 0,5 mℓ of 98% sulphuric acid. The acid was added as rapidly as possible (from a 10 mℓ blow-out pipette) and immediately mixed. After 10 minutes the samples were again mixed, held in a waterbath at 28 - 30°C for 20 minutes, then read at 480 nm and 490 nm.

Blanks were prepared containing water instead of the protein solution. Calibration curves were prepared with glucose and ribose in the range

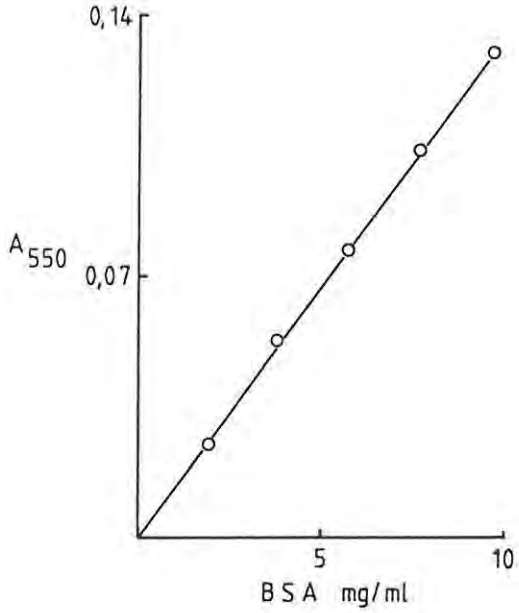

FIGURE 9

Typical Biuret calibration curve. BSA concentration corrected for 3,8% water content.

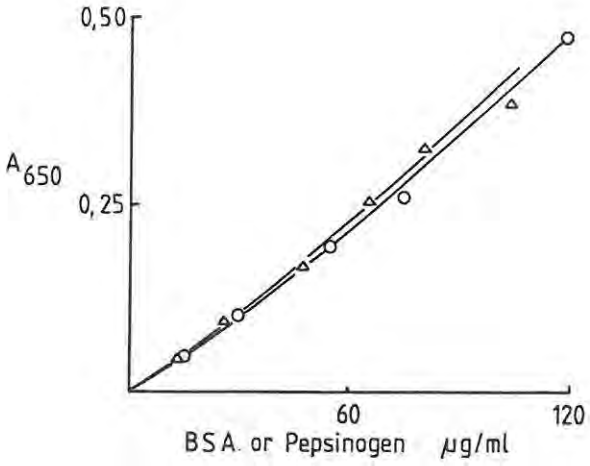

FIGURE 10

Folin-Lowry calibration curve (Hartree modification:1972).
△—△: Pig pepsinogen, corrected for 4% water content (see Appendix 2)
O—O: BSA, corrected for 3,8% water content.

0 - 70µg/mℓ. For comparison, BSA and commercial pig pepsinogen were also assayed for carbohydrate content. See Figures 11 and 12, page64.

3.3.5. Purification of the Zymogens

Ion exchange and molecular exclusion chromatography were carried out in a coldroom at 4°C.

a. Ion Exchange Chromatography

Lyophilised gastric mucosal extracts from all samples of a species were pooled in approximately equal quantities to give a total of $\underline{c}$ 1 g of material (= $\underline{c}$ 300 - 400 mg protein). This was stirred into 100 mℓ of sodium phosphate buffer, 0,02M, pH 7,8, and was dialysed against 4 x 500 mℓ volumes of the same buffer. The dialysed solution was centrifuged for 30 minutes at $\underline{c}$ 3 500G and the small precipitate discarded.

i. Preparation of Dialysing Membrane

Where relatively large quantities of crude protein were being used, the dialysing membrane (Visking : Union Carbide Corporation, or Spectrapor : Spectrum Medical Industries, Los Angeles) was softened in distilled water or buffer and used without further treatment.

Where small quantities of purified enzyme or zymogen were involved, the membrane was first softened and then boiled in an EDTA-SDS solution. See Appendix 6.

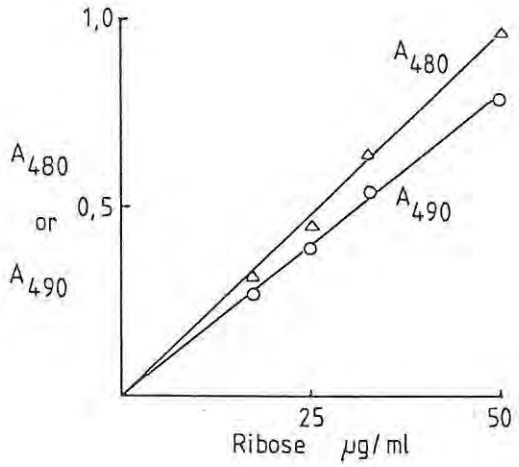

FIGURE 11

Calibration curve for pentose sugars: phenol-sulphuric acid carbohydrate assay.

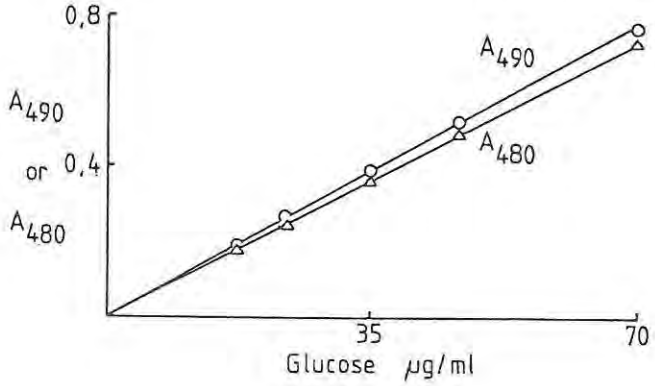

FIGURE 12

Calibration curve for hexose sugars: phenol-sulphuric acid carbohydrate assay.

ii. Preparation of Anion Exchanger

A commercially pre-swollen DEAE cellulose anion exchanger, Whatman's DE52, was used for the main part of the project. The material was prepared by one of the methods recommended by the manufacturer. About 25 g of the exchanger was stirred into c 250 mℓ of 0,2M sodium phosphate buffer, pH 7,8 (= 10 x standard concentration). The slurry was allowed to sediment for 15 mintues, after which fine particles were syphoned off with the supernatant buffer. The exchanger was re-suspended in fresh (concentrated) buffer, allowed to sediment and purged of "fines" three more times. Thereafter the slurry was made to about 120 mℓ with concentrated buffer and poured into a Pharmacia K16/40 column, fitted with a reservoir. The resin was allowed to consolidate under free flow of buffer, at a hydrostatic pressure equivalent to about 2 metres (achieved by raising the column and attaching an extended collecting tube to the outlet). Once packed, a Pharmacia adaptor was fitted to the top of the column and lowered to about 1 cm from the bed, without incorporating bubbles. The system was conventionally linked to a reservoir vessel and gradient-maker (joined by a two-way tap) via a LKB 2120 Varioperpex II peristaltic pump. The reservoir was filled with starting buffer (0,02M) and about 500 mℓ pumped through the column at a flow rate of c 130 mℓ/hour. In all operations buffer was pumped downward through the column.

A 1 mℓ portion of the dialysed mucosal extract was removed for assay, and the remainder introduced into the reservoir and applied to the column at the same flow rate. Column effluent was fractionated on an LKB 7000 Ultrorac fraction collector set in the drop-counting mode. Ordinarily 50-drop fractions (c 3 mℓ) were collected. After application

of the sample a further 100 mℓ of starting buffer was passed through the column. A nominally linear gradient of sodium chloride (0 - 0,6M in 500 mℓ buffer) was then applied to the column. This was followed by 100 mℓ of 0,6M NaCl in buffer, and a final wash of c 50 mℓ 1,5M NaCl in buffer.

Optical densities of the effluent fractions were read manually at 280 nm in 1 cm quartz cells in a Beckman 25 or Pye Unicam SP8-400 dual beam spectrophotometer, against a blank of starting buffer.

To locate peptic activity, 0,1 mℓ samples of the fractions were removed, diluted with two volumes of HCl/KCl buffer, pH 1,8, allowed to activate for 1 - 2 hours at room temperature and then assayed for activity against haemoglobin and APDT as described.

Continuous sequences of fractions exhibiting appreciable peptic activity were individually pooled as indicated in the various graphs.

Prior to the second step of purification (see below), the pooled samples were concentrated. In the early part of the work this was achieved with an Amicon pressurised ultrafiltration system, using a PM30 membrane. (Nominal cut-off = 30 000 daltons.) It was later found more convenient to concentrate samples in a dialysing tube against an external solution of 20 - 30% PEG in 0,02M phosphate buffer. Ryle (1965b) has shown that some polyethylene glycol molecules - apparently a fraction of low molecular mass - pass through 'Visking' dialysis tubing. These, with the larger PEG molecules (molecular mass 15 000 daltons), were substantially separated from Pepsin C (mass 40 700 daltons) by chromatography on G100 Sephadex (Ryle, op. cit.). It may therefore be assumed that a large part of any contaminating PEG was separated from the zymogens in the

present work, during passage through a G150 Sephadex column, as described below.

b. Molecular Exclusion Chromatography

Sephadex G100 has commonly been used for further separation following ion exchange chromatography (Chow and Kassell, 1968; Ryle, 1970; Kassell and Meitner, 1970; Bohak, 1970; Green and Llewellin, 1973; Kageyama et al, 1983). Kageyama and Takahashi (1976a) used G150 to determine molecular masses of Japanese monkey pepsinogens. These workers also used G100 filtration as a purification step following anion exchange chromatography.

In early stages of the present work Sephadex G75, G100 and G150 were used for pilot separations. G150 was found superior to either of the more heavily cross-linked gels in terms of the resolution of activity from overall protein content of the extracts. Sephadex G200 was not used because of the very low flow rate required.

Sephadex G150 molecular exclusion gel was swollen, as recommended by the manufacturer, in sodium phosphate buffer, 0,02M, pH 7,8, degassed, and poured into a Pharmacia K16/100 column. Final bed dimensions were 1,6 x 96 cm. Buffer was routinely filtered through Whatman No. 1 filter paper before being added to the column reservoir. When the system was not in use, sodium azide (0,01%^{m}/v) was added as a preservative.

A suitable volume (c 1,5 - 2 mℓ) of the concentrated material was applied to the top of the gel bed by cautiously introducing the sample beneath the eluting buffer. For this purpose a 2,0 mℓ syringe wsa used, fitted with a 10 cm length of 1 mm polythene tubing. Sodium

chloride was added to the samples ($\underline{c}$ 5% $^{m}/v$) to increase their density.

A constant flow rate of $\underline{c}$ 10 - 12 mℓ/hour was achieved by means of a Mariotte flask with air-intake $\underline{c}$ 20 - 30 cm above the column outlet. Effluent was fractionated in 50-drop volumes.

The elution profiles of protein, and of peptic activity against haemoglobin and APDT were established as described.

Effluent fractions showing peptic activity were pooled and dialysed against 5mM NH_4HCO_3 ($\underline{c}$ pH 8). This ensures a mildly basic solution and hence guards against activation. The volatility of the ammonium carbonate yields salt-free protein on lyophilisation. For convenience of subsequent usage, material from the G150 column was lyophilised in small glass vials each containing $\underline{c}$ 1 - 8 mg of material, as determined by measuring A_{280} values prior to lyophilisation. The vials were sealed under vacuum at the conclusion of freeze-drying, by means of an oxygen-butane blow-torch. They were then stored at -10°C.

3.3.6. Activation of Pepsinogen

Rajagopalan et al (1966b) have shown that the homegeneity or otherwise of pepsin(s) produced from a single zymogen is critically dependent on the activation pH. These workers showed that at pH 2 the activation product is homogeneous as indicated by hydroxylapatite chromatography and end-group analysis.

In the present work, salt-free pepsinogen samples were dissolved in 0,6 mℓ of 5 mM NH_4HCO_3 and chilled to 0°C in crushed ice. For activation, 0,6 mℓ of ice-cold HCl/KCl buffer, 0,2 M, pH 1,8, was added. After two hours, 0,6 mℓ of sodium acetate buffer, 0,1 M, pH 5,2, was added. At this stage 0,05 mℓ was removed, added to c 5 volumes of cold acetone and stored at -10°C for PAGE.

The remaining material was applied to a K16/100 Pharmacia column packed with Sephadex G50 in sodium acetate buffer, 0,05 M, pH 5,2.

It should be noted that the activation time used here (2 hours at 0°C) is proportionately longer than the time (20 minutes at 14°C) used by Rajagopalan et al (op. cit.). Assuming a mean Q_{10} of about 2 in the range 0 - 14°C, one hour of activation at 0°C would be expected to yield approximately comparable circumstances. Earlier work in this project had shown however, that complete activation at pH 1,8 and 0°C is achieved only after about 90 minutes. (See Figure 35, page 139, also Figure 13, page 71.)

In view of the small quantities of material used (c 5 mg), and to increase resolution of the eluate, 34-drop fractions (c 2 mℓ) were collected. These were read in low volume 1 cm light-path cells.

Small samples (0,1 mℓ) of individual fractions were added to 5 volumes of acetone and stored at -10°C for later PAGE.

Material not used immediately was dialysed against 5 mM acetic acid, re-lyophilised and stored at -10°C in vacuum-sealed vials.

3.3.7. Serological Methods

a. Preparation of specific antisera

Four healthy young adult rabbits were used for antibody production. Each rabbit was injected with one of four purified zymogen preparations. Three of the zymogen samples were obtained from the three snake species under investigation, and the fourth was commercial 3 x crystallised pig pepsinogen, which was passed through a column of G150 Sephadex before use. (See Figure 14) The snake pepsinogens used were the PII fractions (see Fig. 18 et seq.) as PI from all three species contained protein impurities. (Mole snake PIII was considered, provisionally, to be identical to PII, and was therefore not used as an immunogen.)

Pre-immune serum was obtained from each rabbit as follows:

The outer dorsal edge of an ear was shaved, rubbed with xylol and lightly smeared with vaseline. A small incision was made, with a new scalpel blade, in the blood vessel in the ear margin. Blood was allowed to run into a centrifuge tube. After standing for two hours, the loose clot was separated from the walls of the tube, and the sample was allowed to stand in the coldroom overnight, for clot

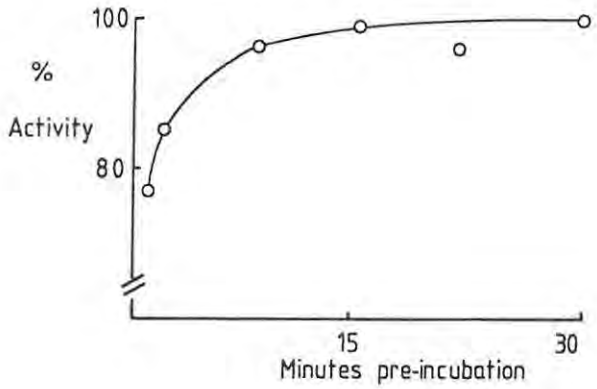

FIGURE 13

Activation of puff adder gastric mucosal (alkaline) extract. Pre-incubation at pH 1,8, 25°C.
Assay: 5 minutes at 37°C. Haemoglobin substrate.

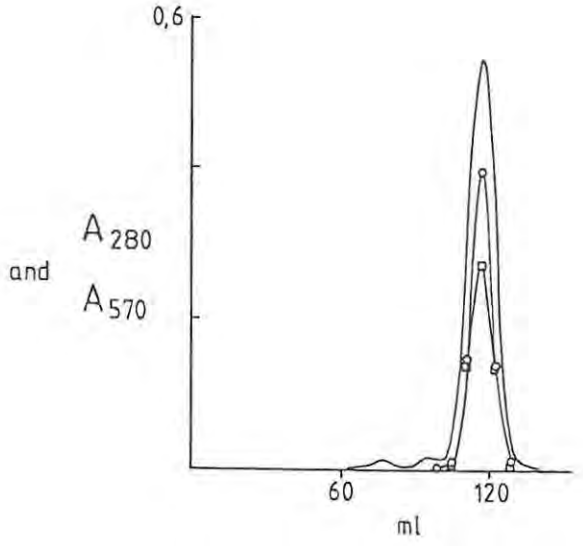

FIGURE 14

Commercial (Sigma) pig pepsinogen chromatographed on Sephadex G150. ——: A_{280}; O—O: activity against haemoglobin (5 minute assays); □—□: activity against APDT (30 minute assays).

retraction to occur. After this the serum was decanted, preserved by the addition of sodium azide to 0,1% (m/v) concentration, and stored in screw-top bottles at -10°C.

The immunogen was prepared by dissolving c 1,5 mg of protein in 1,5 mℓ of PBS. (See Appendix 7.) This solution was drawn into a 2 mℓ syringe and ejected, in c 0,2 mℓ aliquots, through a fine hypodermic needle into 1,5 mℓ of Freund's Complete Adjuvant, in a bijou bottle. After each addition of protein solution the bottle was vigorously shaken on a vortex mixer, producing a water-in-oil emulsion (Garvey et al, 1977). The solution was injected, in two equal volumes, into the gastrocnemius muscles of the rabbit.

All rabbits were given a second injection of antigen three weeks later. The procedure was identical, except that Freund's Incomplete Adjuvant was used.

Post-immunisation sera were collected, as described, two days before the second injection, and again three weeks after the second injection. All sera were preserved with 0,1% azide and stored at -10°C.

b. Ouchterlony double diffusion tests

Oxoid agar (1%) was prepared in PBS, preserved with 0,1% azide. Plates were prepared by pouring 15 mℓ volumes of hot agar into petri dishes on a level surface. They were then autoclaved for 10 minutes and stored, in sealed plastic bags, at 4°C until required.

i. Homologous antigen-antibody reactions

To perform the double-diffusion test, four sets of wells were made in each petri dish. The centre well in each set was charged with antiserum, the concentration of which diminished, in 4-fold serial (PBS) dilutions, from zero dilution (= crude antiserum) to $^{1}/_{32}$ through the four sets. The six peripheral wells in each set were charged with mucosal extract ranging in concentration, clockwise, from 10 mg/mℓ to 0,31 mg/mℓ* in 2-fold (PBS) dilutions. In the case of the pig zymogen, the stock immunogen was 1 mg/mℓ.

ii. Heterologous reactions

Three plates were used. In each plate the centre wells of three of the sets were charged with a single snake antiserum at 4-fold dilution. The three sets of radial cells were charged, respectively, with antigenic material from each of the other two snake species, and with pig pepsinogen. The antigen, in each set, was diluted clockwise in 2-fold steps, from stocks of 10 mg/mℓ (snake mucosae) and 1 mg/mℓ (pig pepsinogen). In this way each snake antiserum was exposed to the other two snake immunogens, and to pig pepsinogen.

The plates were covered, sealed with masking tape and placed in a warm room at 37°C. They were examined by Tyndall illumination after 36 and 48 hours.

---

*10 mg crude mucosa = c 0,5 mg immunogen.

3.3.8. Amino Acid Analyses

Protein for amino acid analyses was weighed and dissolved in distilled water to yield c 1mg/mℓ. the concentration was determined by reference of the A_{280} value to a standard curve prepared with commercial pig pepsinogen.

For each protein, 1mℓ samples (= 1,0mg, puff adder; 0,83mg, cobra) were added to each of three specially prepared, thick-walled, long-stemmed vials. To each was added 1,0mℓ of 11,6M constant boiling HCl, 0,4% in phenol to protect against oxidation of tyrosine (Black and Hogness, 1969). The material was frozen in liquid air and the tubes were evacuated. While under vacuum they were sealed with an oxygen-butane blow torch. The three samples of each protein were then heated at 110°C for 24, 48 and 73 hours respectively (Schroeder,1968).

After hydrolysis the vials were opened, the contents refrozen and the acid removed under vacuum. The dried material was redissolved in 2,0mℓ of sodium citrate loading buffer, pH 2,0, to which was added 200 nmol of norleucine and 200 nmol of AGPA as internal standards.

This solution was applied, in 0,5 mℓ aliquots, to a Beckman 121 M Amino Acid Analyser linked to a Varian CDS 401 "Vista" series computer. Duplicate assays were performed for each hydrolysis time.

Results for each hydrolysis were computed by the machine as nmols of amino acid per mℓ hydrolysate. Values for threonine, serine and cystine, which are progressively destroyed during acid hydrolysis, (Schroeder, 1968) were determined by interpolating concentration values for the three hydrolysis times to zero time.

Calculation of amino acid residues per mole

The method of calculation derives from the fact that a mole of (pure) protein is comprised of whole numbers of its constituent amino acids. For the purpose of calculation, a 'minimum molecular mass' is first determined (Mahler and Cordes, 1966). A stable amino acid* in low concentration is found by inspection (thus histidine, for example, in puff adder PII zymogen : see Table X, p 126), and it is assumed that the protein contains only one residue of this amino acid. The relative amino acid contents of the protein, based on His = 1, are calculated by dividing all the amino acid concentrations by the Histidine value (22,9 nmol/mℓ for puff adder). From these values a minimum molecular mass is derived using the individual residue masses (molecular mass of the amino acid minus 18 daltons for the water removed in synthesis). This minimum molecular mass represents some fraction, x, of the actual mass. When the relative proportions of the amino acids, based on unit histidine, are multiplied, in each case, by this factor x, values for all the residues should approach intergers, representing the actual composition of the protein in residues per mole.

The molecular mass of the protein, determined by other methods, may be used to derive a value for x, and the resulting closeness of residues to whole number assessed by inspection. Alternatively - as adopted in the present work - it is clearly possible by means of a comparatively simple computer programme to derive, 'in the dark', a value for x at which the overall residue composition approaches

---

*Histidine, methionine and phenylalanine are regarded as reliable for such calculations (Naude, pers. comm.)

closest to intergers. (For the practical purpose of the programme, the computer was instructed to determine a best fit value for x, within limits corresponding to molecular masses between 10 000 and 100 000 daltons.) In this way a best fit molecular mass is derived independently from the composition data, and may be compared with the mass as derived by some other method.

3.3.9. Polyacrylamide "Disc" Gel Electrophoresis

a. Preparation of samples

Routinely, protein for PAGE was first precipitated, from a solution of 5 mM NH_4HCO_3 (pepsinogens), or of sodium acetate buffer, 0,02 M, pH 5,2 (pepsins) by adding it to $\underline{c}$ 5 volumes of cold acetone and storing for at least 24 hours at -10°C. (Acetate rather than citrate buffer was chosen for general chromatography in the mildly acidic range because the latter forms a precipitate when added to acetone.) The protein-acetone precipitation mixtures were stored in, or transferred to, 1,8 mℓ Beckman polyethylene microfuge tubes (Serial no. 340196). They were centrifuged for 2 x 5 minute cycles on a Beckman Model B microfuge, and the supernatant acetone was carefully poured off. Residual acetone was allowed to evaporate before dissolving the precipitate.

For routine work the method used was that of Laemmli (1970). See Appendix 8 for reagents and protocol.

To the precipitate ($\underline{c}$ 5 - 20 µg) was added dissociation buffer, water, sucrose and bromophenol blue, to a final volume of $\underline{c}$ 50 µℓ. This was held in a bath of boiling water for 5 minutes.

To study the progress of activation, acetone precipitates were not used. Samples of $\underline{c}$ 50 µg of zymogen in 50 µℓ volumes of 5 mM NH_4HCO_3, in microfuge tubes, were chilled to 0°C in crushed ice, and mixed with equal volumes of HCl/KCl buffer, at a final pH of 2,2. Activation was allowed to proceed for periods between 5 and 100 minutes. Activation was terminated by adding to each tube 25 µℓ of

dissociation buffer and immediately transferred to a boiling water bath for 45 minutes.

The resolving and stacking gels (routinely 10% and 4% respectively) were prepared as indicated in the Appendix. They were allowed to polymerise in slabs between glass plates, 160 x 180 mm, or 136 x 177 mm, separated by 1 mm teflon spacers. Slots 8 mm wide were provided by inserting a teflon "comb" into the stacking gel before polymerisation. The slab gel electrophoresis cells and accessories were made by Hoefer Scientific Instruments, Model SE 600, Serial 0862, or (Biorad) Model 220, Serial Q3HF 3886.

Samples for electrophoresis were introduced, with a Drummond microcap capillary pipette and applicator, or a 100 µl Hamilton syringe, into wells in the stacking gel at a volume of 40 µl per well, giving 50 µl/cm slot width.

Commercial marker proteins of known molecular mass were ordinarily added to two or three of the wells.

Current from a Gelman power source was run at 70 volts, $\underline{c}$ 40 milliamps, until samples were "stacked" at the stacking gel/resolving gel interface, after which the potential difference was increased to 100 volts for the separating run.

The run was terminated when the tracking dye had come within 1 cm of the lower edge of the gel ($\underline{c}$ 6 hours).

The resolving gels were stained, for 4 hours at ambient temperature or overnight at 4°C, in Coomassie Brilliant Blue, and were destained with several changes of destaining solution. After destaining they were photographed on 125 ASA panchromatic film, which was processed in Agfa developer and fixer. Gels were dried, under vacuum, on a

porous plate covered with Gladwrap (Union Carbide).

b. Derivation of Molecular Masses

As has been indicated by Shapiro et al (1967), and more fully demonstrated by Weber and Osborne (1969), mobilities of SDS-treated proteins in SDS-PAGE systems are related in a consistent manner to their molecular masses. This provides a simple and rapid means of obtaining mass data with a reported accuracy of ±10% (Weber and Osborne, op. cit.). These workers obtained a smooth curve or a straight line when the logarithms of protein molecular masses were plotted against electrophoretic mobilities. Although they used rather elaborate corrections to derive mobilities from different gels - made necessary by the differential swelling of gels, during staining and destaining, according to degree of cross-linkage - the procedure may be simplified by using several marker proteins, with the experimental material(s), on single multi-lane slab gels. In such cases the distance of migration of markers from the origin is plotted against log mass, yielding a smooth curve from which masses of the experimental materials may be derived.

3.3.10. Tryptic Peptide Mapping

The methods used were essentially those of Elder et al (1977). For a detailed list of reagents see Appendix 9.

a. Preparation

Dried polyacrylamide slabs containing separated, Coomassie-stained proteins, were rehydrated in Laemmli destaining solution, after which individual protein bands were cut out with a razor blade. Slices were placed in small beakers and washed first with 40% methanol (3 x 50 mℓ) and then with 10% methanol (3 x 50 mℓ). They were then transferred to the hydrophilic side of a Gelbond membrane and dried in a desiccator over silica gel in a 37°C warm room overnight.

b. Radioiodination

Dried slices were treated separately in small test tubes. To each was added 20 µℓ of sodium phosphate buffer, 0,5 M, pH 7,5, followed by 10 uℓ of $Na^{125}I$ (c 100 µCi) and 5 µℓ of chloramine T (1 mg/mℓ). After one hour 1,9 mℓ of sodium bisulphite (1 mg/mℓ) was added. After c 15 minutes each slice was transferred to a 2 ℓ flask and washed with 5 x 500 mℓ volumes of 10% methanol. Slices were then individually transferred to 1,8 mℓ microfuge tubes and dried under vacuum for c 3 hours.

c. Tryptic digestion

To each tube was added 0,5 mℓ of 0,05 M ammonium bicarbonate, followed by 50 µℓ of trypsin solution (1 mg/mℓ in distilled water), and 20 µℓ of toluene. The tubes were sealed with parafilm and incubated at 37°C for 20 hours. After digestion each solution was transferred to a 70 x 10 mm test tube and dried under a stream of

nitrogen.

d. Electrophoresis

The dried hydrolysate from each gel slice was redissolved in 20 µl of Buffer I (Appendix 10), and was spotted onto the lower right-hand corner of a 20 x 20 cm pre-coated TLC cellulose plate (Merck). At the opposite lower corner was spotted c 5 µl of 2% Orange G/1% acid fuchsin tracker dye solution.

The plate was arranged on a Shandon electrophoresis cell, fitted with a cooling plate carrying running tap water. The system was levelled with a spirit level, and the cellulose plate was sprayed with Buffer I immediately before applying the current from an LKB 2197 power supply unit set to constant power mode, at 11 watts for a pre-set time of 70 minutes. At the end of this period the leading edge of the tracker dye had almost reached the opposite side of the plate. The plates were allowed to dry, horizontally, in a fume cupboard overnight.

e. Ascending Chromatography

Prior to introducing the plates, the inner walls of the Shandon chromatography tanks were covered with Whatman No. 1 filter paper cut to size and saturated with Buffer II. (See Appendix 10) The buffer was also added to each tank to a depth of about 1 cm. One to two

hours was allowed for the vapour pressure in the sealed tanks to stabilise.

The dried plates were introduced to the equilibrated tanks, two per tank, with the axis of the first-dimension separation at the bottom. Each tank was sealed with a heavy glass lid, and ascending chromatography allowed to proceed until the solvent fronts were within $\underline{c}$ 1 cm of the top of the plates ($\underline{c}$ 6 hours).

The plates were again dried, horizontally, in a fume cupboard.

f. Radiography

Each plate was wrapped in Glad Wrap, secured on the reverse side with masking tape. A clean glass plate was placed over the coated side of the experimental plate, and "hinged" along one edge with masking tape. In the darkroom a sheet of Cronex 4 X-ray film (Dupont Corporation) was sandwiched between the two glass plates, and the whole wrapped in three layers of heavy-duty aluminium foil. The wrapped plates were set aside for periods of 24 to 100 hours (experimental plates) or 30 to 240 hours (blanks), after which the films were developed in Kodak GBX developer (Cat. no. 190 0984), and fixed in Kodak GBX fixer (Cat. no. 190 2485). When dry, they were photographed, by transmitted light, on 35 mm panchromatic film (125 ASA) which was conventionally developed. Contact tracings of the plates were also made.

g. Control

A slice was cut from clear sections of two of the stained electrophoretograms. The two slices were pooled and treated exactly as described for the protein-containing slices.

CHAPTER FOUR

EXTRACTION AND PURIFICATION OF ZYMOGENS

CHAPTER FOUR

EXTRACTION AND PURIFICATION OF ZYMOGENS

4.1. Introduction

Any formal study of enzymes or their precursors requires that they be purified to at least some extent. In the context of the main purpose of this study - viz. the investigation of possible adaptive evolution of the gastric proteases in response to a highly proteolytic venom - it was considered also important to determine the activities of individual crude mucosal extracts.

This chapter reports the origin of mucosal samples, their extraction and assay, and purification of the pepsinogens.

4.2. Crude material : General data

4.2.1. Extraction of activity

To test the effect of prolonged stirring time on the amount of pepsinogen extracted from the gastric mucosa under the conditions adopted, the following experiment was performed on one puff adder:

The mucosa was removed and homogenised as described, and was immediately centrifuged. A small volume of the supernate was removed and assayed for proteolytic activity and for soluble protein as described in the

relevant sections. The sedimented material was then resuspended and stirred at c 4°C overnight, after which it was again centrifuged and the supernate assayed. The specific activity* of the material removed immediately after homogenisation was 170 units mg^{-1}. That removed after c 16 hours of stirring exhibited a specific activity of 675 units mg^{-1}.

This indicates that prolonged stirring results in an approximately 4-fold enrichment of the enzyme extract.

4.2.2. Loss of activity on lyophilisation

In the early part of the work a pooled sample of puff adder gastric mucosal homogenates was subjected to the haemoglobin assay before and after lyophilisation. Post-lyophilisation specific activity was 90% of the original. Another before-and-after assay on a different set of mucosae indicated 94 - 95% retention of specific activity. These and other data indicate that the zymogens are resistant to denaturation by freezing. Fourie et al (1974) found that pig pepsin showed a significant loss of activity only after four freeze-thaw cycles.

---

*A unit of activity is that causing an increase of 0,001 A_{280} units above the blank per minute under the defined conditions of the assay (see Methods), and in the presence of excess substrate.
Specific activity is defined as the number of such activity units per milligram of the catalytic material.

4.2.3. Storage at 4 - 5°C

Figure 17 shows the keeping properties, at 4 - 5°C, of crude puff adder mucosal extracts at pH values as indicated. No provision was made (sodium azide, toluene etc.) to inhibit the growth of organisms. This may account for the apparently superior keeping properties at pH 8,9 over pH 7,5.

4.2.4. Specific activities against various protein substrates

Preliminary assays with various substrates were carried out, using cobra gastric mucosal extract, to ascertain whether any substrate type offered significant advantages. The results of trials involving five substrates are set out in Table III. All assays were carried out with a single stock solution of mucosal extract. All substrates were made to 1,7% (m/v) in 0,035 M HCl. These yielded pHs varying from 1,91 to 2,12. All were adjusted to pH 1,91, ±0,1 unit. Incubation time was 5 minutes; temperature : 37°C.

Although not included in the assays summarised in Table III , bovine globin was also investigated as a substrate in four (duplicate) assays. A_{280} values were $\leq$ 120% of those obtained with enzyme substrate powder.

The results presented in Table III are consistent with an observation by Berstad (1970), that human haemoglobin is a more sensitive substrate for (human) gastric juice and for commercial pig pepsin, than is bovine haemoglobin. Berstad also noted that blank values for human haemoglobin were "considerably lower" than those for bovine

haemoglobin. This finding was not supported in the present study. Berstad also prepared human haemoglobin by the method of Nyman (op. cit.).

The finding, illustrated in Table III , that NaOH/urea-denatured (bovine) haemoglobin is less susceptible to peptic hydrolysis than is the unmodified haemoglobin, is broadly consistent with that of Schlamowitz and Peterson (1959), who found that NaOH/urea-denatured haemoglobin produces only about 85% of the A_{280} value generated by pig pepsin on the unmodified substrate at pH 1,8. (These workers also demonstrated, however, that the pH optimum for peptic hydrolysis of the denatured material is shifted to c pH 3,4, at which pH its susceptibility to digestion is closely comparable with that of the unmodified substrate at pH 1,8.)

TABLE IV summarises various details of mucosae used in the main study.

4.3. Purification of Zymogens

4.3.1. Anion exchange chromatography

Two independent separations were performed for each of the three snake species. The two elution profiles of the cobra and the puff adder extracts were very similar. Those of the mole snake differed, and both profiles are presented. See Figures 16 to 18 , pages 88 - 90.

In all cases the A_{280} elution profiles were rather simple. Three groups of proteins were resolved, corresponding to an unretarded fraction, a fraction displaced in a sodium chloride gradient between

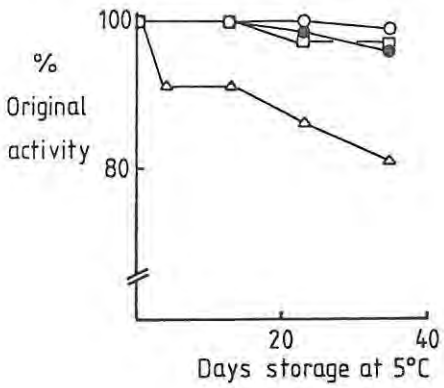

FIGURE 15

Stability of peptic or potential peptic activity of mucosal extracts stored at various pH values. Storage temperature: 5°C.

pH 7,5
pH 8,9
pH 10,5
Piperazine-glycine buffer: pepsinogen.
pH 3,0: HCl/KCl buffer: pepsin.

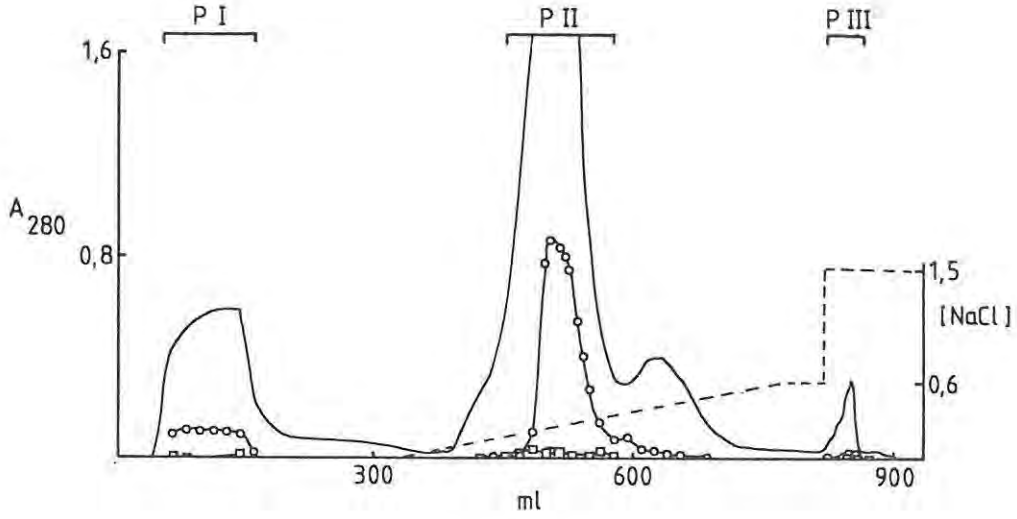

FIGURE 16

Cobra gastric mucosa separated on Whatman DE 52 anion exchanger. ———: A_{280}; O—O: activity against haemoglobin (5 minute assays); □—□: activity against APDT (30 minute assays); - - - - -: NaCl gradient.

Following the practice of Kageyama et al (1976a, etc.), the zymogens are designated P I, II and III according to the order of their elution.

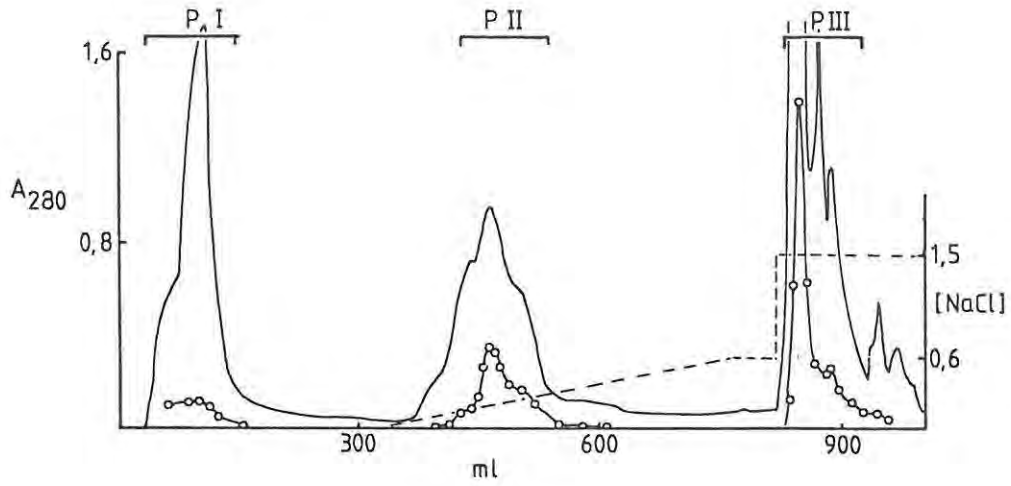

FIGURE 17a

Mole snake gastric mucosa separated on DE 52 anion exchanger. ———: A_{280}; O—O: activity against haemoglobin (5 minute assays); – – – – –: NaCl gradient.

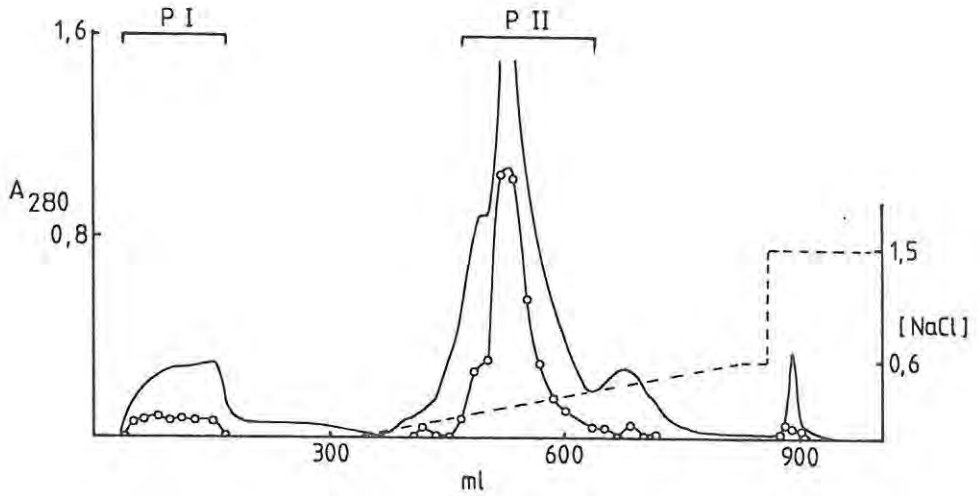

FIGURE 17 b

Mole snake gastric mucosa: second separation of stock material on anion exchanger. Details as for Figure 17a.

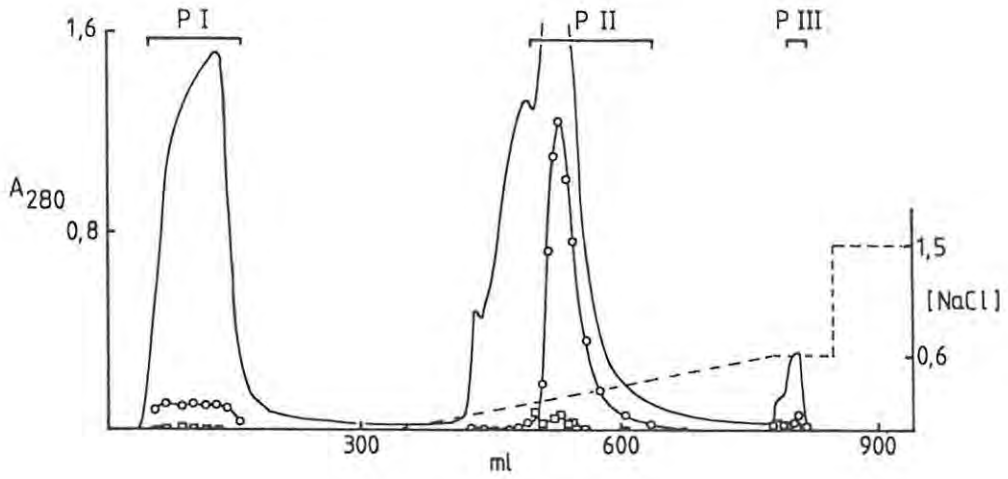

FIGURE 18

Puff adder gastric mucosa separated on Whatman DE 52 anion exchanger. ———: A_{280}; O—O: activity against haemoglobin (5 minute assays); □—□: activity against APDT (30 minute assays); — — —: NaCl gradient.
(Preliminary work on puff adder gastric mucosae, using Cellex D (Biorad) and Whatman DE 22 anion exchangers, gave very similar elution profiles.)

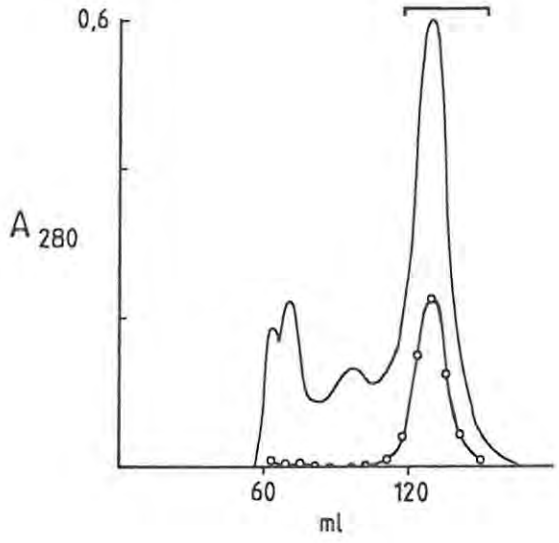

FIGURE 19

Cobra P I zymogen fraction separated on Sephadex G150 molecular exclusion gel. ———: A_{280}; O—O: activity against haemoglobin (5 minute assays).

TABLE III

COMPARISON OF VARIOUS SUBSTRATES.

SOURCE OF ENZYME : CRUDE COBRA GASTRIC MUCOSA.

ASSAY TIME : 5 MINUTES; pH : 1,9; TEMPERATURE : 37°C.

SUBSTRATE	BLANKS + MEAN A_{280} and COEFFICIENT OF VARIATION (%)*	ACTIVE SAMPLES +* MEAN INCREMENT IN A_{280} and COEFFICIENT OF VARIATION (%)
Purified Bovine Haemoglobin	0,092 ± 4,3	0,212 ± 2,8
As above, NaOH/urea-denatured	0,790 ± 0,5	0,119 ± 4,2
Bovine Haemoglobin enzyme substrate powder	0,258 ± 2,4	0,198 ± 3,4
Human Haemoglobin dialysed and lyophilised	0,345 ± 2,0	0,270 ± 2,7
Human plasma, dialysed and lyophilised	0,137 ± 2,6	0,012 ± 36

\+ Twelve Blanks in each test system ; A_{280} values recorded against 5% TCA.

* Coefficient of variation $= \frac{\sigma}{M} \times 100\%$ where σ = standard deviation $= \sqrt{\frac{\Sigma(M-x)^2}{n-1}}$

+* Ten active samples, read against 10% TCA and corrected for mean of Blanks.

TABLE IV

DETAILS OF MUCOSAE EXTRACTED FROM SNAKES

Notations in the Table (+, * etc.) refer to the Notes to TABLE IV, overleaf.

SPECIES	SPECIMEN NO.	MASS	WET MASS OF GASTRIC MUCOSA (g)	MASS OF LYOPHILISED MUCOSA (g) +*	SPECIFIC ACTIVITY units mg^{-1}	WET MUCOSAL MASS AS % OF BODY MASS ++*	MEAN MASS AS % OF BODY MASS AND STANDARD DEVIATION
Cobra	1	630	6,5	0,80 (28,9)	882	1,03	
	2	523	5,4	0,75 (35,0)	1093	1,03	
	3	464	3,9	0,50 (31,3)	1026	0,84	1,06g
	4	514	6,7	0,98 (31,7)	837	1,30	S.D.:
	5	446	4,5	0,77 (40,7)	1495	1,00	0,157
	6	475	5,6	0,78 (31,5)	660	1,17	
Mole Snake	1	568	5,6	0,80 (27,1)	540	0,98	
	2	531	4,3	0,66 (29,2)	746	0,80	
	+3	580	6,5	0,88 (27,4)	709	1,12	0,93g
	4	692	6,2	1,63 (22,6)	1214	0,89	S.D.:
	5	930	8,0	1,24 (35,3)	1313	0,86	0,124

(Continued overleaf)

TABLE IV contd.

SPECIES	SPECIMEN NO.	MASS	WET MASS OF GASTRIC MUCOSA (g)	MASS OF LYOPHILISED MUCOSA (g)	SPECIFIC ACTIVITY units mg^{-1}	WET MUCOSAL MASS AS % OF BODY MASS	MEAN MASS AS % OF BODY MASS AND STANDARD DEVIATION
Puff adder	1	965	5,5	0,90 (30,5)	1318	0,56	
	2	570	5,0	1,06 (20,8)	750	0,87	0,62g
	3	792	3,4	0,45 (39,0)	696	0,42	
	++4	917	5,9	1,05 (28,0)	300	0,64	S.D.: 0,156
	*5	390	2,8	0,37 (25,9)	520	0,71	
	**6	1355	7,3	1,15 (35,0)	493	0,53	

Notes to TABLE IV:

\+ Perforated gastric ulcer. Small local nematode infestation.

++ Grahamstown specimen.

* Port Elizabeth specimen. Small rodent in stomach.

+* Lyophilised masses include buffer salts. Figures in brackets indicate percentage, by mass, of protein.

++* The snakes listed in this TABLE, used in the main body of the work, are designated "Group I". During the 4 years before formal commencement of this work, several puff adders were obtained both locally and from Port Elizabeth. Although their mucosae were not included in the final study, their masses, as percentage of body mass, are listed, as "Group II", in TABLE VII, p. . Also appended to TABLE VII is an analysis of the gastric mucosal masses of specimens in TABLES IV and VII.

$\underline{c}$ 0,1 M and $\underline{c}$ 0,4 M, and a fraction displaced from the column at a chloride concentration of 0,6 M or greater. Peptic activity against haemoglobin was associated with all three of these fractions.

In the cases of both cobra and both puff adder separations, and of the second mole snake separation, the peptic activity associated with the third eluted peak (PIII) was minor. The cobra and puff adder PIII fractions were further purified by molecular exclusion chromatography on Sephadex G150, and subjected to PAGE; but, in view of the very small quantities obtained, were not investigated further. The first mole snake separation yielded a very large peak of peptic activity in the PIII fraction. This was collected for further study.

The major peak of activity, in cobra and puff adder chromatograms, and in the second mole snake chromatogram, emerged within the gradient in the range $\underline{c}$ 0,2 - 0,3 M chloride.

The cobra and puff adder elution profiles also show that the material is essentially devoid of activity towards the synthetic dipeptide substrate APDT. The mole snake DE52 eluate was not assayed for APDT activity, but the three mole snake fractions PI, PII and PIII were each assayed against APDT after passage through G150 gel (see below) and were shown also to be insignificantly active against this substrate.

It should be remarked that the profile of the chloride gradient was not determined empirically. As delivered from a gradient-maker comprised of two identical cylindrical vessels, such a gradient is theoretically linear (Williams and Wilson, 1975), and has been

illustrated as such.

4.3.2. Molecular exclusion chromatography

Column fractions, pooled as indicated in the figures, were concentrated 10 - 20 x and samples of the concentrate applied to a column of G150 Sephadex. Typical elution profiles are illustrated in Figures 19 to 27.

Also shown (Figure 28) is the elution profile of a purified commercial preparation of pig pepsinogen. (See also Figure 14 for a different commercial preparation.)

In all G150 elutions, peptic activity was confined to a single group of fractions emerging in the greatest elution volume. In the cases of the PII fractions from all three species, and of the PIII fraction of the mole snake extract, the forms of the protein elution profile, and the symmetrical distribution of peptic activity, suggest a high degree of purity within the pooled fractions.

Elution volumes of the most active fractions in the various peaks of pepsin activity are set out in Table V, page 101.

It is apparent that, in all three snakes, PI emerges in a somewhat larger elution volume than PII, providing slender preliminary evidence that the PI and PII zymogens differ in their masses, and that PI is the smaller of the two in each case. Further, mole snake PII and PIII elute in the same volume, suggesting close similarity or identity. (The second mole snake DE52 elution profile also suggests that MS PII

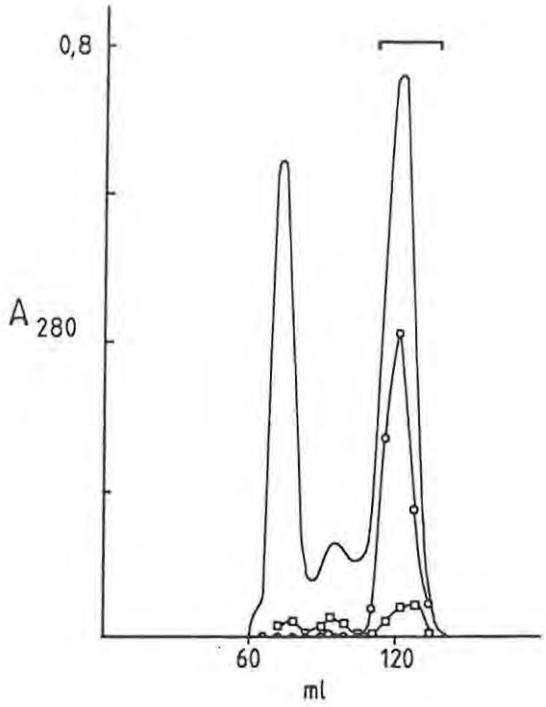

FIGURE 20

Cobra P II zymogen fraction separated on Sephadex G150. ———: A_{280}; O——O: activity against haemoglobin (5 minute assays); □——□: activity against APDT (30 minute assays).

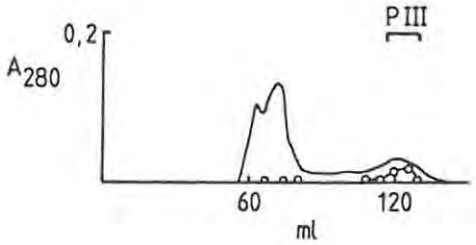

FIGURE 21

Cobra P III zymogen on Sephadex G150. ———: A_{280}; O——O: activity against haemoglobin (5 minute assays).

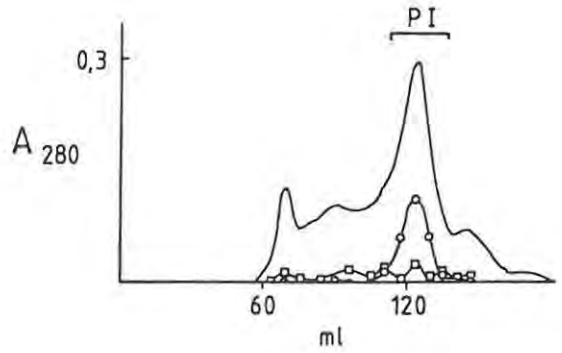

FIGURE 22

Mole snake P I zymogen on Sephadex G150. ———: A_{280};
O—O: activity against haemoglobin (5 minute assays);
□—□: activity against APDT (30 minute assays).

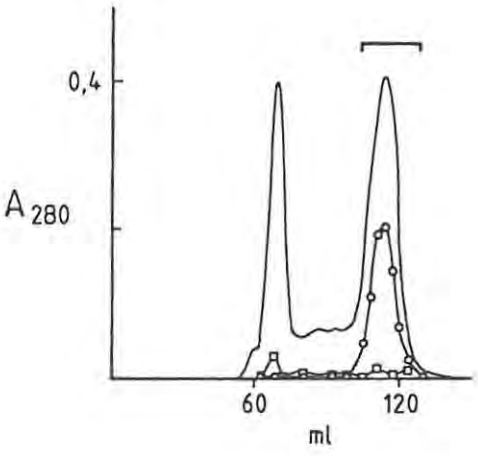

FIGURE 23

Mole snake P II zymogen on Sephadex G150.———: A_{280};
O—O: activity against haemoglobin (5 minute assays)
□—□: activity against APDT (30 minute assays).

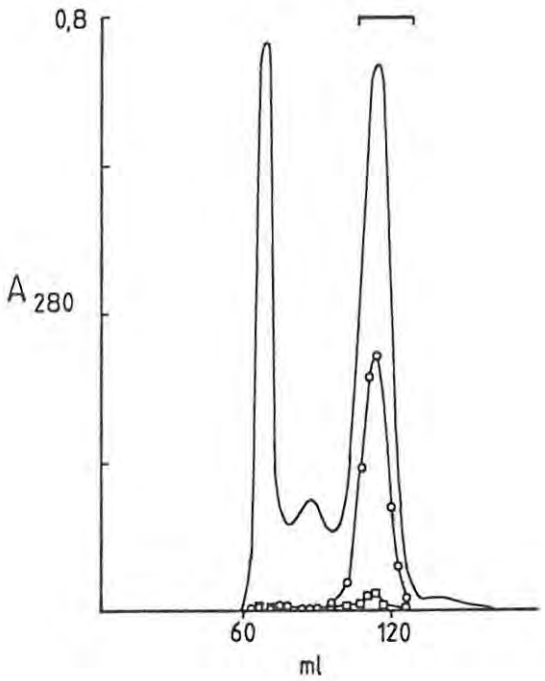

FIGURE 24

Mole snake P III zymogen on Sephadex G150. ———: A_{280};
O—O: activity against haemoglobin (5 minute assays);
□—□: activity against APDT (30 minute assays).

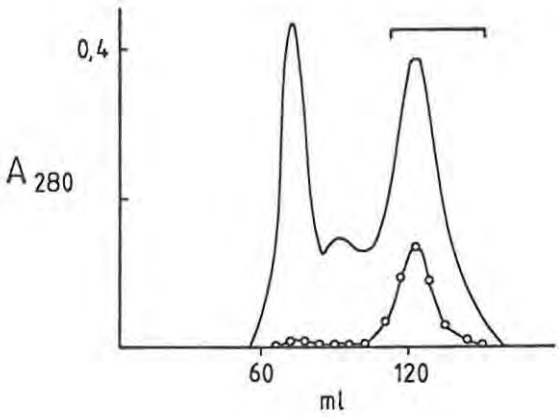

FIGURE 25

Puff adder P I zymogen on Sephadex G150. ———: A_{280};
O—O: activity against haemoglobin (5 minute assays).

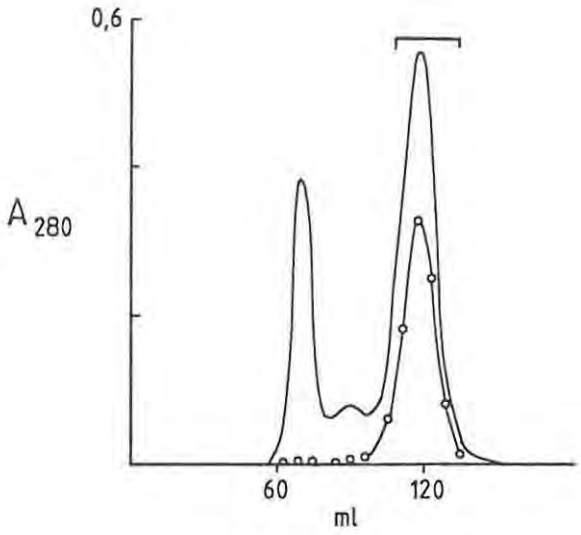

FIGURE 26

Puff adder P II zymogen on Sephadex G150. ———: A_{280};
O—O: activity against haemoglobin (5 minute assays).

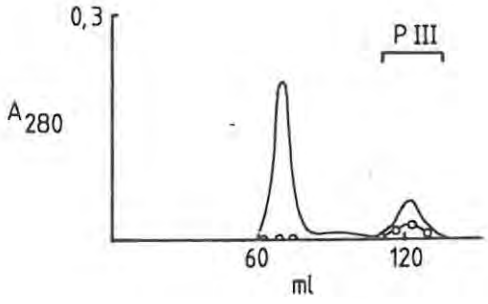

FIGURE 27

Puff adder P III zymogen on Sephadex G150. ———: A_{280};
O—O: activity against haemoglobin (5 minute assays).

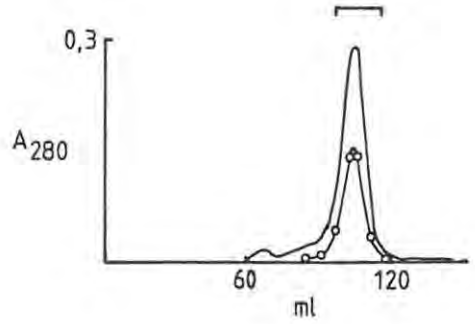

FIGURE 28

Commercial (ICN) pig pepsinogen on Sephadex G150. ———: A_{280}; O——O: activity against haemoglobin (5 minute assays). (See also Figure 14.)

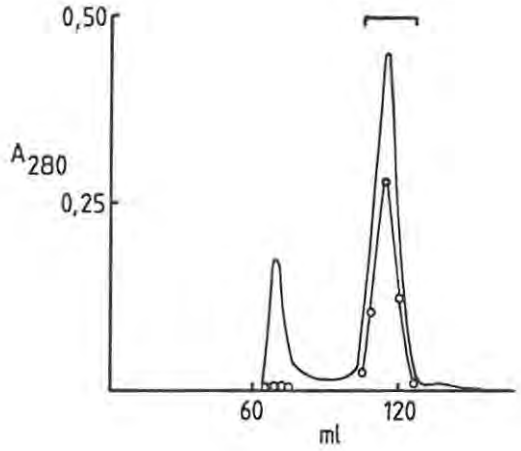

FIGURE 29

Puff adder P I zymogen fraction precipitated in 63% saturated ammonium sulphate solution, and separated on Sephadex G150. ———: A_{280}; O——O: activity against haemoglobin (5 minute assays).

and PIII, derived from the first DE52 separation may be identical.) The data also indicate that the mass of commercial pig pepsinogen is closer to those of the PII snake zymogens than to the PI group.

TABLE V

ELUTION VOLUMES OF POTENTIAL PEPTIC ACTIVITY FROM G150 SEPHADEX COLUMNS

MATERIAL		ELUTION VOLUME (mℓ)
Cobra	PI	141
	PII	126
	PIII	126
Puff adder	PI	122
	PII	114
	PIII	123
Mole snake	PI	126
	PII	111
	PIII	111
Pig (Sigma) Pepsinogen		117

4.3.3. Precipitation in ammonium sulphate solutions

The G150 elution profiles of PI (all three snakes) indicate the presence of substantial remaining impurities. In pilot attempts to purify the puff adder PI fraction further, a sample of the pooled PI material from the DE52 elution was precipitated into three fractions

at 50%, 63% and 80% saturation of ammonium sulphate. The precipitates were redissolved, dialysed and assayed for protein and activity. Material reclaimed from the 63% precipitate showed the greatest increase in specific activity. This was applied to the G150 column. See Figure 29, page 100.

The specific activity of the centre-peak fraction of the G150 eluate was approximately 3 000 units mg^{-1} - about 1,7 x that of the non-precipitated centre-peak G150 fraction of the PI zymogen.

Although substantially pure after G150 separation, a sample of puff adder PII from the DE52 elution was also subjected to 63% ammonium sulphate precipitation and subsequently applied to the G150 column. See Figure 30. Enhancement of the final specific activity in this case was only $\underline{c}$ 1,1 x.

TABLE VI summarises increments in specific activities of the mucosae during purification. (See page 104.)

4.4. Disc electrophoresis in SDS polyacrylamide slab gels

4.4.1. Cobra

Plate I shows heavy contamination of the PI zymogen fraction (lane 2). PII (lane 3) is essentially free of impurities. (Page 106.)

4.4.2. Mole snake

Plate II reveals similar impurity in the PI fraction (lanes 2 and 3). There is also an indication that the PI zymogen band may comprise more than one proenzyme.

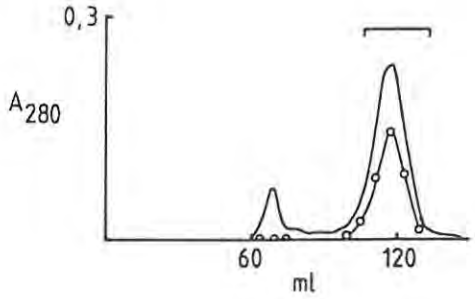

FIGURE 30

Puff adder P II zymogen fraction precipitated in 63% saturated ammonium sulphate solution and separated on Sephadex G150. ———: A_{280}; O—O: activity against haemoglobin (5 minute assays).

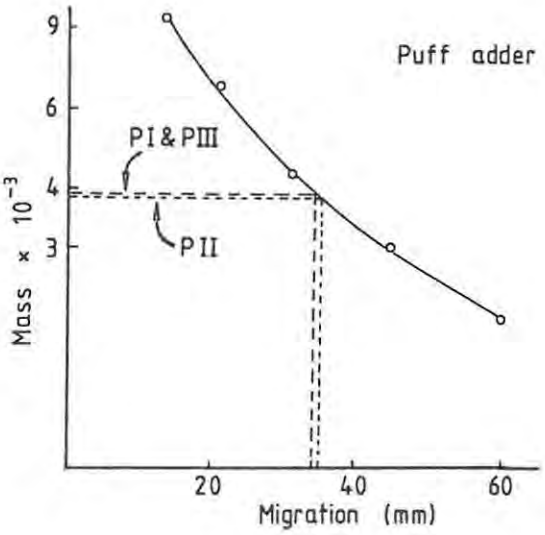

FIGURE 31

Typical plot of mass vs migration distance for marker proteins on PAGE. Phosphorylase b: 94 000 daltons; BSA: 67 000; ovalbumin: 43 000; Carbonic anhydrase: 30 000; Soybean trypsin inhibitor: 20 100. (Data from Pharmacia kit. cf. Weber & Osborn (1969) - BSA: 68 000.) Broken lines on the graph indicate the migration distances and masses of puff adder pepsinogens.

TABLE VI

SUMMARY OF TYPICAL PURIFICATION DATA

FOR MUCOSAE OF THE THREE SNAKES

(Specific activities of commercial (pig) pepsinogen are included for comparison.)

SPECIES	CRUDE MATERIAL*	ANION EXCHANGE	MOLECULAR EXCLUSION
Cobra	Specific activity: 1037 units mg^{-1}	PI Specific activity: 2 000 units mg^{-1}	Specific activity: 3250 units mg^{-1}
	Total activity: 471,8 x 10^3 units = 100%	Total activity: 78,4 x 10^3 units Yield = 17%	Total activity: 69,7 x 10^3 units Yield = 15%
		PII Specific activity: 1870 units mg^{-1}	Specific activity: 2312 units mg^{-1}
		Total activity: 260,3 x 10^3 units Yield = 55%	Total activity: 244,6 x 10^3 units. Yield = 51%
Mole Snake	Specific activity: 1106 units mg^{-1}	PI Specific activity: 885 units mg^{-1}	Specific activity: 2 600 units mg^{-1}
	Total activity: 365 x 10^3 units = 100%	Total activity: 57,7 x 10^3 units Yield = 16%	Total activity: 49 x 10^3 units Yield = 13%
		PII Specific activity: 2 100 units mg^{-1}	Specific activity: 2729 units mg^{-1}
		Total activity: 105,8 x 10^3 units Yield = 30%	Total activity: 95,2 x 10^3 units Yield = 26%

(Continued overleaf)

TABLE VI contd.

SPECIES	CRUDE MATERIAL	ANION EXCHANGE	MOLECULAR EXCLUSION
Mole Snake (contd.)		PIII Specific activity: 1770 units mg^{-1} Total activity: 153 x 10^3 units Yield = 42%	Specific activity: 2590 units mg^{-1} Total activity: 133 x 10^3 units Yield = 36%
Puff adder	Specific activity: 1250 units mg^{-1} Total activity: 354 x 10^3 units = 100%	PI Specific activity: 650 units mg^{-1} Total activity: 50 x 10^3 units Yield = 14%	Specific activity: 1714 units mg^{-1} Total activity: 41 x 10^3 units Yield = 12%
		PII Specific activity: 2 250 units mg^{-1} Total activity: 216 x 10^3 units. Yield = 61%	Specific activity: 3 666 units mg^{-1} Total activity: 192,2 x 10^3 units Yield = 54%
Pig - Commercial purified (Sigma)			Specific activity: 4 365 units mg^{-1}
Pig - Commercial purified (ICN)			Specific activity: 3 052 units mg^{-1}

*
Specific activities of crude materials were determined after dialysis against 0,02 M phosphate buffer, pH 7,8. Such dialysis ordinarily yielded a small precipitate and increment of specific activity of c 1,1 - 1,2 x over that of crude material prior to dialysis.

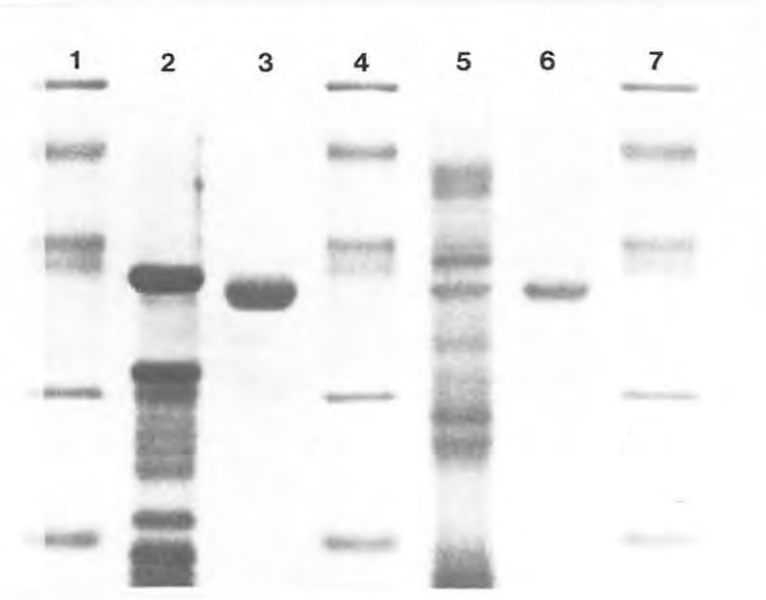

PLATE I

COBRA PEPSINOGENS

Lanes 1, 4 and 7 : Markers.
2 : Pooled PI fraction ex G150 gel.
3 : Centre Peak PII ex G150 gel.
5 : PII ex DE52 anion exchanger.
6 : PIII ex G150 gel.

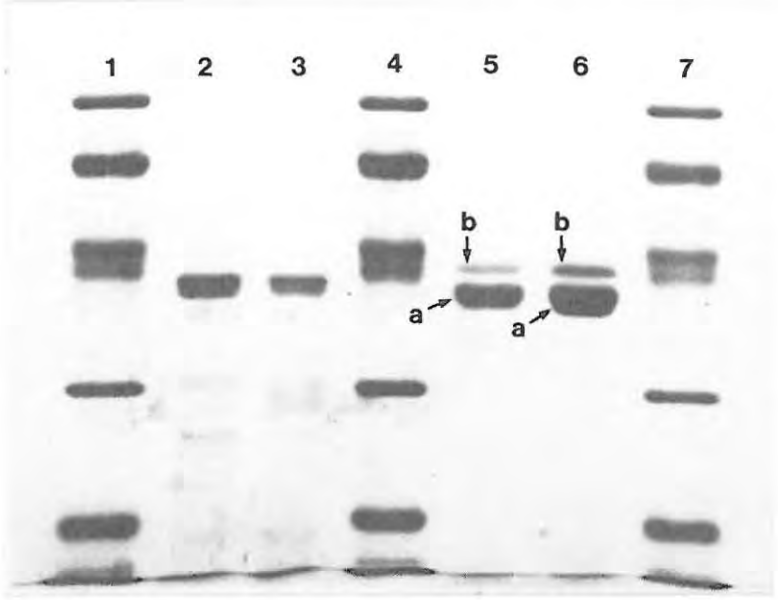

PLATE II

MOLE SNAKE PEPSINOGENS

Lanes 1, 4 and 7 : Markers.
2 : Centre Peak PI, ex G150 gel.
3 : Pooled PI fraction ex G150 gel.
5 : Centre Peak PII ex G150 gel.
6 : Centre Peak PIII ex G150 gel.

PII and PIII (lanes 5 and 6) are essentially free of impurities, but both reveal a duplex structure. The two components in each case are here designated a and b, the former being the smaller (faster migrating) molecular species. The question whether both of these bands are pepsinogens is considered in a later section.

The PIIa and b pepsinogens are probably identical to the PIII pair. (See comment on ion-exchange separations of crude mole snake mucosa, and Figures 17a and 17b page 89.) The PII and PIII materials, mixed and added to a single lane on a slab gel, were found to co-migrate.

4.4.3. Puff adder

The PI material (Plate III, lanes 2 and 3) is again impure, while PII (lane 5) is largely free of contaminants. Plate IV shows substantially increased purity of the PI fraction after precipitation in 63% ammonium sulphate (See also Figure 29, page 100).

4.5. Discussion

4.5.1. Comparison of relative gastric mucosal masses (TABLES IV & VII, pp.92 &109)

These data, rather than establishing any general facts, suggest certain trends and possibilities - viz. that puff adder gastric (digestive) processes require less secretory tissue, per unit of body mass, than is required for the same processes in the other two species.

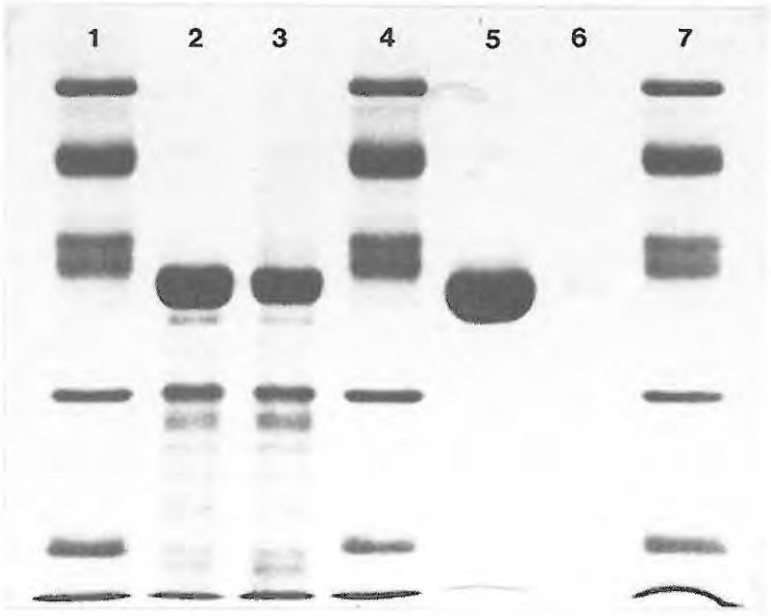

PLATE III

PUFF ADDER PEPSINOGENS

Lanes 1, 4 and 7 : Markers.
2 : Centre Peak PI ex G150 gel
3 : Pooled PI fraction ex G150 gel.
5 : Centre Peak PII ex G150 gel.
6 : Pooled PIII fraction ex G150 gel.

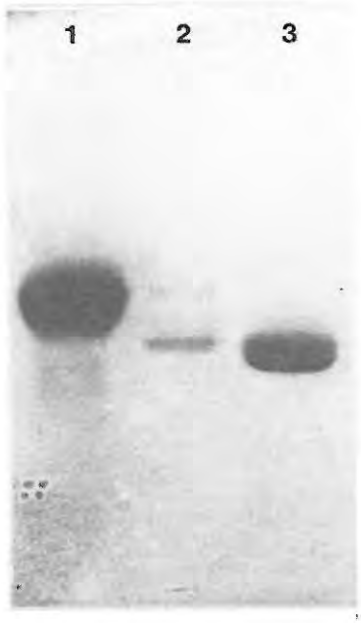

PLATE IV

PUFF ADDER PI, AMMONIUM SULPHATE FRACTION (See Figure 31)

. Lane 1 : Ovalbumin.
2 : Pooled PI fraction, 63% saturated ammonium sulphate precipitate.
3 : The same; heavier loading.

TABLE VII

MASS DATA FOR "GROUP II" PUFF ADDERS

MASS OF SNAKE (g)	WET GASTRIC MASS (g)	% OF BODY MASS
377	3,0	0,79
593	6,6	1,10
340	2,1	0,61
675	3,9	0,57
601	4,2	0,69
768	4,8	0,62
721	5,3	0,73
363	3,1	0,85
545	3,3	0,60
1062	5,8	0,54
778	6,5	0,83
308	3,5	1,10
455	4,6	1,00

(see also Notes to TABLE IV (+++), pages 92 and 93)

Notes to TABLES VII AND IV:

Inspite of the smallness of the samples, including data presented in TABLE IV, it is worth noting that nominal differences exist between the specimens in terms of the relative masses of their gastric mucosae. Using Student's t distribution as appropriate for small samples (Daniel, 1983), and with confidence levels as indicated, it may be shown that:

1. The mole snake and cobra mucosal masses, expressed as fractions of body mass, do not differ significantly ($P > 0,9$) (TABLE IV).

2. The mole snake relative mucosal masses differ from those of the Group I puff adders ($P < 0,01$).

3. The Group II puff adder relative mucosal masses do not differ from those of the Group I puff adders ($P > 0,1$).

4. The combined Group II and Group I puff adder relative mucosal masses differ from those of the mole snakes ($P < 0,05$).

5. The Group II puff adder relative mucosal masses do not differ from the mole snake relative mucosal masses ($P > 0,1$).

These data will be considered in the General Discussion.

This possibility is consistent with the hypothesis, outlined in the Introduction, that the contribution of proteolytic (viperid) venom to the digestive process results in some complementary adaptation in the gastric mucosae and/or their secretions. A rigorous investigation of this putative relationship - insofar as it may be reflected in relative mucosal masses - will require not only larger numbers of specimens, but also a more complete knowledge of periodic changes - seasonal/hormonal - in individual snake gastric mucosal tissue.

4.5.2. Choice of substrate, and specific activity data

a. Substrate

In spite of the demonstrated superiority of human haemoglobin over the other substrates tested, (TABLE III), the difference was not considered sufficient to justify the preparation of large quantities of this material for the entire project. Similarly, the marginal superiority of pure haemoglobin over the "substrate powder" was considered insufficient justification for the substantial cost difference.*

Globin, although providing a slightly more sensitive substrate than

---

*(Miles) Seravac report (December 1985) that they no longer produce bovine haemoglobin. The quoted Sigma price for equivalent material is over 20 x that of the (haemoglobin) substrate powder.

haemoglobin substrate powder, is tedious and time consuming to prepare. The maximum theoretical yield from (pure) haemoglobin is about 96%, but the actual recovery obtained in this work was approximately 70%. This adds significantly to the cost of the material. Globin was found to dissolve well in distilled water, but tended to develop cloudiness in 0,15 M NaCl solution. This apparent instability may interfere with assays.

The variation in substrate susceptibility indicates that, for comparability of such data, the haemoglobin substrate should be closely specified. A further, more general weakness in the use of TCA-soluble products of protein hydrolysis for precise characterisation of activity is that, as noted by Lin et al (1969), the criteria for solubility in TCA are not well understood, and there is no simple relationship between the enzymatic cleavage of peptide bonds in the substrate and the accumulation, in the TCA-soluble fraction, of hydrolysis products. Provided substrate remains in excess, there is a generally linear development of optical density in the (filtered) supernate with increasing incubation time, but the relationship is often somewhat curvilinear during the first 10 - 15 minutes. This effect is also apparent when BSA is used as a substrate (Schlamowitz and Peterson, op. cit.). As noted by Lin et al (op. cit.) the curvilinear time/A_{280} relationship in the early stages of the incubation is presumably due to the production, from each cleavage, of products either or both of which remain TCA-insoluble. Since it is assumed that size of product moiety is an important criterion of TCA-insolubility, a necessary consequence of progressive enzyme activity is the production of an increasing proportion of small, and

hence TCA-soluble, species in the digestion mixture.

Notwithstanding the apparent generality of this mechanistic explanation of curvilinearity, it was found in the present work that the effect was more marked with pure commercial pig pepsin than with the snake pepsins.

The "classical" Anson assay is based on a 10-minute incubation. This incubation time has been used, or recommended, by Ryle (1970, op. cit.); Bohak (1970, op. cit); Sanchez-Chiang (1982); Raufman et al (1984) and Green and Llewellin (op. cit.). A 5-minute incubation time has been used by Kassel and Meitner (op. cit.), and by Chow and Kassel (op. cit.). Rajagopalan et al (1966 b) used 4 minutes. Clearly, from graphical data supplied by Schlamowitz and Peterson (op. cit.) for hydrolysis of BSA, and from data obtained from haemoglobin in the present study, activity is not linear within these times, hence standardisation of incubation time is important if activities are to be closely compared. Kageyama and Takahashi (1976a) used 5 - 40 minutes incubation: "The amount of enzyme used was so adjusted that the activity was proportional to the enzyme concentration, and that the reaction proceeded linearly for about 1 hour".

While incubation time has, at least generally, been standardised on an arbitrary 10 minutes, other parameters of the assay - volumes and concentration of substrate and of TCA - have varied so widely among workers as to effectively remove any potential benefit conferred by the standardisation of incubation time.

To offset some of the difficulty inherent in the use of native proteins,

Lin et al (op. cit.) investigated the use, as substrates, of proteins the free amino groups of which had been dimethylated. This permits the assay of newly-formed amino groups following upon hydrolysis. These workers used the TNBS method of amino group assay, but remark that the ninhydrin method should give similar results. Their data show dimethylated proteins (casein and haemoglobin) to provide highly sensitive protease substrates, the hydrolysis of which is a linear function of time.

Notwithstanding this, such modified protein substrates do not appear to have been widely adopted in routine pepsin investigations. Fox et al (1976), noting Lin's paper (op. cit.), and the general non-acceptance of dimethylated substrates, used dimethyl casein and dimethyl haemoglobin to determine pH optimum and K_m data for sheep pepsin.

Part of the reason for eschewing dimethylated proteins is perhaps that they are time consuming and tedious to prepare, and that, inspite of its inherent limitations, unmodified haemoglobin has an indisputable role in pepsin investigation. (See, for example, the extensive work of Marciniszyn et al, 1976b.)

More significantly, several synthetic dipeptide and oligopeptide substrates have been prepared which offer advantages over the unmodified and the dimethylated protein substrates.

Early attempts to make dimethylhaemoglobin for the present work, both by the method of Means and Feeney (1968), and the slightly modified procedure of Lin et al (op. cit.), were unsuccessful. The extent of dimethylation remained low, and differences between blank and

experimental values in hydrolysis mixtures were inadequate. As use of these substrates did not appear to be essential to the project, the synthesis was not pursued.

b. Comparison of specific activities (TABLE IV, page 92.)

The variables on which observed activities depend, as discussed in the previous section, have been standardised in deriving the specific activities listed in the Table.

Comparison of these activities reveals nothing which might contribute positively or negatively to the arguments concerning a possible complementary relationship between venom and peptic activity. The intraspecific variation is high in all three cases, and indicates that the population variation within each species is very large. This consideration suggests that specific activity data, as an indicator for or against the hypothesis presented here, would have to be based on such large numbers as to be impractical or injudicious in the case of animals which are feral and in ecological jeopardy.

4.5.3. Final purification of the PI zymogens

Although, as reported later, the incompletely purified PI zymogens have been used for kinetic studies (after activation and passage through a Sephadex G50 gel column), and also for tryptic peptide mapping (by excision of the zymogens from stained gel slabs), further work on

these zymogens will require that they be more fully purified.

Data reported in the previous sections indicate that such purification may be achieved by ammonium sulphate precipitation. This was not routinely done in the present work because the gastric mucosae contain relatively little of the PI zymogens, and ammonium sulphate precipitation was shown to result in a loss of at least 50% of the active material. Bohak (1969), working on chicken pepsinogens, reported a loss of 60 - 80% of activity after ammonium sulphate precipitation. In certain circumstances such losses may be tolerable. When some 3 - 6g (wet mass) of mucosa is obtained from the stomach of a single member of a feral species, the continued existence of which is in any case questionable, one reads with a certain envy of other workers obtaining 9,1 kg of "hashed mucosa" from an abbattoir (Chow and Kassell, op. cit.), or 400g of chicken stomachs from a poultry farm (Green and Llewellin, op. cit.).

A more modern and superior method of final - or at least further - purification involves the use of affinity chromatography. Harboe et al (1974) used polylysine-Sepharose 4B for the purification of bovine pesinogen. Rechromatography on DEAE cellulose is also useful, but again involves rather substantial losses.

4.5.4. Multiplicity of zymogens

The indication, by PAGE, that more than one pepsinogen is present in the mucosa of each of the three species, is consistent with the

findings in other groups. In all cases investigated, analysis has revealed a multiplicity of gastric zymogens. Thus there are at least four in the dog fish (Bar-Eli and Merret, 1970); six in Japanese Monkey (Kageyama and Takahashi, 1976b); at least five in chickens (Green and Llewellin, 1973); five in Asiatic Black Bear (Kageyama et al,1983b; three in domestic dog (Cavadore et al, 1979); six in domestic (white) rabbit (Kageyama and Takahashi, 1984); five(?) in Japanese Quail (Esumi et al, 1980); six in Man (Taggart et al, 1979): eight in Crab-eating Monkey (Kageyama and Takahashi, 1980).

The heterogeneity of snake pepsinogens is reported more fully in a later section of this work.

The degree of purity achieved and demonstrated in the previous sections allows the determination of some physico-chemical properties of the pepsinogens.

CHAPTER FIVE

PHYSICOCHEMICAL STUDIES ON THE ZYMOGENS

CHAPTER FIVE

PHYSICOCHEMICAL STUDIES ON THE ZYMOGENS

5.1. Introduction

Any genetically-based character showing intra- or inter-group variation is potentially useful in determining degrees of heterogeneity within the group, or of relatedness between groups. Such data may fall, of course, into the overlapping provinces of the geneticist, the taxonomist and the ethologist.

Since evolution is essentially a stochastic process (Bateson, 1979) the majority of characters are the results of adaptive interaction with the environment. Facilities for - and perhaps a human proclivity towards - reductionist exploration, dictate the accumulation of factual minutiae as an antecedent to the more satisfying apprehension - however dimly - of pattern.

The purpose of the next phase of the investigation, as reported in this Chapter, was the collection of physico-chemical data allowing some formal biochemical quantification and comparisons.

5.2. Molecular masses of the pepsinogens

5.2.1. Snake pepsinogens

Measurements of migration distances were made on the destained gels

prior to drying.

Weber and Osborn (1975) note that "the plot obtained for log molecular weight vs mobility is usually a straight line, but with some gel systems it may be slightly concave".

It is unfortunate that in some cases doubt exists about the position of the 43 000 dalton marker protein (ovalbumin).* See plates I, II and III.The duplex nominally ascribed to ovalbumin was found to be a fault of one of the Pharmacia kits. Other workers in the laboratory obtained the same result. Another marker kit gave satisfactory results. To determine which of the paired bands corresponded to the true ovalbumin position, the migration distance of this protein on the satisfactory gels was measured relative to the distance moved by the 68 000 dalton marker (BSA). The ratio (mean : 1,43) was used to locate the correct position of ovalbumin on the defective gels. In this way the corrected ovalbumin position was found to correspond closely with the position of the slower migrating component of the duplex. This value was used in plotting the relevant graphs. The masses obtained for the zymogens of the

---

*
The actual mass ascribable to ovalbumin is itself an experimental variable. The Pharmacia kit quotes a mass of 43 000 daltons. Sigma (and Pierce) kits quote 45 000.

Castellino and Barker (1968) determined a mass for ovalbumin of 44 620 ($\pm$300) daltons, by the method of Osmometry, and 43 040 by Sedimentation Equilibrium. As indicated by the differing masses cited in commercial mass calibration kits, it appears that some doubt exists as to the most appropriate operationally-determined mass to use for PAGE analytical systems. In the results obtained in the present work, the values fitting most naturally into the curve appeared in some cases to be $\underline{c}$ 43 000, in others $\underline{c}$ 45 000.

three snakes are given in TABLE VIII. Some of the values reported in the Table were obtained from gels used in studies on activation. These studies revealed microheterogeneity of the pepsinogens. See Section 5.7., page 144 et seq.

5.2.2. SDS-PAGE-derived mass of commercial pig pepsinogen

Pig pepsinogen was run with several gels. In all cases the zymogen moved with, or fractionally more slowly than, ovalbumin, suggesting the disturbingly erroneous mass of 44 000 daltons or more. The mass of the pig zymogen, derived from amino acid sequence data, is 39 630 (Narasinga Rao et al, 1977).

One of the Sigma kits of Marker Proteins - Code MW-SDS-70 (not used in the present study) - contains pig pepsin. A note in the Catalogue indicates that this kit is not for use with the Laemmli system, because " ... the pepsin component migrates abnormally slowly, approximately at the same rate as ovalbumin (Albumin. Egg)". As references to the section in which this comment appears, the Sigma catalogue cites papers by Weber and Osborn (1969), Davies and Stark (1970) and Laemmli (op. cit.). None of these papers refers directly to the anomalous migration of pig pepsin, and it must provisionally be assumed that this remains unexplained. The present study indicates that this applies also to commercial pig pepsinogen. Interestingly, however, it apparently does not apply to the snake pepsinogens (or their pepsins - see later section).

TABLE VIII

MASSES OF ZYMOGENS AS DERIVED FROM PAGE DATA

(See p118 *et seq.*)

SPECIES	ZYMOGEN	MASS (daltons)
Puff adder	PI	39 000
	PII	38 000
	PIII	39 000
Puff adder	PII	38 000
	PIIa	36 800
	PIIb	38 000
Cobra	PI	39 000
	PII	38 000
	PIII	38 000
Cobra	PIIa	38 500
	PIIb	40 500
Cobra	PI	40 000
	PII	38 - 39 000
Mole snake	PI	40 000
	PIIa	39 000
	PIIb	43 000
	PIIIa	39 000
	PIIIb	43 000
Mole snake	PI	-
	PIIIa	37 000
	PIIIb	40 000

From the PAGE data alone, however, the possibility does exist that the actual masses of the snake pepsinogens are themselves substantially less - by about 10 - 12% - than their masses as derived from the electrophoretograms. That this is probably not so, at least in the case of puff adder PII zymogens, is indicated by earlier work (Robertson, 1979), in which the mass of the puff adder PII zymogen was determined by elution from a column of G150 gel, calibrated with marker proteins. The mass ascribed to the PII zymogens was 39 000 (± 2 000) daltons (Figure 34). This is in close agreement with the PAGE findings, and suggests that the latter are not seriously in error for the other two snake species. Esumi et al (op. cit.) used the Laemmli SDS-PAGE method to determine the molecular mass of one of the quail pepsinogens, obtaining a value of 40 000.

These findings suggest that at least certain snake and bird pepsinogens (and pepsins) differ in some respect - presumably related to tertiary structure - from the principal pig zymogen and pepsin. Weber and Osborn (1975) report that, since SDS does not bind to carbohydrate, "Glycoproteins cannot be expected to behave normally on SDS gels". Pig pepsinogen contains c 1,2% carbohydrate (Neumann et al, 1969) - substantially more than is found in the snake (PII) material (see later section). That this is not the cause of the anomalous migration is suggested by the fact that pig pepsin contains little or no carbohydrate (Neumann et al, op. cit.), yet also migrates slowly.

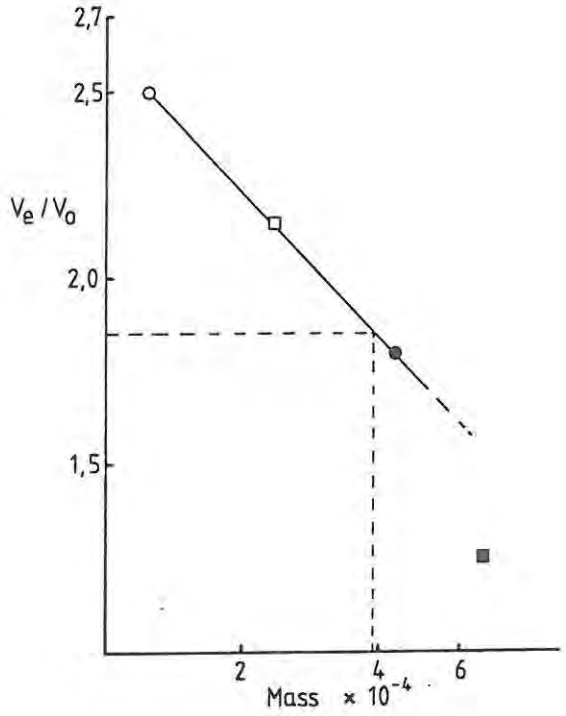

FIGURE 32

Mass of puff adder P II pepsinogen derived by column chromatography on Sephadex G150, according to the method of Whitaker (1963). Masses of marker proteins: BSA: 68 000 daltons (■) (Weber and Osborn,1969); Ovalbumin: 43 000 (●) (Weber and Osborn, op. cit.); a Chymotrypsinogen: 23 650 (□) (CRC Handbook of Biochemistry, 2nd Ed., 1970); Cytochrome c (bovine heart): 12 327 (○) (Sigma Chemical Co., catalogue April 1976).
V_e : elution volume of protein; V_0 : column void volume. V_0 determined with Pharmacia Blue Dextran 2 000, average molecular mass 2×10^6. Sephadex G150 fractionation range: 1×10^3 - $1,5 \times 10^5$ (Pharmacia product brochure).

5.3. Amino acid composition of the pepsinogens

Only the PII pepsinogens of the cobra and puff adder, and the PII and PIII pepsinogens of the mole snake, were used for composition analyses, as the PI fractions from all three species were shown to be substantially impure.

TABLES IX and X show composition data for cobra PII and puff adder PII pepsinogens, as nmol amino acid per mℓ, derived from 24, 48 and 72 hour hydrolysis times.

General notes on the data in TABLES IX and X.

Schroeder (op. cit.) notes that the amino acids valine, isoleucine and leucine (with large aliphatic chains) "have been shown to be released more slowly from peptide linkage than are other residues". He further remarks that they are nonetheless likely to be almost completely released in 24 hours of hydrolysis, but that the completeness depends on sequence in the protein. T. Haylett* (pers. comm.) reports that valine and isoleucine are difficult to release, and that 72 hour hydrolysis values are usually used. This practice has accordingly been adopted here. In the case of leucine, the data from the three hydrolysis times do not appear to differ sufficiently to call for selection.

Haylett also notes that cystine (and tryptophan) values are always very unreliable and must be determined separately. Moore (1972) notes

---

* National Chemical Research Laboratory, CSIR, Pretoria.

TABLE IX

COBRA PII PEPSINOGEN(S) AMINO ACID COMPOSITION

AMINO ACID	HYDROLYSIS TIMES (HOURS) Figures are n mol/mℓ						AVERAGE n mol/mℓ
	24		48		72		
	a	b	a	b	a	b	
Asp	215,3	211,3	211,4	213,3	216,4	215,2	213,8
Thr*	120,1	115,4	114,2	114,2	111,3	110,9	127,0
Ser*	162,5	162,3	145,7	149,4	133,4	131,3	178,0
Glu	201,9	197,0	195,5	197,3	206,7	203,8	200,3
Pro	101,6	-	100,3	-	93,5	95,6	97,7
Gly	186,1	200,3	183,6	203,4	187,1	182,9	190,5
Ala	99,6	98,5	98,5	99,2	100,5	100,2	99,4
Val[a)]	119,2	119,6	121,6	124,6	125,9	124,3	125,1
Cys*	17,5	18,1	11,6	15,6	-	9,5	22,0
Met	(5,9)	12,6	10,3	13,2	12,4	18,3	13,5
Ile[a)]	123,0	120,6	126,2	125,5	127,9	126,0	126,9
Leu	166,2	164,7	166,6	164,1	167,3	165,8	165,7
Tyr	121,4	119,7	121,2	121,6	123,4	122,7	121,6
Phe	116,3	110,4	114,1	113,5	116,9	113,1	114,0
NH_3	221,8	234,9	241,5	250,9	257,5	264,9	-
His	21,6	22,4	22,1	22,7	22,1	22,1	22,1
Lys	54,2	56,0	54,9	56,7	54,9	50,7	54,5
(AGPA)≠	91,9	98,2	95,5	99,9	95,9	101,8	-
Arg	30,0	30,9	30,3	31,3	30,5	31,0	30,6

* Extrapolated to zero incubation time.

a) 72-hour hydrolysis data used.

≠ AGPA added at 100 n mol/mℓ.

( ) Value not included in average.

TABLE X

PUFF ADDER PII PEPSINOGEN(S). AMINO ACID COMPOSITION

AMINO ACID	HYDROLYSIS TIMES (HOURS) Figures are n mol/mℓ						AVERAGE n mol/mℓ
	24		48		72		
	a	b	a	b	a	b	
Asp	(372,9)	385,3	393,6	388,9	381,6	-	387,3
Thr*	205,6	206,6	208,5	202,5	199,7	-	210,0
Ser*	302,7	318,2	286,6	286,6	255,4	-	350,0
Glu	352,6	360,9	379,9	371,2	398,9	-	372,7
Pro	158,1	-	166,9	-	164,8	-	163,2
Gly	318,7	357,5	337,3	(363,1)	328,0	-	335,3
Ala	168,0	173,9	176,6	176,5	183,2	-	175,6
Val[a)]	187,3	194,4	200,2	205,7	206,1	-	206,1
Cys*	34,0	36,2	28,5	33,1	-	-	40,0
Met	16,2	24,9	17,7	27,6	19,1	-	21,1
Ile[a)]	214,9	219,3	231,2	231,3	229,8	-	229,8
Leu	292,5	295,4	307,2	304,0	294,2	-	298,6
Tyr	210,2	213,8	219,8	221,7	221,8	-	217,4
Phe	(205,9)	(207,6)	216,5	216,5	219,7	-	217,5
NH_3	357,1	385,1	402,6	431,3	476,0	-	
His	32,2	22,9	22,6	23,7	22,5	-	22,9
Lys	77,9	81,5	80,9	84,7	77,4	-	80,4
(AGPA)≠	92,6	98,2	93,3	95,3	102,5	-	
Arg	38,6	40,4	39,6	40,8	39,1	-	39,7

* Extrapolated to zero incubation time.

a) 72-hour hydrolysis data used.

≠ AGPA added at 100 n mol/mℓ.

( ) Value not included in average.

TABLE XI

AMINO ACID COMPOSITION, IN RESIDUES PER MOLE, OF COBRA PII AND PUFF ADDER PII PEPSINOGENS

	COBRA		PUFF ADDER	
	Unrounded	Rounded	Unrounded	Rounded
Asp	35,2	35	40,5	41
Thr	20,7	21	22,1	22
Ser	29,0	29	36,7	37
Glu	32,7	33	39,1	39
Pro	16,0	16	17,0	17
Gly	31,2	31	35,0	35
Ala	16,3	16	18,7	19
Val	20,3	20	21,3	21
Cys	2,5	3	3,3	3
Met	2,2	2	2,1	2
Ile	20,3	20	24,2	24
Leu	27,2	27	31,2	31
Tyr	20,0	20	23,3	23
Phe	18,5	19	22,8	23
His	3,6	4	2,4	2
Lys	8,7	9	8,4	8
Arg	5,9	5	4,1	4
Trp	N.D.	-	N.D.	-
Residues		310		328
Mass (daltons)		33 719		38 045

Note: The number of residues is calculated from the amino acid concentrations (TABLE X) as described in the text.

TABLE XII

AMINO ACID COMPOSITION, IN RESIDUES PER MOLE, OF MOLE SNAKE PII AND PIII PEPSINOGENS*

	M.S. PII		M.S. PIII	
	Unrounded	Rounded	Unrounded	Rounded
Asp	44,1	44	41,1	41
Thr	30,1	30	30,0	30
Ser	46,8	47	39,1	39
Glu	44,2	44	43,1	43
Pro	25,2	25	24,9	25
Gly	43,9	44	38,8	39
Ala	23,7	24	21,7	22
Val	26,7	27	24,2	24
Cys	N.D.	-	N.D.	-
Met	10,9	11	8,9	9
Ile	27,5	27	25,2	25
Leu	34,7	35	31,8	32
Tyr	26,1	26	23,6	24
Phe	22,3	22	25,1	25
His	4,0	4	3,9	4
Lys	10,8	11	10,3	10
Arg	7,9	8	6,9	7
Trp	N.D.	-	N.D.	-
Residues		429		399
Mass (daltons)		47 694		44 692

*Chemical and computerised analyses performed by the National Chemical Research Laboratory.

that cystine or cysteine "can be most precisely determined after derivatization".

The Analyser's failure to produce data for the second 72 hour puff adder hydrolysis time is unfortunate, and cannot be accounted for. Likewise, certain blanks appeared (as indicated) in the other analyses.

The composition data presented in TABLE XI were derived as described in Section 3.3.8., Methods, page74. Mole snake PII and PIII material was analysed by the National Chemical Research Laboratory, and was subjected to a minimization programme essentially similar to that described.

5.4. Serology*: Ouchterlony tests

a. Pre-immune sera

Pre-immune sera did not yield any detectable reactions against the four immunogens used in the study.

b. Homologous reactions

i. Pig pepsinogen

Serum of the rabbit challenged with pig pepsinogen failed to show

---

*In addition to the general abbreviations used throughout this work, the following contractions are used in this section:

C : MS : PA : cobra : mole snake : puff adder.

detectable antibody when exposed to homologous immunogen.

ii. Snake pepsinogens

Sera of all three rabbits exposed to snake pepsinogens proved to be antibody positive. Plates V and VI show homologous and heterologous reactions with cobra and mole snake antisera (centre wells).
(See page 131.)

c. Heterologous reactions

In addition to the cross-reactions shown in Plates V and VI, Plates VII to X (pages 132 and 133) show reactions between the snake pepsinogens and antipepsinogens at various antigen dilutions. Quoted dilutions are from stocks of crude mucosal extracts at $\underline{c}$ 10 mg/mℓ.

As is anticipated, cobra antibody reacts strongly with homologous antigen (Plate V), and less strongly with the heterologous puff adder and mole snake antigens. There is some tentative evidence that the heterologous puff adder antibody : cobra antigen reaction may be somewhat stronger than the homologous puff adder reaction.

The stronger precipitin reaction between puff adder antipepsinogen and cobra pepsinogen than between puff adder antipepsinogen and mole snake pepsinogen, suggests greater similarity between puff adder and cobra pepsinogens than between puff adder and mole snake (see Plates IX and X).

No precipitin reaction was observed between pig pepsinogen and any of the snake antipepsinogens. Stock pig pepsinogen was made at

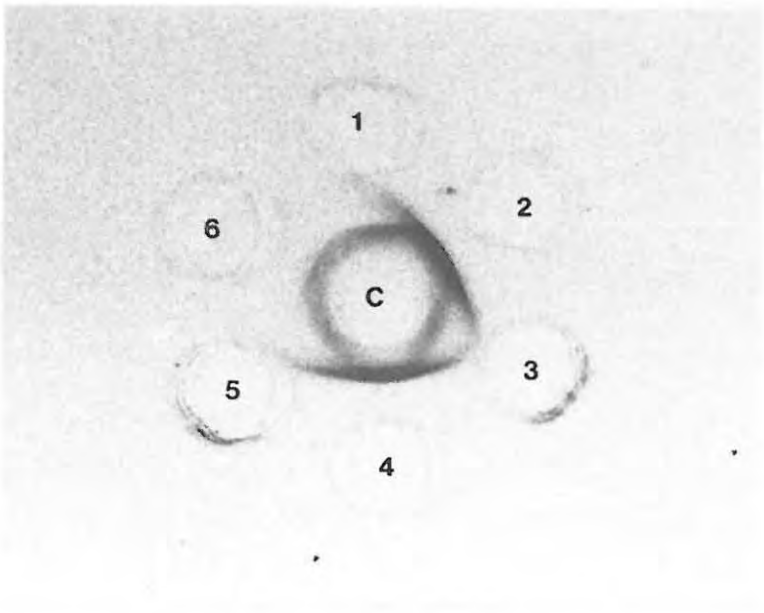

PLATE V

DOUBLE-DIFFUSION TEST. COBRA ANTISERUM

Centre well : antiserum (1/4 dilution)
Radially : 1 and 6 : mole snake
2 and 4 : cobra
3 and 5 : puff adder

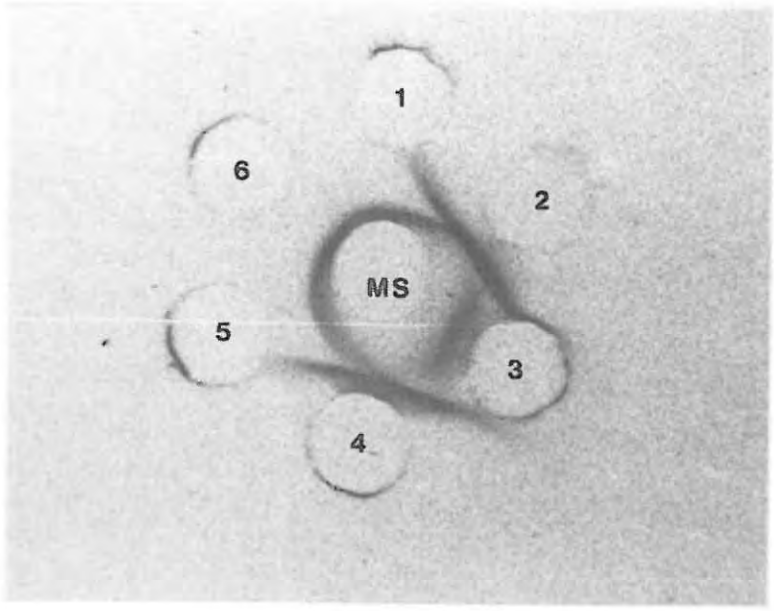

PLATE VI

DOUBLE-DIFFUSION TEST. MOLE SNAKE ANTISERUM

Centre well : antiserum (1/4 dilution)
Radially : 1 and 6 : cobra
: 2 and 4 : mole snake
: 3 and 5 : puff adder

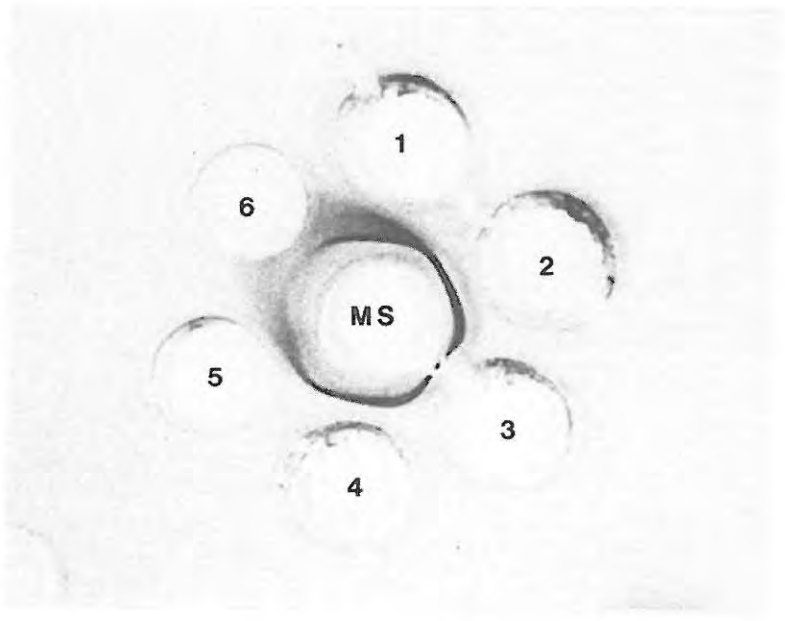

PLATE VII

DOUBLE-DIFFUSION TEST.
MOLE SNAKE ANTISERUM

Centre well : antiserum (1/4 dilution)
Radially : puff adder antigen wells 1 to 6, 1/1 to 1/32 in 2-fold dilutions

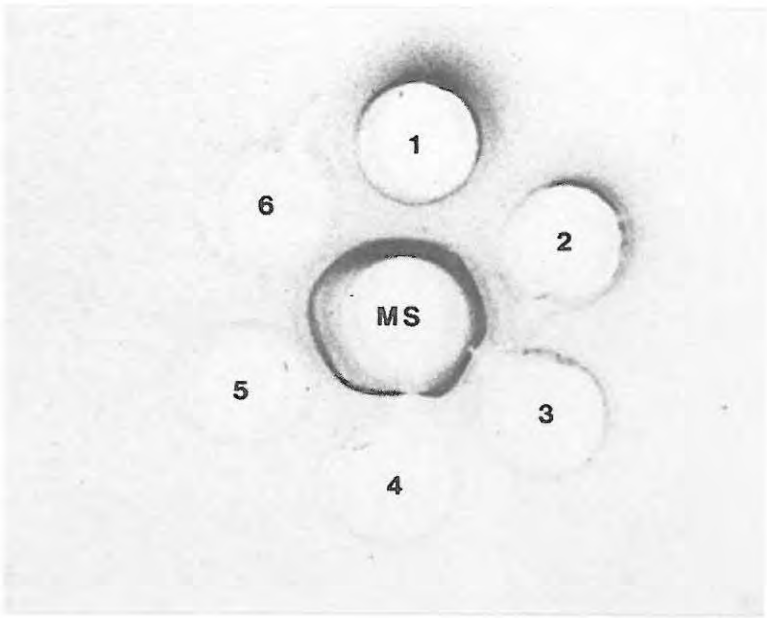

PLATE VIII

DOUBLE-DIFFUSION TEST.
MOLE SNAKE ANTISERUM

Centre well : antiserum (1/4 dilution)
Radially : cobra antigen wells 1 to 6, 1/1 to 1/32 in 2-fold dilutions.

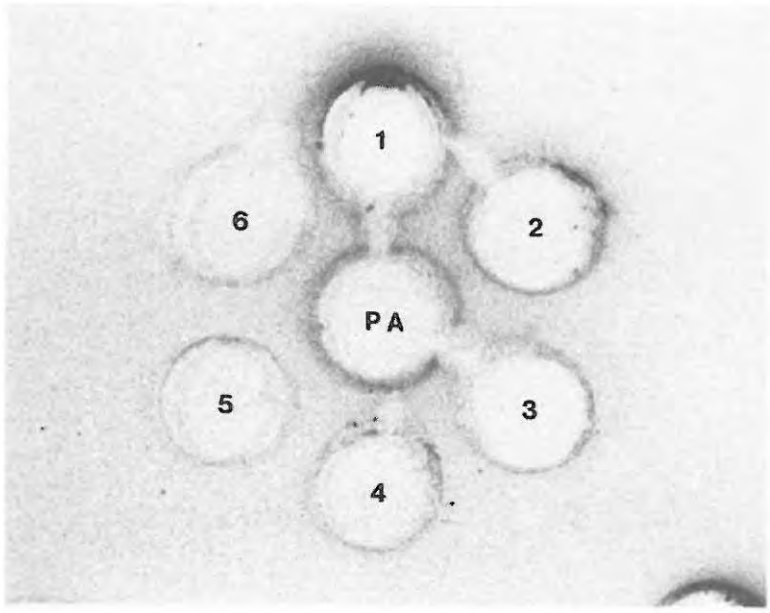

PLATE IX

DOUBLE-DIFFUSION TEST.
PUFF ADDER ANTISERUM

Centre well : antiserum (1/4 dilution)
Radially : mole snake antigen wells 1 to 6,
1/1 to 1/32 in 2-fold dilutions.

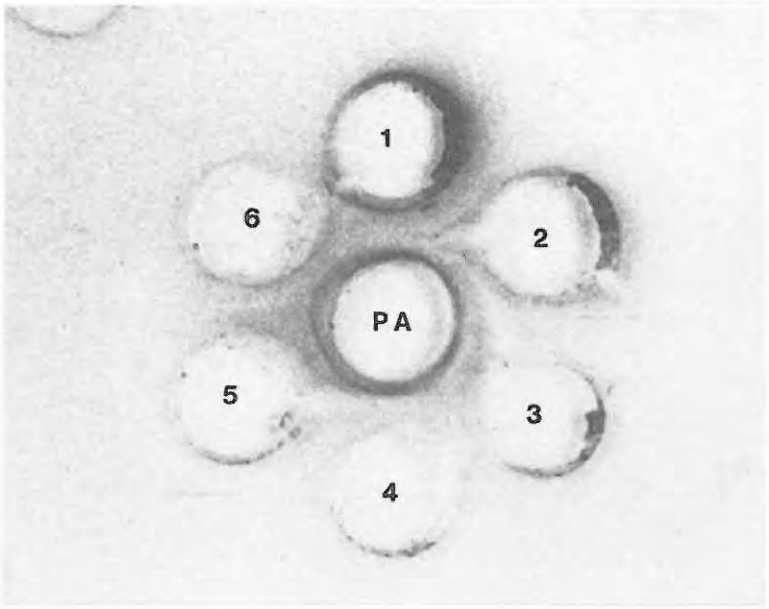

PLATE X

DOUBLE-DIFFUSION TEST.
PUFF ADDER ANTISERUM

Centre well : antiserum (1/4 dilution)
Radially : cobra antigen wells 1 to 6,
1/1 to 1/32 in 2-fold dilutions.

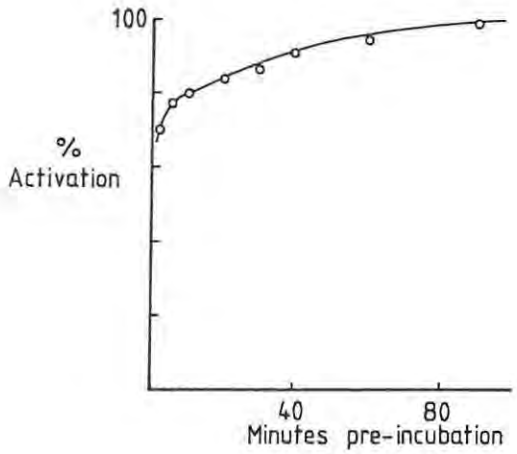

FIGURE 33

Mole snake pepsinogen P III activated at pH 2,0, 0°C, for indicated times to 90 minutes. Assay, against haemoglobin, 5 minutes at 37°C.

1 mg/mℓ and used in the dilution range 1/1 to 1/32.

5.5. Carbohydrate assays

Calibration curves

Calibration curves for ribose and for glucose are presented in Figures 11 and 12 (page 64). TABLE XIII gives data on A_{480} and A_{490} absorbances for phenol-sulphuric acid carbohydrate assays of the zymogens examined, and for BSA.

Hodge et al (1962) note that hexoses, including methylated hexoses, should be read at 490 nm. Pentoses, uronic acids and methylated derivatives are read at 480 nm.

The data for snake pepsinogens summarised in TABLE XIII indicate that one or more components of the PII zymogens are glycoproteins, and that the total carbohydrate on the cobra, puff adder and pig PII zymogens is about 0,2%, while that in the mole snake PII group is about 0,4%. The ratio of absorbances at 490 and 480 nm suggests that the sugars are at least predominantly pentoses or pentose derivatives.

The Bovine serum albumin used in this study (see Materials) also yielded a value of c 0,2% carbohydrate. See Discussion (Section 5,8,4,).

5.6. Tryptic Peptide Mapping

"Fingerprinting" of enzyme hydrolysates of purified proteins offers

a means of characterising, and hence of comparing them (Brust and Fasold, 1981; Elder et al, 1977; Kew et al, 1980). As the systems for such analyses have been developed to incorporate the high-resolution technique of SDS-PAGE, and the exquisitely sensitive process of detection by means of radio labelling, they provide a useful means of investigating such small quantities of proteins as are studied here.

Plates XI and XII show autoradiographs of cobra PII and puff adder PII pepsinogens. Figures 34 and 35 (page 140)are 2-fold reduced contact tracings of the original autoradiographs. Similarly, Figures 36 to 41 (pages 140- 141) show tracings of the other radiographs. The trace in Figure 42 (page 142) is that of a control autoradiograph, prepared as described in the Methods section.

Cobra PII and puff adder PII pepsinogen maps reveal greater similarities than do any of the other radiographs.

The labelled peptides from the various proteins may be compared in terms both of their number and of their two-dimensional distribution.

The PI zymogens differ from the PII group in all three species. This difference is reflected in a smaller number of fragments in the PI zymogens than in the PII group (most striking in the cases of mole snake and puff adder), and also in the distribution of such fragments. TABLE XIV lists the approximate numbers of labelled fragments on the autoradiographs of the various zymogens. These numbers are necessarily estimates, for three reasons. Firstly, the unsatisfactorily high "background" (see Discussion) in many areas may obscure individual

spots. Secondly, some of the spots, most of which are asymmetric, may represent two or more partly overlapping fragments. Thirdly, the control autoradiograph indicates that foci of radiation exist on the plates independently of the labelled protein fragments. In a note added to their paper in proof, Elder et al, (1977) ascribe this to diffusible contaminants in the acrylamide which are radio-iodinated in the course of protein labelling, and "can complicate mapping of minor protein components".

The counting of faint spots on the experimental plates therefore introduces the possibility of including artefactual material. Ideally, each experimental plate should be accompanied by a control, produced with protein-free polyacrylamide from the experimental slab gel, and exposed for the same time to a photographic plate.

Another circumstance reflected in TABLE XIV is the larger number of fragments, overall, derived from the mole snake zymogens than from those of the cobra and puff adder.

TABLE XIII

PHENOL-SULPHURIC ACID CARBOHYDRATE ASSAYS

The absorbances listed were obtained as described in the Methods Section. The carbohydrate content (see text, page 135) is derived from the calibration curves in Figures 11 and 12, page 64.

ZYMOGEN		A_{490}	MEAN	A_{480}	MEAN	$A_{490}:A_{480}$	MEAN
Cobra PII	1	0,045	0,045	0,045	0,045	1,0	1,0
	2	0,045		0,045		1,0	
	3	0,045		0,045		1.0	
Mole snake PII	1	0,085	0,086	0,087	0,089	0,977	0,963
	2	0,086		0,091		0,945	
	3	0.088		0.091		0,967	
Puff adder PII	1	0,026	0,031	0,030	0,035	0,866	0,902
	2	0,033		0,035		0,942	
	3	0,036		0,040		0,900	
Pig	1	0,030	0,035	0,033	0,038	0,909	0,028
	2	0,039		0,041		0,951	
	3	0,037		0,040		0,925	
BSA	1	0,030	0,031	0,029	0,030	1,034	1,032
	2	0,033		0,032		1,031	

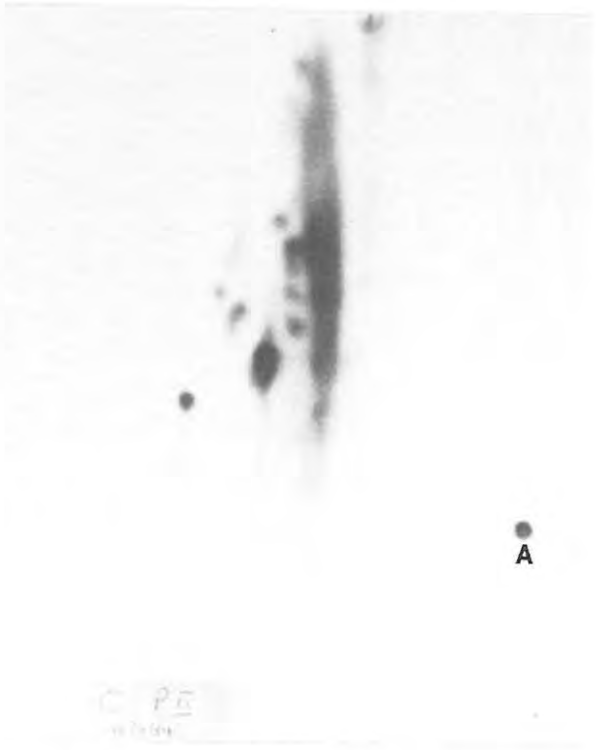

PLATE XI

TRYPTIC PEPTIDE MAP :
COBRA PII PEPSINOGEN

"A" : Point of application of hydrolysate. See text, also Figure 34.

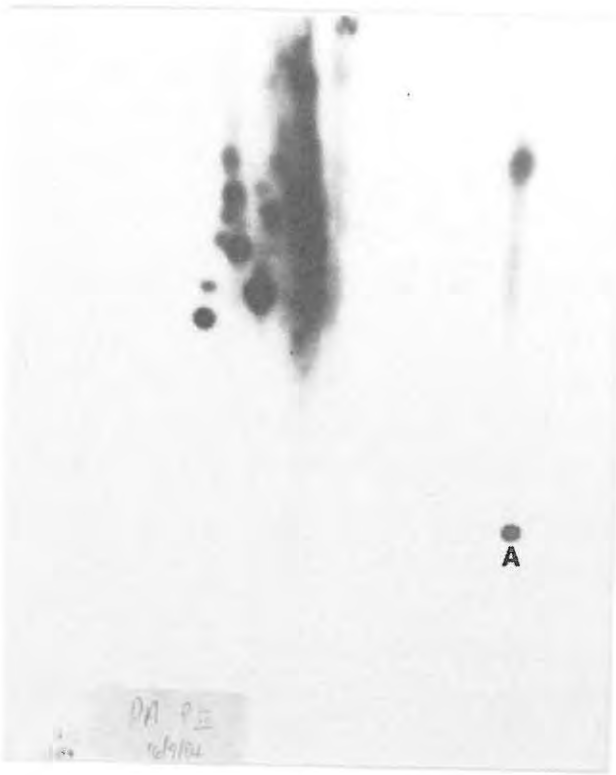

PLATE XII

TRYPTIC PEPTIDE MAP :
PUFF ADDER PII PEPSINOGEN

"A" : Point of application of hydrolysate. See text, also Figure 35.

FIGURES 34 TO 42

Figures 34 to 41 are tracings, c 2,3-fold reduced, of autoradiographs of trypsin hydrolysates of the zymogens, as indicated below each Figure. Figure 42 is a control. Electrophoresis was carried out along the x axis, with polarity as shown; ascending chromatography along the y axis. Co-ordinates are centimetres. 'A' on each Figure is the point of application of the hydrolysate.

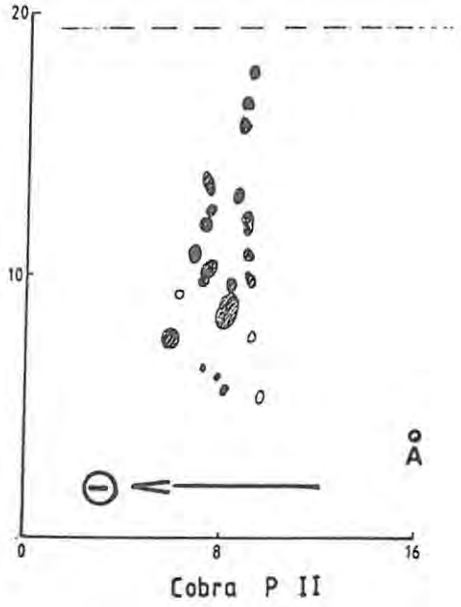

FIGURE 34

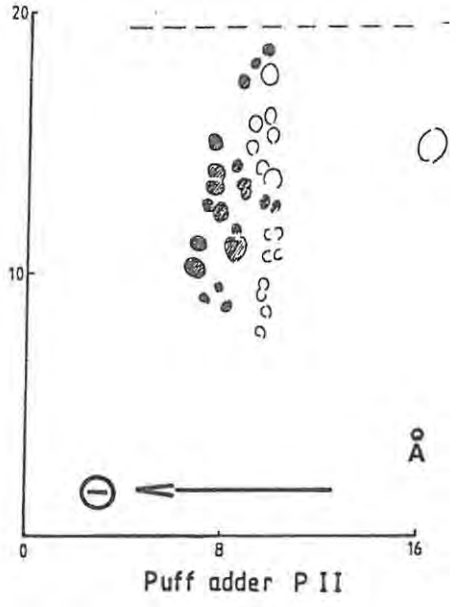

FIGURE 35

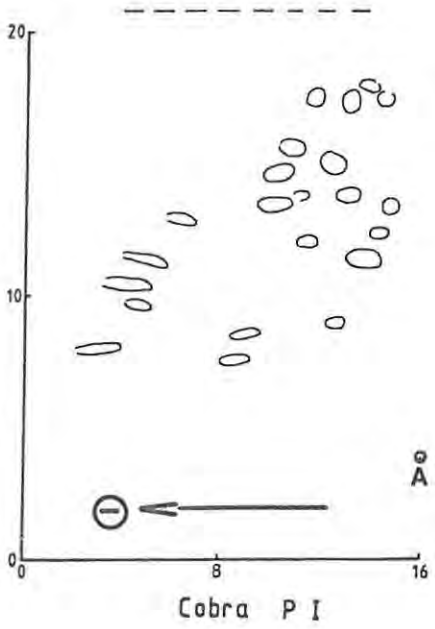

FIGURE 36

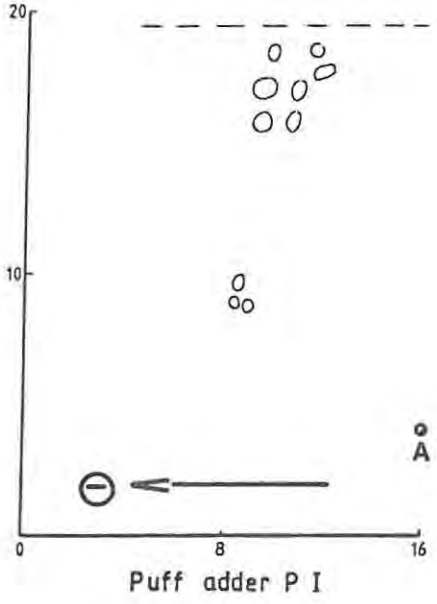

FIGURE 37

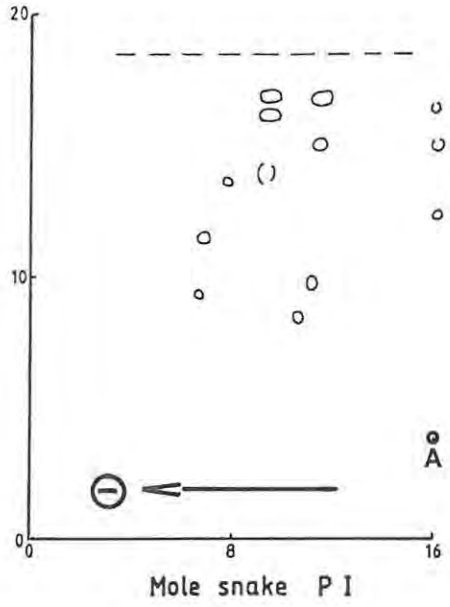

FIGURE 38

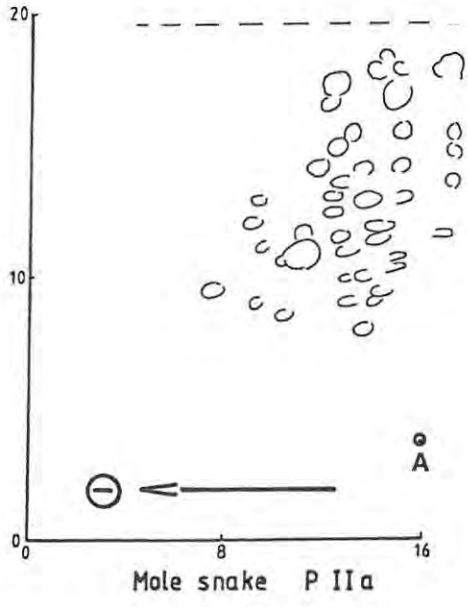

FIGURE 39

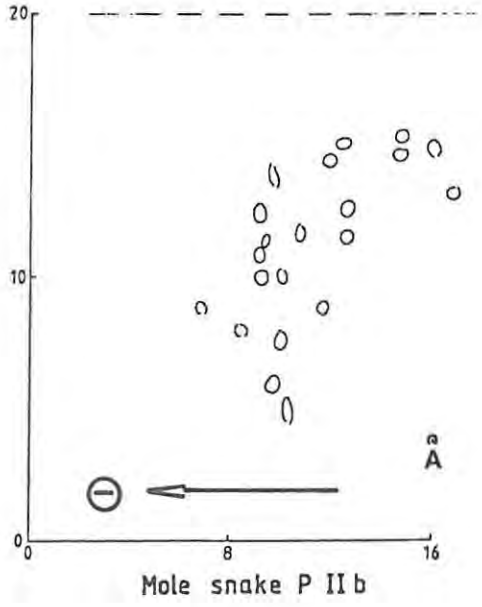

FIGURE 40

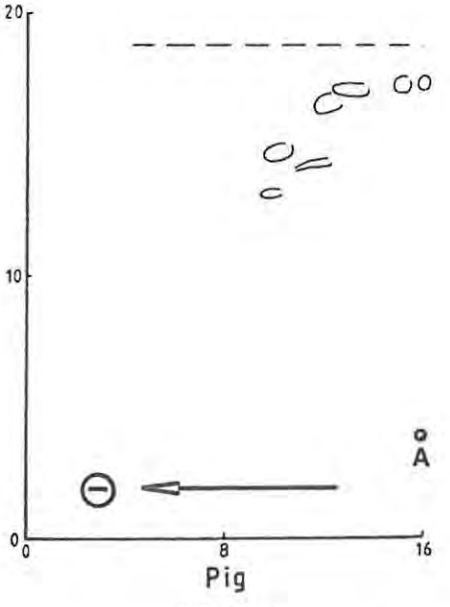

FIGURE 41

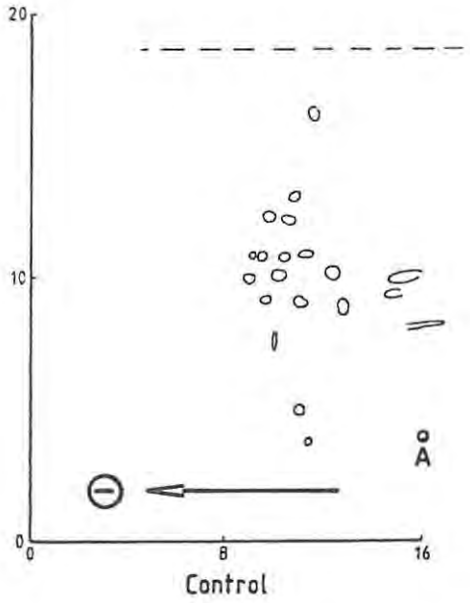

FIGURE 42

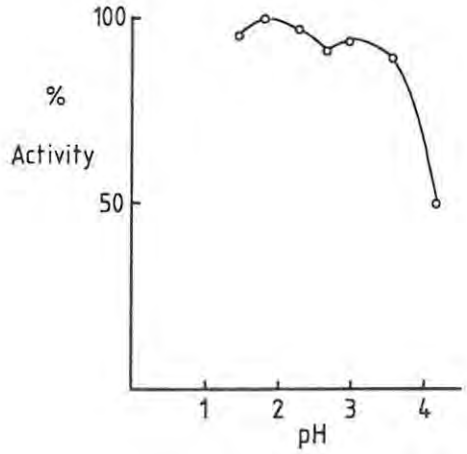

FIGURE 43

Cobra crude gastric mucosal extract: pH optimum profile. Substrate: haemoglobin. Incubation time: 5 minutes; temperature: 37°C.

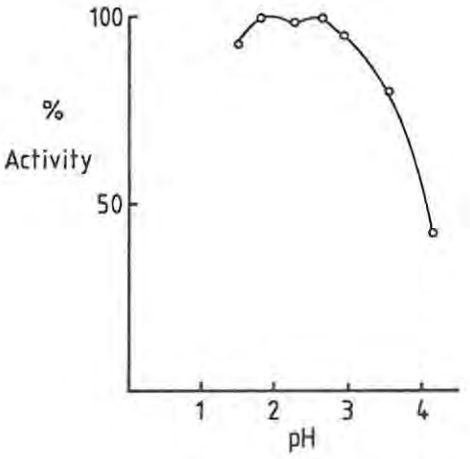

FIGURE 44

Mole snake crude gastric mucosal extract: pH optimum profile. Details as in Figure 43.

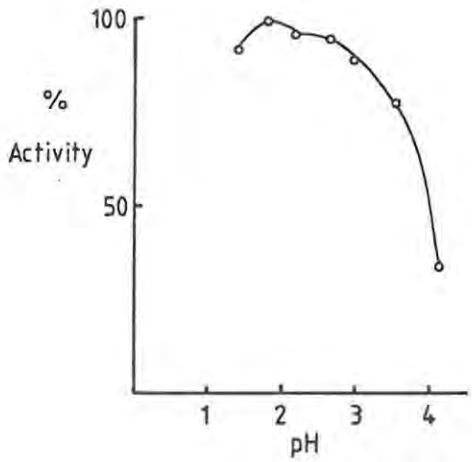

FIGURE 45

Puff adder crude gastric mucosal extract: pH optimum profile. Details as in Figure 43.

TABLE XIV

APPROXIMATE NUMBER OF IODINATED FRAGMENTS ON TRYPTIC PEPTIDE MAPS

MATERIAL		APPROXIMATE NUMBER OF FRAGMENTS
Pig		7
Puff adder	PI	10
	PII	36
Cobra	PI	22
	PII	24
Mole snake	PI	13
	PIIa	43
	PIIb	21
Control		20

5.7. Activation of the zymogens

5.7.1. The PII pepsinogens (cobra and puff adder), and mole snake PIII pepsinogens

Activation was achieved as described earlier, and the resulting products were passed through a column of Sephadex G50 at pH 5,2.

a. Cobra

Plate XIII shows the pepsinogens, and activation up to 40 minutes. The (unactivated) control zymogen (lane 6) reveals two components. As for the mole snake (Chapter IV, p.106) these are here designated PIIa (smaller, faster-migrating molecular species), and PIIb. These two components are also visible in the incompletely activated material in Lane 7.

Three pepsin moieties arise in the course of activation. This suggests either that one of the precursors is in fact heterogeneous, or that, under the conditions of activation, one zymogen moiety gives rise to two pepsins.

Note that activation is not quite complete at 40 minutes, as indicated by traces of remaining zymogens. The presence of pepstatin (Plate XIII, lane 7) does not alter the development of pepsins. This suggests that the activation process is essentially unimolecular.

The unactivated material (Lane 6) includes a band corresponding with one of the pepsins which appears on subsequent activation. This was

presumed to be pepsin present in the original PII purified fraction. Since the original extraction, and all subsequent procedures prior to formal activation, were carried out at pH 7,8 or in freshly boiled distilled water or 5mM NH_4HCO_3, it was assumed that this pepsin was denatured, and hence did not contribute to the activation process. To test the assumption that no active pepsin was adventitiously present in the zymogen solutions prior to controlled activation, a sample of salt-free cobra PII zymogen, previously dialysed against boiled distilled water, was divided into two equal volumes. To one was added an equal volume of TRIS buffer, pH 8,0, 0,05 M. To the other was added the same volume of HCl/KCl buffer, pH 1,8. After ten minutes incubation at 37°C, three volumes of HCl/KCl were added to each solution. Both samples were conventionally assayed for peptic activity. Assays were performed in duplicate, for 5 and 10 minutes. No difference existed between the activities of the two samples. Formal studies on alkaline stability indicated that, at pH 7,5, 50% of the activity of cobra PII pepsin is irreversibly lost after 10 minutes.

It was concluded that the small amount of protein identified as pepsin in the unactivated sample(s) was enzyme present in the (extracellular) mucosal tissue at the time of extraction, and promptly denatured by the ambient pH. If this interpretation is correct, it is interesting to note that only one pepsin species appears to be present in the unactivated material, rather than the three which arise in the controlled activation system used in this study.

(During the course of preparatory assays for the experiment referred

to above, it was found that the zymogen itself is unstable at pH 10. After 6 hours at ambient temperature, the sample at pH 10 showed 80% of the activity of a control sample held at pH 1,8.)

b. Mole snake

Plate XIV shows unactivated pepsinogens, PIII, and the appearance of activation products to 90 minutes. As noted earlier (Chapter IV, 4.4.2. and Plate II), PIII material is comprised of a and b zymogens. Three pepsins are produced. These three are more clearly seen in Plate XVI, lanes 3 and 4, respectively before and after passage through the G50 Sephadex column.

As with the cobra, the apparent development of three pepsins from two precursor zymogens suggests a concealed heterogeneity in the latter, or the production of two enzymatic moieties from a single zymogen.

c. Puff adder

Plate XV shows unactivated PII zymogens, and the appearance of pepsins up to 100 minutes. Again the unactivated material is seen to be comprised of a and b components, but here the larger, slower-migrating molecular species is in greater quantity than the smaller enzyme molecule.

Comparison of Lanes 2 and 3 indicates that zymogen PIIa has lost a part of the molecule during the first five minutes of activation. No change is apparent in PIIb during this period. At 20 minutes (Lane 5), the 5-minute intermediate PIIa has largely disappeared, and the substantive enzyme species are present. At 100 minutes only very faint traces remain of the PIIa and b zymogens. There are at least two, probably three and possibly even four enzymes. (See TABLE XV) Plate XVI (Lanes 1 and 2) shows another sample of puff adder pepsins derived from the PII zymogens. These pepsins are from samples of activated material before (lane 1) and after (Lane 2) passage through a column of Sephadex G50. Two pepsin species are clearly apparent, and neither band gives evidence of heterogeneity. It appears probable that the migration distance in this gel was insufficient to show the greater heterogeneity indicated in Plate XV. (See Discussion.)

It is interesting to note (Plate XV) that activation of the a and b PII zymogens appears to proceed by different mechanisms. PIIb does not give evidence of passing through a 5 minute, clearly defined intermediate.

The final molecular masses of the pepsins described in a to c, above, are set out in TABLE XV, page 148.

5.7.2. The PI pepsinogens : all three species

Pepsins arising from the PI zymogens were subject to PAGE after

TABLE XV

MASSES OF PEPSINS DERIVED FROM COBRA AND PUFF ADDER PII ZYMOGENS, AND FROM MOLE SNAKE PIII ZYMOGENS*

SPECIES	MASS (daltons)	PLATE NO.
Cobra PII	31 000 33 500 35 000	XIII (p. 164)
Mole Snake PIII	31 000 33 000 34 000	XIV (p. 164)
Puff adder PII	31 200 (?) 32 800 33 200 (?) 35 000	XV (p. 165)

* Masses are as reported by Robertson (1984).

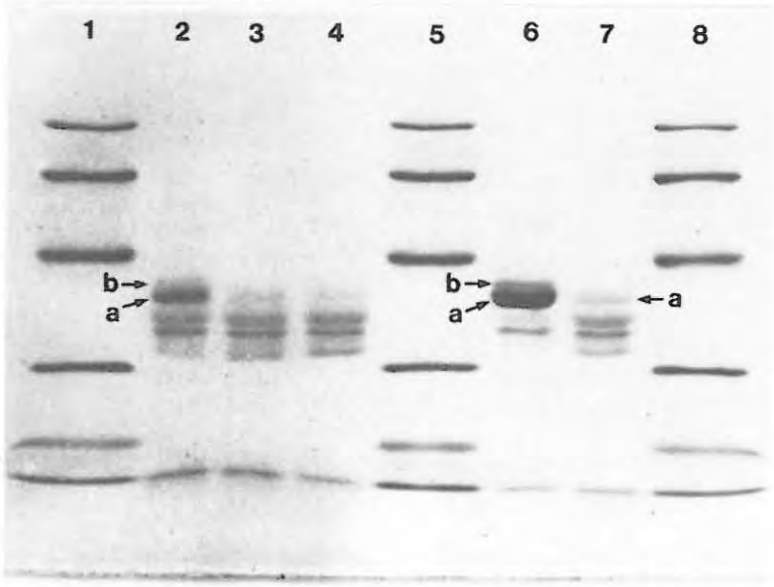

PLATE XIII

COBRA PII PEPSINOGENS : ACTIVATION

SDS-PAGE

Lanes 1, 5 and 8 : Markers.

2, 3 and 4 : 5, 20 and 40 minutes activation at pH 2.

6 : PII pepsinogen in TRIS, pH 8 (= control)

7 : Pepsin(ogen), 40 minutes at pH 2, with pepstatin, c 1 : 1 molar ratio.

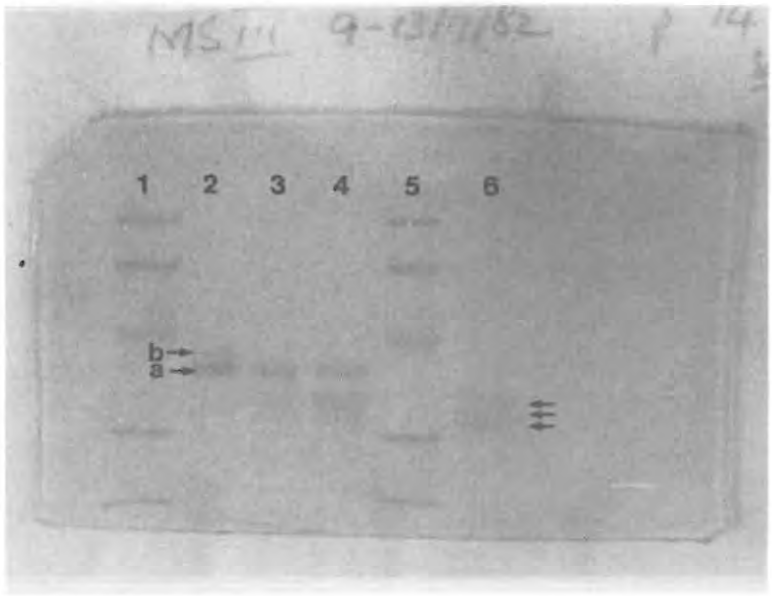

PLATE XIV

MOLE SNAKE PIII PEPSINOGENS : ACTIVATION

SDS-PAGE

Lanes 1 and 5 : Markers.

2 : PIII pepsinogen at pH 8.

3, 4 and 6 : 5, 20 and 90 minutes activation at pH 2

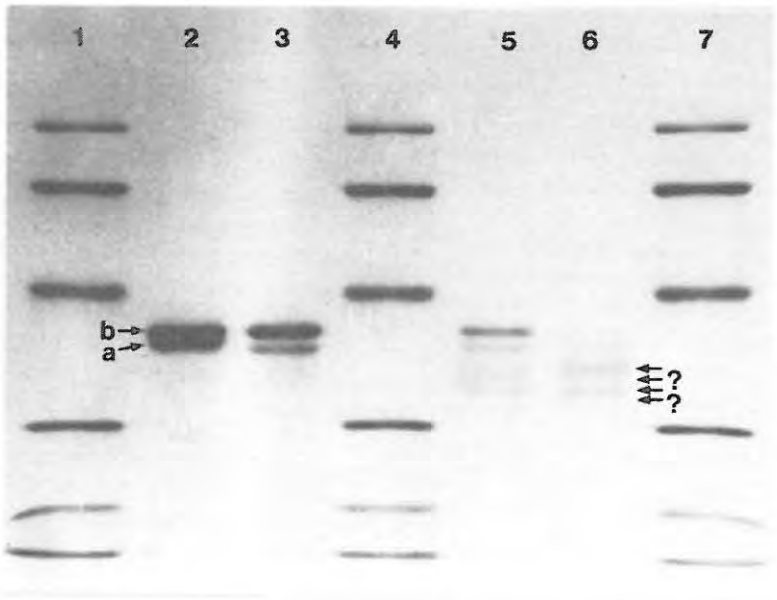

PLATE XV

PUFF ADDER PII

PEPSINOGENS : ACTIVATION

SDS-PAGE

Lanes 1, 4 and 7 : Markers.

2 : PII pepsinogen at pH 8.

3, 5 and 6 : 5, 20 and 100 minutes activation at pH 2.

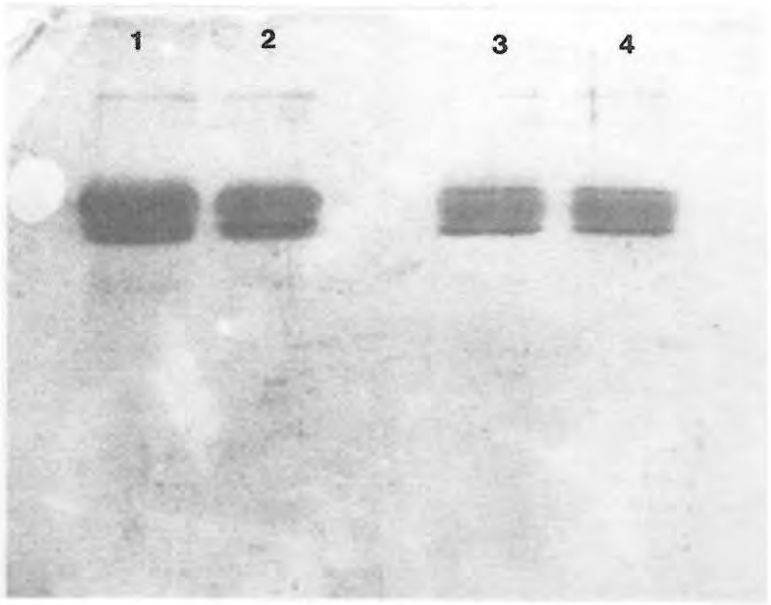

PLATE XVI

PUFF ADDER AND MOLE SNAKE PEPSINS.

SDS-PAGE

Puff adder PII (Lanes 1 and 2) and mole snake PIII (Lanes 3 and 4) pepsins on 15% polyacrylamide gel,

Lanes 1 and 3 : Before passage through Sephadex G50,

Lanes 2 and 4 : After passage through Sephadex G50.

separation on Sephadex G50. Results were generally equivocal and disappointing. Cobra PI gives no clear evidence of comprising more than one protein, but the possibility of heterogeneity cannot be excluded.

Mole snake PI and puff adder PI similarly may or may not comprise two (or indeed more) pepsins.

Of the relative molecular masses of Pepsins I and II it may be noted that in cobra and mole snake, Pepsin(s) I are apparently heavier than Pepsins II. This appears also to be true of puff adder Pepsin(s) I and II, but the difference here, if such difference exists at all, is smaller. These gels have not been used to derive molecular masses.

5.7.3. Enzyme Activity developed during Zymogen Activation

Formal investigation of activation is best carried out by the milk-clotting assay of McPhie (1977), or the alkaline inactivation method used by Al-Janabi *et al* (1972). To derive an operationally useful measure of activation time a simpler and admittedly less satisfactory method was used in the present work. The zymogen was exposed to a pH of 2,0 at 0°C for periods of up to 90 minutes, followed by a 5 minute assay against haemoglobin at 37°C. The data (Figure 33) show that, in these circumstances, observed activity of the mole snake PIII material increased to a maximum after 90 minutes.

The particular weakness of the procedure is that an undetermined but

substantial proportion of the activation occurs in the course of the assay at 37°C, and the time-activation profile is thus not a good reflection of activation occurring at 0°C. This carries the direct implication that activation at 0°C will lag behind what is measured in the assay system used here. The information provided by the method is nonetheless adequate to show minimum activation time required to avoid artificially low results in routine activity assays of newly-activated material. (Failure to make specific provision for full activation led to inconsistent results in activity assays during early stages of the work.)

There is a broad correspondence between the progress of activation (e.g. as in Figure 33) and the development of pepsin bands on PAGE (Plates XII and XV). It may be argued that development of full enzyme activity before the complete disappearance of those PAGE bands nominally ascribed to zymogen(s) would strongly suggest that one or more of the latter were wholly or partly contributed by contaminant protein. Notwithstanding the limitation of the present activation procedure, the data do not suggest such contamination. Plate XIII, lane 7, (40 minutes activation at 0°C) clearly shows traces of remaining material in the position of the zymogen (lane 6). If it is assumed that the rate of activation of cobra PII zymogen (Plate XIII) is similar to that shown by mole snake PIII in Figure 33, it may be accepted from the activation time indicated, that at 0°C an indeterminate but significant proportion of the zymogen remains to be activated. This material must exist in the faint band observable in the zymogen position in lane 7, and the amount of alien protein, if any, must surely be very small indeed. Comparable arguments may be applied to

the puff adder material (Plate XV) and the mole snake PIII material (Plate XIV), though far less clearly in the latter case.

Some further evidence that the zymogens do not contain major contaminants is perhaps provided by the disappearance of these bands pari passu with the development of pepsin bands on PAGE. This is best seen in Plate XIII, lane 7, (page 149) and Plate XV, lane 6, (page 150). The possibility of nascent enzyme immediately hydrolysing, and thus removing, a larger contaminant protein, which might then be erroneously identified, on PAGE, as an activating zymogen, appears remote. At pH 2 pepsinogen activates by a predominantly unimolecular (first order) reaction (see Review, section 2.3.3.c.). Activation is thus likely to be much faster at 0°C than bimolecular enzyme-substrate proteolysis at the same temperature.

A useful general means of identifying electrophoretically separated bands of protein with specific enzymes is by demonstrating enzymatic activity directly associated with such bands. These techniques involve use of the native protein. Ryle and Hamilton (1966) demonstrated activity of pepsin in starch gels.

Attempts made in the present study to recover potential peptic activity from polyacrylamide gel electrophoretograms of the (native) proteins were disappointing. Resolution of the native proteins was poorer than that routinely attainable with SDS preparations. (This was found also to be true of snake plasma proteins, and it appears, from reports, that SDS-treated preparations generally yield higher resolution (Newman, per. comm.; see also Weiner et al, 1972). Recovery of enzyme activity was also extremely low - c 18% of that applied in several trials. This

very low recovery was also found when gels were prepared with riboflavin instead of ammonium persulphate. (It was established that the acetone precipitates of the zymogens used for electrophoresis yielded good activity.)

Reasons for these poor results are by no means clear, more especially as Naude* (pers. comm.) reports successful recovery of potential peptic activity of ostrich pepsinogen from PAG-electrophoretograms of the native material.

---

*R.J. Naude, Department of Biochemistry, University of Port Elizabeth.

5.8. Discussion

5.8.1. Molecular masses of the pepsinogens : Chromatography gel elution volumes and PAGE data

The elution volumes (TABLE V) of PI and PII zymogens suggest that, in all three species, the former are smaller than the PII group. This is in *prima facie* conflict with most of the PAGE findings. (The exception is mole snake PIIb, which is heavier, on PAGE, than PI. (See TABLE VIII, and Plate II.)

Mass is not the only molecular parameter relevant to the performance of proteins during exclusion chromatography. The molecular radius influences K_{AV} - the fraction of the gel volume available to a given set of particles and hence affects V_e - the elution volume of the species (Laurent and Killander, 1964). If the permeating molecule is non-spherical, the effect on V_e is complex. (See Netter, 1969, for discussion.) The influence of molecular shape on elution volume has prompted use of the term "steric exclusion chromatography" (Ravenscroft *et al*, 1985).

These considerations may account for the discrepancy in the relative masses of PI and PII zymogens, as indicated by the two methods.

While the theoretical basis for understanding the migration rates of SDS-modified proteins in slab gels is not yet fully developed, it appears that molecular radius bears a different relationship to the molecular mass as determined in these systems, from that which obtains in the elution of essentially unmodified proteins from conventional

chromatography gels. Notwithstanding this, and as has been noted earlier, the mass of puff adder PII material, as determined by molecular- or steric-exclusion chromatography, agrees quite well with masses derived from PAGE.

5.8.2. Amino acid analyses

No general contrast appears to exist between the compositions of the three snake pepsinogens and those of several other species presented for comparison. (See Chapter Two : Review) The considerable preponderance of acidic over basic amino acids appears to be a general feature of gastric zymogens (Foltmann, op. cit.).

A high ratio of glutamic to aspartic acid has been noted as one of the characteristics of the Pepsinogen C group (= gastricsins, Foltmann, op. cit.; Chiang et al, 1967). In the cobra, mole snake and puff adder PII zymogens the Glu : Asp ratios are respectively 1 : 1,06 1 : 1,00; 1 : 1,05. On these grounds, therefore, the snake PII pepsinogens are not to be equated with the pepsinogen C group of other vertebrates.

The cobra material yielded two small unidentified peaks on the analyser; the puff adder three. These may represent amino sugars. This suggestion is consistent with the finding that cobra PII and puff adder PII pepsinogens contain carbohydrate. (See Section 5.5., Chapter 5). Galactosamine has been found in one of the chicken pepsinogens (Green and Llewellin, op. cit.).

The masses of the cobra and puff adder zymogens, derived from amino acid composition data as described, are 33 719 and 38 045 daltons respectively. These masses do not include the tryptophan component. TABLE I (page 20) indicates that the number of tryptophan residues in pepsinogens from a wide variety of species is between 4 and 6 per mole. If an arbitrary allowance is made for 5 such residues in the cobra and puff adder pepsinogens, the corrected mass values are 34 650 and 38 976 daltons. This mass for the puff adder zymogen correlates well with the mass derived from PAGE (38 000 daltons : TABLE VIII, page 121). This, with the closeness of calculated residues to whole numbers, would ordinarily suggest a high degree of homogeneity. In the present case, however, the puff adder PII zymogen sample is apparently not homogeneous, as is indicated by SDS-PAGE. (See TABLE VIII and Plate XV, lane 2, page 150). The composition data thus provide evidence that the components of the PII material are very similar to each other, and, by implication, are free of major contamination.

The mass of the cobra PII material (34 503 daltons with the estimated tryptophan component) agrees less well with the value (38 000 daltons) derived from SDS-PAGE. The raw composition data for cobra and puff adder zymogens were also processed by the National Chemical Research Laboratory with their own, more comprehensive, error minimization programme. The best-fit masses obtained were 34 930 (cobra) and 37 718 (Puff adder)*. Noting the difference

---

*
These values do not include any estimated tryptophan content. The values, adjusted for 5 such residues each, are 35 861 and 38 649 daltons respectively.

between the composition-derived mass for the cobra and the SDS-PAGE value, Haylett (pers. comm.) remarks that a statistical treatment of the composition data indicates the presence of two proteins which are closely related.

Hence it may be concluded, for cobra and puff adder PII material, that both are essentially free of contaminant proteins, and that both are comprised of more than one pepsinogen moiety.

The mole snake PII and PIII data (TABLE XII, page 128), provided and analysed by the National Chemical Research Laboratory, are somewhat problematic in the light of the SDS-PAGE results. The molecular masses, from composition, are calculated to be 47 694 and 44 692 daltons respectively*. In both cases SDS-PAGE reveals the zymogens to be composed of a and b moieties with masses of 39 000 and 43 000 daltons (Plate II, page 106 , and TABLE VIII, page 121). There are some grounds, discussed later, for believing the MSII and III zymogens to be the same. The NCRL analyses do not support such a belief, but if the masses of MS PII and PIII differ by some 3 000 daltons, as indicated by their composition, it is surprising that this is not reflected in the SDS-PAGE data. As noted, the PII and PIII mole snake pepsinogens comigrate. The PAGE results in general suggest strongly that proteins so distinct in mass would show clear differences in migration.

It may be suggested that small amounts of contaminant(s), present

---

*Unadjusted for tryptophan.

in both, but differing in quantity and/or kind between the PII and PIII samples, could give rise to the observed variations in amino acid content between them, and hence to the computed difference is mass. In the same context it seems probable that if either component of the doublet - i.e. a or b in PII or PIII material - were an entirely unrelated protein, this would introduce major differences in the composition analyses, and in the masses derived from an error minimization programme. The mole snake PII and PIII compositon data thus lend some support to the belief (argued elsewhere) that both a and b components are pepsinogens and are free of major impurities.

5.8.3. Serology

The purpose of the tests is to investigate relatedness between the zymogens studied. Antigenic similarity has been shown to correlate well with sequence homology in globular proteins (White et al, 1978)

Ryle (pers. comm.) notes that pig pepsinogen and pepsin are very poor immunogens. This is consistent with the failure of pig pepsinogen in the present study to stimulate production of detectable antibody in the test rabbit, and may to some extent reflect the general antigenic similarity between mammalian pepsinogens. In the same context the positive antigenic response of rabbits to all three snake pepsinogens may be indicative of the greater phyletic separation.

It is interesting that the reaction between puff adder antipepsinogen

and cobra pepsinogen is, as noted, stronger than that between puff adder antipepsinogen and mole snake pepsinogen (Plates IX and X, page 133). Plate V, page 131) provides some small indication that here too the cobra - puff adder reaction is stronger than the cobra - mole snake reaction.

The general inference to be drawn from these data has been noted.

5.8.4. Carbohydrate content

Assay of the carbohydrate content of pepsinogens and pepsins is not always undertaken as part of routine studies, and generalisations are therefore difficult.

Green and Llewellin (1973) reported the presence of neutral sugar in chicken pepsin. Of eight pepsinogens in Crab-eating Monkey, two (Pepsinogens Ia and Ib) contain c 4,5% carbohydrate, which is assumed on indirect evidence to be attached to the pepsin moieties of the zymogens (Kageyama and Takahashi, 1980).

One of five pepsinogens from Asiatic Black Bear contains 3,7% carbohydrate; the others contain none (Kageyama et al, 1983b).

Neumann et al (1969) found pig pepsinogen to contain c 1,2% neutral sugar, the major component of which was D glucose. The pepsin derived from this source contained no detectable carbohydrate.

Neumann and co-workers (op. cit.) note that the sugar is bound to the zymogen by an acid labile bond, but that the sugar release appears to

precede release of activation peptides from the activating zymogen. They conclude, tentatively, that the sugar in pepsinogen stabilises the pro-enzyme in a conformation in which the active site is protected. If this is so, it raises the question of the conformation or stability of those pepsinogens from which carbohydrate is apparently absent.

It will be interesting to assay the PI pepsinogens for carbohydrate. This was not done in the present work because the available material was used for assays considered more instructive. (See Chapter 6.) Positive results of such determinations would in any case be equivocal due to the incomplete purity of the PI zymogens.

Some comment is required on the finding, in the present study, that BSA contains $\underline{c}$ 0,2% carbohydrate. Serum albumin " ... consists of a carbohydrate-free polypeptide chain ... "*. The material used for the assay, however, (Sigma, Cat. no. A4378) may contain 1 - 3% globulins (Sigma Catalogue). Several of the (bovine) globulins contain significant amounts of carbohydrate : IgA, 3%; IgM, 14%, etc.**

---

*Merck Index, 9th ed., 1976. Editor M. Windholz. Merck and Co. Inc., Rahway, New Jersey.

**Handbook of Biochemistry, 2nd ed., 1970. Editor H.A. Sober. Chemical Rubber Company, Cleveland, Ohio.

5.8.5. Tryptic peptide maps

It is difficult to draw even tentative conclusions from the distribution of labelled fragments. The plates best lending themselves to comparison are those of the puff adder PII and cobra PII zymogens. Spots suggesting some broad correspondence of relative position are shaded in the contact tracings. If the two autoradiographs are superimposed and moved to a "best fit" of spot patterns the small similarity is somewhat enhanced, suggesting that overall migration rates, on both axes, of homologous, or nearly homologous, peptides may have differed on the two plates - further reducing a correspondence at best tenuous.

The maximum number of labelled spots depends, theoretically, on the number of trypsin-sensitive peptide bonds. This limiting number may be reduced according to the number of residues susceptible to iodination, and their distribution among the potential hydrolysis products.

According to Schroeder (op. cit.), trypsin limits its hydrolytic action almost exclusively to bonds associated with the carboxyl groups of lysine and arginine. The rate of cleavage of Lys - Pro and Arg - Pro bonds is reported to be zero, while cleavage of Arg - Lys and Asp - Arg proceeds very slowly. Elder et al (op. cit.)quote Krohn et al (not seen by the present writer) as stating that the chloramine T method of iodination iodinates histidine and phenylalanine residues as well as tyrosine. The zymogens here studied are rich in these three residues, hence high levels of iodination might be expected.

As noted, however, the number of spots is limited by the number of cleavages. Where there are x potential sites of hydrolysis, and assuming that enzymatic hydrolysis proceeds to completion (see below), x + 1 fragments may be produced. In view of the bond specificities noted above for trypsin, and disregarding the possibility of Lys - Pro and Arg - Pro couplets, the maximum number of fragments which may be generated from the three PII zymogens appears, at first sight, to be 17 (cobra); 20 (mole snake); and 14 (puff adder).(See TABLE XIV, page 143.)

Even allowing for significant error in the experimentally determined numbers of residues, the number of spots on all three of the PII autoradiographs is considerably in excess of these figures.

The answer to this superficially anomalous finding perhaps lies in the heterogeneity of the PII zymogens. The composition data are derived, as is revealed by the PAGE studies, from a mixture of two or three proteins in each species. The individual polypeptides (pro-enzymes) inevitably differ in the distribution of their susceptible bonds, hence the resulting fragments will possess different mobilities.

If the provisional assumption is made that the lysine and arginine content of the PI zymogens is similar to that found in the PII groups, the foregoing argument explains the greatly reduced numbers of spots in the PI maps, since the PI zymogens reveal little or no heterogeneity on PAGE.

Another possibility, as pointed out by D.A. Hendry (pers. comm), is

that tryptic hydrolysis did not proceed to completion in the gel extracts, hence producing a large number of fragments of differing migration characteristics. If this is the cause, or a contributory cause, of the large number of spots on the PII autoradiographs, the very much smaller number on the PI maps (trypsin-digested under nominally identical conditions), provisionally suggests substantially different lysine and arginine contents in the PI zymogens.

While comparison of the autoradiographs is difficult, a possible vague similarity between the puff adder PII and cobra PII zymogens has been noted. It is less easy to find correspondences of pattern between mole snake PIIa and/or PIIb and either puff adder or cobra PII autoradiographs. However equivocal these findings, they are broadly consistent with the data - also tentative - derived from serology.

The composition data indicate a larger number of arginine and lysine residues in the mole snake PII (a and b) zymogens than in cobra or puff adder PII material. This difference may be sufficient to account for the larger number of labelled peptides obtained from the mole snake PIIa map. This map shows a relatively large number of spots (43 - TABLE XIV), yet the protein is not obviously heterogeneous. In addition to the possibility of incomplete tryptic hydrolysis, mentioned above, the greater number may be a reflection of cryptic homogeneity within the PIIa fraction. This possibility was noted in respect of mole snake PIII, since the PIIIa and b complex yields three pepsins on activation. In view of the fact, as noted, that mole snake PII and PIII zymogens comigrate on PAGE, it is possible that they are identical (see General Discussion).

Autoradiographs of the PI zymogens unfortunately contribute nothing to the question of relatedness.

Apart from the question of reproducibility (see below) it may be that the tryptic peptide maps are too narrowly specific to be useful in comparing proteins beyond a certain structural/phyletic separation. In a sense, such analyses are "three-dimensional" - depending upon susceptibility to tryptic cleavage, electrophoretic mobilities and chromatographic solvent partition coefficients. To these may be added a fourth variable: susceptibility to iodination of the SDS-treated protein.

Kew et al (op. cit.) working on poliovirus coat proteins, found that small numbers (8 - 13) of amino acid substitutions between two proteins produced a 7% change in electrophoretic mobility. In attempting to account for what they see as a large mobility change for relatively small structural differences in the proteins, they refer to a report by de Jong et al (1978 : not seen by the present writer), showing that the substitution of a single hydrophilic residue for a hydrophobic one can increase the electrophoretic mobility of a 20 000 dalton protein by 3%.

In the matter of iodination, Elder et al (op. cit.) note that the conformation of a protein can affect the extent of labelling of certain peptides. They add that "no doubt this result would vary from protein to protein". It is relevant to remark here that a further question concerns the marked differences in exposure times required amongst the labelled and hydrolysed protein samples, to produce autoradiographs of comparable density.

Before questions of this sort can be resolved, the reproducibility of the technique should be examined in detail. In the present work, mole snake PI material was labelled, digested and mapped in two independent experiments. The results of the two bore some general similarities, but were not decisive enough to provide good evidence of repeatability. Tryptic peptide maps of certain viral proteins, prepared as parts of graduate research projects in the Department of Microbiology, Rhodes University, suggest that results are generally repeatable. The technique suffers the drawback that assessment of degrees of difference between autoradiographs is essentially subjective.

Another cause for concern is the status of control autoradiographs. In the present study, as noted, the control plate was prepared with pooled protein-free gel slices from the slab gels used for mole snake PI and cobra PI maps. The exposure time required to yield faint artefactual spots (240 hours) was substantially longer than that used for exposing the experimental plates, with the exception of mole snake PIIb (also 240 hours). Of the other plates, the longest exposure times were 120 hours (mole snake PIIa and cobra PII). Both these plates yielded spots considerably darker than those produced on the Control after 240 hours. This suggests that artefactual spots are unlikely to contribute significantly to the experimental plates, barring that of mole snake PIIb.

In the light of comments by Elder *et al* (*op. cit.*), the 240-hour experimental mole snake PIIb plate may be seen as an anomaly. Since all gel slabs used for the mapping studies were prepared from a single

stock of acrylamide, it was assumed, following Elder, that one control plate would suffice. The spots developed on the Control plate should therefore have been common to all the experimental plates, albeit, in most cases, at very much lower densities due to the generally shorter exposure times. The mole snake PIIb plate, however, should exhibit a "blank" pattern of spots similar to those of the control, and in addition to those of the labelled polypeptides. In fact there is no discernable pattern on this plate which corresponds with the control result, and only three spots show any equivalence of position.

An undoubted weakness in the present method is that, beyond general standardisation of the iodination technique, the activity applied to the chromatography plates was not standardised. This refinement would greatly facilitate the preparation of photographic plates, and would perhaps lead to easier interpretation of the results.

Ryle (pers. comm.) has suggested that the problem of artefactual spots arising from gel constituents would be avoided by iodinating the protein before electrophoresing it. If such spots do indeed arise from gel constituents, this would surely be so.

After the present work had been completed, however, C. Eve* investigated the provenance of spots on control autoradiographs. She examined polyacrylamide from seven commercial sources and concluded that this is not the source of such spots. Her studies led to the tentative conclusion that autodigestion of

---

*Unpublished Honours project, 1985, Department of Microbiology, Rhodes University.

adventitiously-labelled trypsin may be responsible. It appears that Ryle's suggestion, noted above, would also serve to reduce or eliminate artefactual spots arising in this manner. Another finding of Eve's work was that the use of sodium iodide as a quenching agent after radioiodination of experimental samples results in far less "background" radioactivity on the plates, and hence a lower level of fogging of the autoradiograph negatives.

5.8.6. Mechanism(s) of activation

A point of interest arising from the results, noted en passant in Section 5.7.1. of this chapter, is the activation sequence of puff adder PII zymogens. The 5-minute activation (Plate XV , lane 3) shows the existence of an intermediate formed, apparently, from the smaller of the zymogens (PIIa, as here designated). This observation is qualitatively consistent with the release, as the first stage in (intramolecular) activation of pig pepsinogen, of a short peptide. The displacement of this intermediate PIIa relative to its position in the unactivated state (lane 2) - about 1 millimetre - corresponds with a reduction in mass of, at most, about 1 000 daltons, and hence suggests the loss of a smaller fragment than the 16 residues removed from pig pepsinogen, or of the 17 residues removed from bovine pepsinogen (Harboe et al, op. cit.). It is further interesting that the heavier, slower-moving pepsinogen (PIIb) does not appear to convert to an equivalent intermediate moiety, but instead disappears progressively from the electrophoretogram (Lanes 5 and 6 : activation times 20 and 100 minutes respectively) as the substantive enzymes appear. This effect is not apparent in comparable electrophoretograms of activating zymogens in the other two species. Kageyama et al (1983b) used SDS-PAGE to analyse the activation of Black bear pepsinogen II-1, and report that "no intermediate form between pepsinogen and pepsin was observed".

As noted in the Review (Chapter Two, 2.3.3.c.), Kageyama and Takahashi (1983a) have shown that activation of pig pepsinogen at pH 2 proceeds simultaneously by two pathways. In the case of the snake materials,

it appears that the puff adder PII a and b pepsinogens activate by different pathways, which are presumably comparable with the two pathways noted for (reportedly homogeneous) pig pepsinogen. The intermediate "pseudopepsin" was not observed in the activation sequences of the cobra and mole snake.

5.8.7. Separation of activation peptides from pepsins

Ion exchange methods, either alone or in conjunction with molecular exclusion chromatography, have routinely been used to separate activation peptides from each other, and from the catalytic moieties. (See, for example, Kegeyama and Takahashi, 1976b; Kageyama et al, 1983b)

In order to effect only a separation of enzyme(s) from the activation peptides, molecular exclusion chromatography alone may suffice. This procedure was adopted here to avoid the rather large losses of material often attendant on ion exchange techniques. Rajagopalan et al (1966b),quoting work by Herriott (1938 : not seen by the writer), note that "these cleavage products of pepsinogen activation , which are basic, form complexes with pepsin at low pH, but ... these complexes tend to dissociate between pH 4 and pH 5". Rajagopalan et al (op. cit.) found that passage through a Sephadex G25 (30 cm) column at pH 4,4 "did not effect complete separation, as could be shown by amino acid analysis. The protein component often had slightly more than the theoretical quantity of 1 lysine residue per molecule".

Bohak (1969) used an 83 cm column of Sephadex G100 (equilibrated with

0,001 M HCl) to separate activated chicken pepsinogen from its activation peptides.

In the present work, to test whether any indication could be obtained, on PAGE, of activation moieties and their removal after passage through a G50 column, puff adder and mole snake activated material was subjected to disc electrophoresis on a 15% acrylamide gel before and after chromatography. (Plate XVI) The results unfortunately do not show activation peptides, and hence give no indication of the extent of their removal. Laemmli (op. cit.) found that small peptides were not retained in electrophoresis gels.

Elution profiles of the G50 column eluates indicated that material of low molecular mass was removed from the main (symmetrical) peak. Increase of specific activity was ordinarily c 1,2-fold for the PII (and mole snake PIII) materials, and rather more for the PI enzymes, suggesting substantial removal of activation peptides.

The activated enzymes, essentially free of activation moieties and other impurities, were used for kinetic studies.

CHAPTER SIX

KINETIC STUDIES ON THE PEPSINS

CHAPTER SIX

KINETIC STUDIES ON THE PEPSINS

6.1. Introduction

Investigation of the catalytic properties of enzymes provides a powerful means of characterising and comparing them. The information so obtained, viewed in the broader context of the whole organism, may provide insight into the processes of adaptation and selection.

Work on the pepsinogens of the three snake species has yielded general information on these proteins, and has provided some indication of relatedness between them. As described in the Introduction, such matters are part of the study's raison d'être.

The other declared purpose of the work is to seek evidence of a possible relationship between gastric proteases and venom activity. The activated enzymes are more likely to reflect such a relationship than are the pepsinogen proenzymes themselves, in addition to providing an extended base for characterisation, as noted above. This chapter reports some of the kinetic characteristics of the pepsins derived, as described, from the purified zymogens. Also reported here is an investigation of the concentration of pepsinogen in the blood plasma of the three snakes. The rationale for this investigation is outlined in the appropriate section.

6.2. pH Optima

6.2.1. Crude mucosal extracts

Figures 43 to 45 show the pH-dependent activities of snake gastric mucosae against haemoglobin, as percentages of their respective maxima. Figure 46 shows comparable data for pig pepsin.

All three snake mucosae show a rather broad range of optimal or near-optimal activity between c pH 1,8 and 2,6 - 3,0. In all cases, but most clearly in those of cobra and mole snake, there is evidence of two optima, one of which appears to be at about pH 1,8, and the other at about pH 3 (cobra), pH 2,6 (mole snake) and pH 2,5 (puff adder).

Derived from these graphs, the activities at pH 4,0, as percentages of their respective maxima, are approximately 60% (cobra), 50% (mole snake), 44% (puff adder) and 5% (pig).

Since these differences in relative activity at high pH may have certain implications (see Discussion, Section 6.9.6., and General Discussion) these findings were tested in an independent set of experiments.

Snake mucosal extracts, and pig pepsin, were assayed against stock solutions of haemoglobin at pH 2,0 and pH 4,0. Ten incubations of each enzymatic sample were carried out at each of the two pH values. Six blank assays were performed at each pH, and their respective means used to correct the means of the active samples. In another, parallel set of assays, conditions were identical, except that the haemoglobin solution at pH 4,0 had sodium chloride added to give a chloride

concentration equivalent to that existing in the stock solution adjusted to pH 2,0. (After addition of sodium chloride the pH was checked and found to be unchanged.) This set of assays was included because of the report by Schlamowitz and Peterson (1961) that ionic strength influences the susceptibility of BSA to cleavage by pepsin in the pH range 1 - 5.

Results are summarised in Figure 47, page 175.

Activities of the (unadjusted) extracts at pH 4, as percentages of activities at pH 2 for the same extracts, are: cobra 42; mole snake 40; puff adder 31; pig (pure pepsin) 8.

These data are in broad agreement with those derived from Figures 43 to 45 in that, of the three snakes, puff adder mucosa is least active at pH 4, while the relative activity shown by pig pepsin is very much lower.

An earlier, independent experiment, using BSA as substrate, indicated negligible activity by all three mucosae at pH 4,0. (Pig pepsin was not tested.) Again in this experiment, however, the relative activity of puff adder mucosa at pH 3,0 was substantially less than that shown by the other two species. (Figure 48.)

6.2.2. Purified pepsins

Figure 49 shows the activity of crude cobra mucosa (from Figure 43) with activity profiles obtained from the purified PI and PII pepsins.

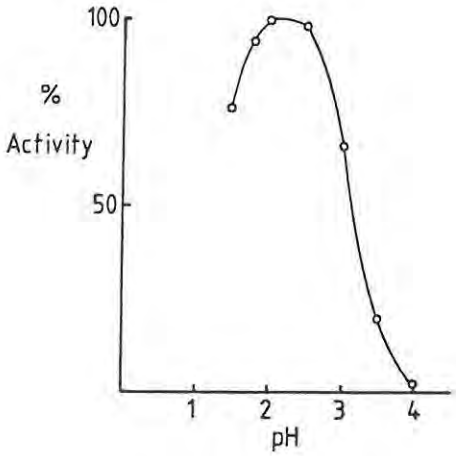

FIGURE 46

Commercial pig pepsin: pH optimum profile. . Details as in Figure 43.

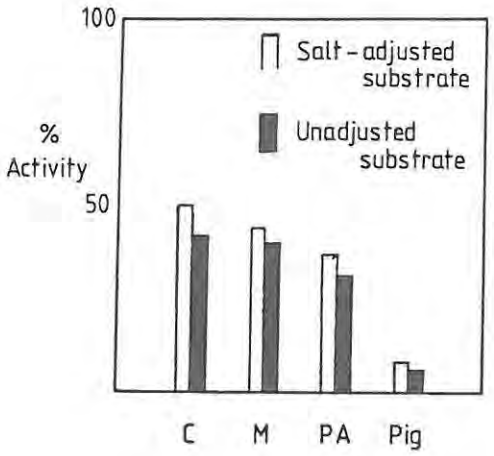

FIGURE 47

Activities of crude mucosae, and of pure pig pepsin, at pH 4,0, as percentages of their respective activities at pH 2,0. The substrate (haemoglobin) was made to $\underline{c}$ 0,04 M in NaCl for the 'salt-adjusted' series.

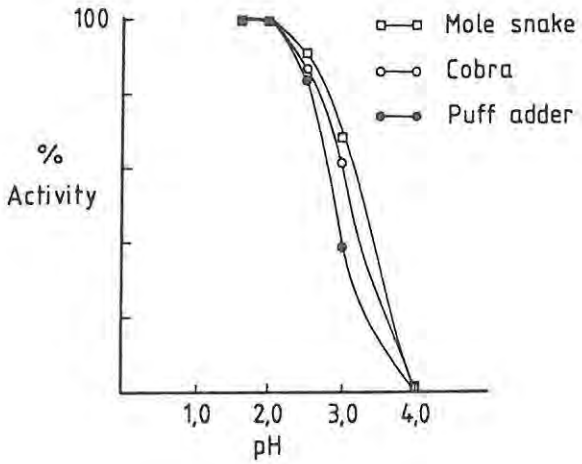

FIGURE 48

Gastric mucosal extracts: pH dependencies for the hydrolysis of bovine serum albumin.

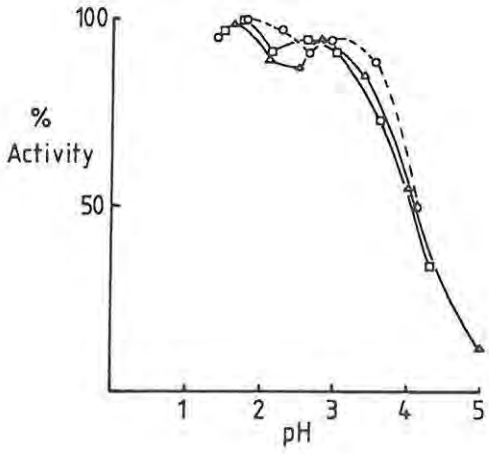

FIGURE 49

Cobra: pH optimum profiles. O - O:crude extract; △—△: P I; □—□: P II. Haemoglobin substrate.

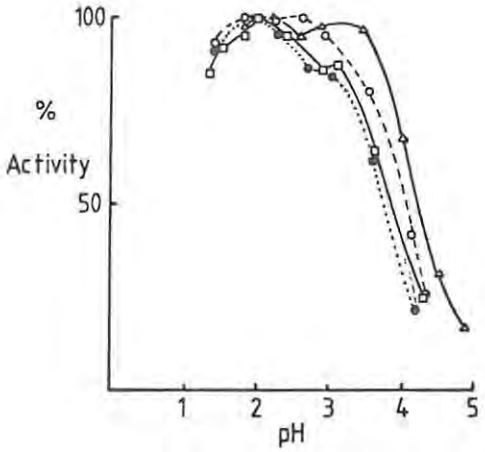

FIGURE 50

Mole snake: pH optimum profiles. O--O: crude extract; △—△ : P I; □—□: P II; ●·····● : P III. Haemoglobin substrate.

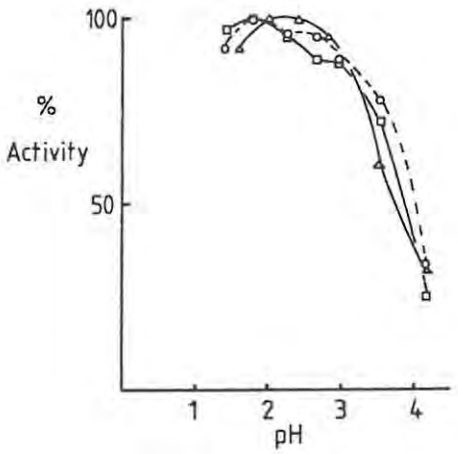

FIGURE 51

Puff adder: pH optimum profiles. O - O: crude extract; △—△: P I; □—□: P II. Haemoglobin substrate.

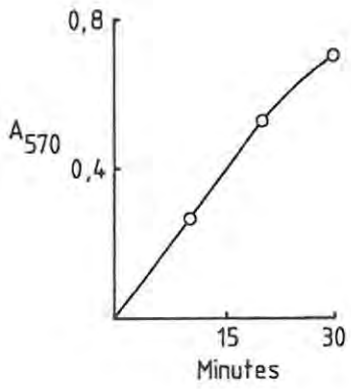

FIGURE 52

Activated commercial (ICN) pig pepsinogen assayed against APDT. Specific activity: 160 nmol DIT $mg^{-1}min^{-1}$. See DIT calibration curve, Figure 8.

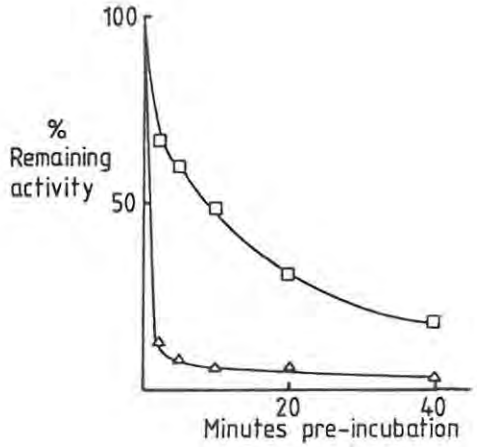

FIGURE 53

Cobra P I and P II pepsins: alkaline stability. Assayed on haemoglobin after pre-incubation at 37°C for the indicated times. △—△: P I pre-incubated at pH7,1; □—□: P II pre-incubated at pH7,5. Phosphate buffers.

Figures 50 and 51 show similar composite activity profiles for mole snake and puff adder. (In both cases the profiles of crude mucosal extracts are derived from the same data as are shown in Figures 44 and 45, page 142.

It may be noted that, while puff adder PI shows a single optimum (between c pH 2 and 2,4), PII shows a bimodal activity profile.

6.3. APDTase activity

Lack of significant activity against this substrate by pepsins of the three snake species is indicated in the DE52 elution profiles (cobra and puff adder), and the G150 elution profiles (mole snake PI, PII and PIII).

To establish that the assay method, as here applied, is successful in revealing APDTase activity, commercial pig pepsin was used. (Figure 14 shows such activity to be clearly detectable in the molecular exclusion column eluates.)

As noted by Ryle (pers. comm.), difficulties exist in applying a fully satisfactory correction for the APDT assays.

Contaminant proteins in impure fractions, acting as endogenous substrates, compete with APDT, thus reducing, to an indeterminate extent, hydrolysis of the latter. The APDT (substrate) blanks, however, exhibit more hydrolysis of endogenous substrate than occurs in the active (APDT) samples, where APDT in turn competes with such endogenous material. Corrections made by subtracting A_{570} values of ninhydrin-positive blanks from active samples, as applied here, will thus yield spuriously low results. Ryle notes that there is no easy solution. Clearly the problem

exists only where fractions contain appreciable quantities of endogenous potential substrates. This condition certainly applies in the cases of the APDTase assays of cobra and puff adder material from anion exchange columns. Assays of the substantially purer fractions of mole snake material eluted from G150 columns (see p. 97 - 98, Figures 22 to 24) are presumably less susceptible to errors of the sort described. In these, more reliable cases, APDTase activity is also very low.

Figure 52 shows the activity at pH 2,0, of ICN pepsin against APDT. (See also Figure 8 for DIT calibration curve.) Pilot assays using a citrate/HCl buffer, established that, under the assay conditions used, hydrolysis of the substrate occurred optimally at pH 1,8 - 2,0, essentially in agreement with the data first reported by Baker (1951), and later by Jackson et al (1965).

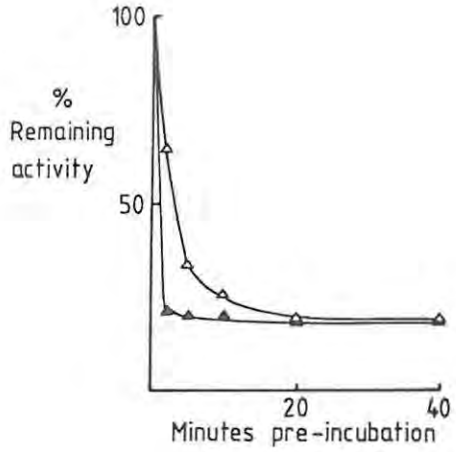

FIGURE 54

Mole snake PI pepsin: alkaline stability. Assayed on haemoglobin after pre-incubation at 37°C for the indicated times. △——△:pH 7,2; ▲——▲:pH 7,6. Phosphate buffers.

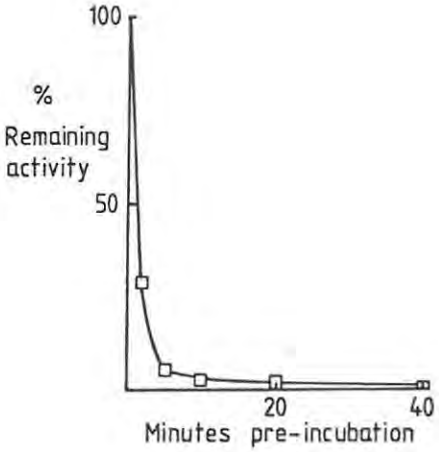

FIGURE 55

Mole snake P II pepsins: alkaline stability. Assayed on haemoglobin after pre-incubation at 37°C for the indicated times. pH 7,4. Tris buffer.

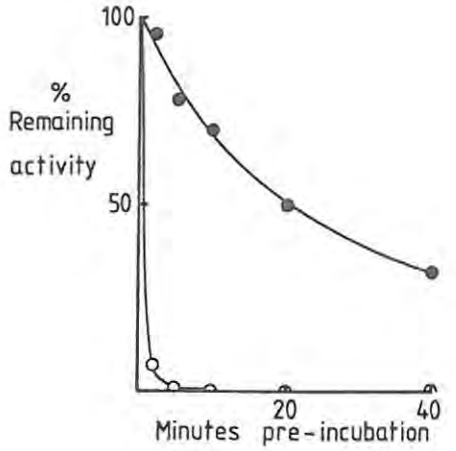

FIGURE 56

Mole snake P III pepsins: alkaline stability. Assayed on haemoglobin after pre-incubation at 37°C for the indicated times. ●——●:pH 7,3; ○——○:pH 8,1. Tris buffers.

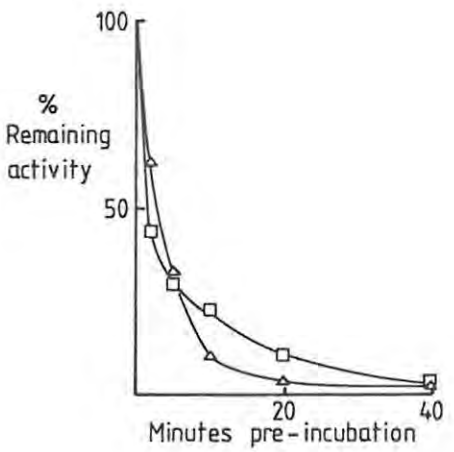

FIGURE 57

Puff adder P I and P II pepsins: alkaline stability. Assayed on haemoglobin after pre-incubation at 37°C for the indicated times. △——△:P I: pH 7,1; □——□: P II: pH 7,2. Phosphate buffers.

6.4. Alkaline stability

Figures 53 to 57 show loss of activity of the purified pepsins with increasing time of pre-incubation at 37°C, at pH values indicated.

In the cobra, and to a smaller extent the puff adder, the data suggest that the PII group is more alkali-stable than PI. Mole snake PII pepsin is quickly destroyed at pH 7,4. In view of the probability (discussed later) that mole snake PIII is the same zymogen complex as PII, the result for PIII (Figure 56) is suspect. It appears unlikely that a difference of 0,1 pH unit between the two incubations would cause the observed difference in rates of loss of activity. (Figures 55 and 56)

A further point of doubt, also discussed later, is the result for mole snake PI (Figure 54).

Figure 58 is included to attest to the well-documented instability of pig pepsin under conditions of mild alkalinity.

While alkaline stability studies on purified enzymes give indications of structural differences between them, *in vivo* exposure to mild alkalinity occurs in a complex and variable *milieu*. Crude mucosal extracts, previously activated by acidification, were incubated at pH 8,1, 37°C, for periods up to 40 minutes. The temperature chosen is probably a little above the preferred temperature range of the animals (Fitzsimons, *op. cit.*), while pH 8,1 is also probably somewhat above the limit of alkalinity which may be encountered in "physiological" circumstances - occasioned, perhaps, by duodeno-gastric reflux. (In

dissections, bile stains were sometimes noted on the distal gastric mucosa, suggesting the ingress of duodenal fluid.

Notwithstanding the choice of these conditions, the assays with crude mucosal material do not reflect in vivo circumstances in any comprehensive sense. No effort was made to study the effects of changing ionic strength. This latter variable is known to influence the rate of alkaline denaturation (Steinhardt, 1937; Buzzell and Sturtevant, 1952, not seen by the present writer, quoted by Herriott, 1962).

Figure 59 shows the loss of activity of crude mucosal extracts in TRIS buffer at pH 8,1, (page 184.).

All the crude extracts retain some residual activity. Such retained activity appears greater, in the case of puff adder mucosa, than that retained by the purified PI and PII puff adder pepsins. It may be that some reversible association occurs between the pepsins and other proteins during the alkaline incubation, and that such association stabilises the pepsins. It is also possible that the residual activity results from a degree of renaturation of the pepsin(s), since Northrop (1931, not seen by the writer, quoted by Herriot, 1962), has shown that alkaline denaturation of pepsin is to some extent reversible.

Ryle (pers. comm.) has drawn attention to the possibility that crude mucosal extracts may contain cathepsin(s), which could thus contribute to the observed residual activity of alkali-inactivated samples.

It is very probable that crude extracts do indeed contain cathepsins, but the likelihood of their significantly influencing the residual activity profiles appears, on indirect grounds, rather small. While cathepsins

are very widely distributed in animal tissues (Barrett, 1969), and hence are almost certainly present in snake gastric mucosae, the main form, Cathepsin D (EC 3.4.23.5) is present in highest concentration in kidney, spleen and liver of several vertebrates (Barrett, op. cit.). No detailed information appears to be available on cathepsins in gastric mucosal tissues of reptiles.

Press et al (1960) purified Cathepsin D from bovine spleen. They determined the pH optimum, against haemoglobin, to be 3,0. Insofar as their activity data may be compared, it seems that the specific activity of crude spleen, against haemoglobin at pH 3,0, is no more than about 1% of that determined in the present study for gastric mucosae against haemoglobin at c pH 2,0. This is a small fraction in the present context, but what is perhaps more significant is that Press et al (op. cit.) note that activity of Cathepsin D "was rapidly lost below pH 2,5 ...". Their graphic data indicate, in fact, that no residual activity remained at pH 2,0 (37°C). These workers also note that loss of activity in acid solutions appeared to be irreversible. In an earlier study, Mirsky et al (1952) noted that cathepsins are inactivated by incubation at pH 1,5, 3 - 5°C, for 30 minutes. It will be recalled that, in the present work, material was routinely assayed at pH 1,8, after activation at the same pH.

6.5. Pepstatin inhibition

Figures 60 to 63 show inhibition of snake and pig pepsins by pepstatin. The data indicate that snake PI pepsins are in all cases more susceptible than PII pepsins to inhibition. Pig pepsin appears to resemble the cobra and mole snake PI pepsins more closely than the

PII group in pepstatin sensitivity. In the case of the puff adder the similarity of PI to pig pepsin is less marked.

TABLE XVI lists the approximate pepstatin : pepsin molar ratios at which 50% inhibition occurs. (See page 186.)

6.6. Temperature-Activity studies

Figures 64 to 69 show the peptic activities, at pH 1,8, of crude mucosae from all three snake species, and of the purified pepsins, as a function of temperature. Comparable data for pig pepsin are given in Figure 70.

TABLE XVII shows Arrhenius activation energy (E_a) data derived from points in the temperature range 15 - 25°C. Figures 71 to 77 show plots of the logarithm of the rate against 1000/Absolute temperature. Such plots yield the activation energy from the relationship:

$$\text{Slope} = \frac{-E_a}{2{,}303R}$$

(Piszkiewicz, 1977)

where R = Universal Gas Constant
= 1,987 cal mol^{-1} deg^{-1}

and E_a = Arrhenius activation energy.

Derivation of E requires that the plot of log k against $\frac{1}{T}$ be linear. Figures 71 to 77 show that the experimental data satisfy this condition only over the first four temperatures (to 24,3°C : crude material and pure pig pepsin), and the first three temperatures (to 25,6°C : purified snake pepsins). See Discussion.

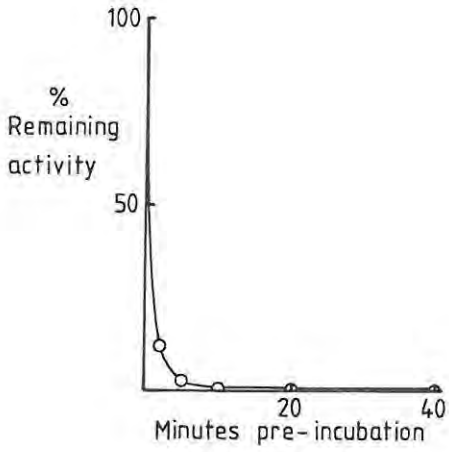

FIGURE 58

Commercial pig pepsin: alkaline stability. Assayed on haemoglobin after pre-incubation at 37°C for the indicated times. pH 7,2. Tris buffer.

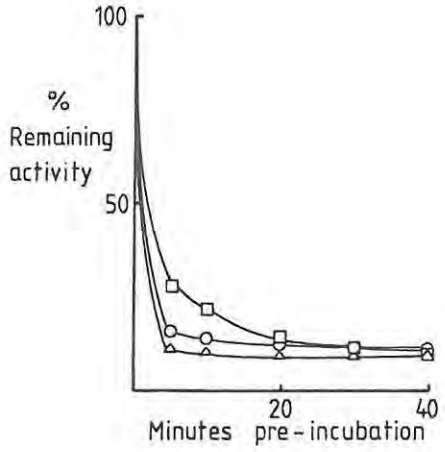

FIGURE 59

Crude mucosal extracts: alkaline stability. Assayed on haemoglobin after pre-incubation at 37°C for the indicated times. O—O:cobra; Δ—Δ:mole snake; □—□:puff adder. pH 8,1. Tris buffer.

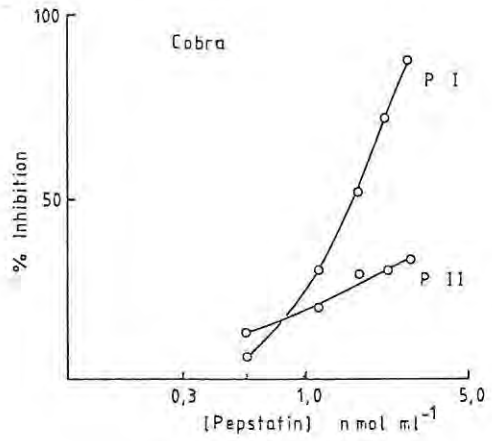

FIGURE 60

Cobra P I and P II pepsins: inhibition by pepstatin.

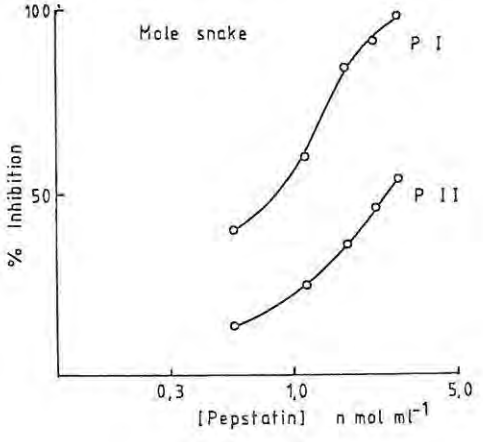

FIGURE 61

Mole snake P I and P II pepsins: Inhibition by pepstatin.

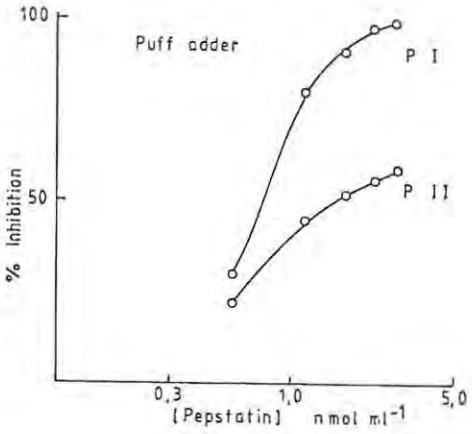

FIGURE 62

Puff adder P I and P II pepsins: inhibition by pepstatin.

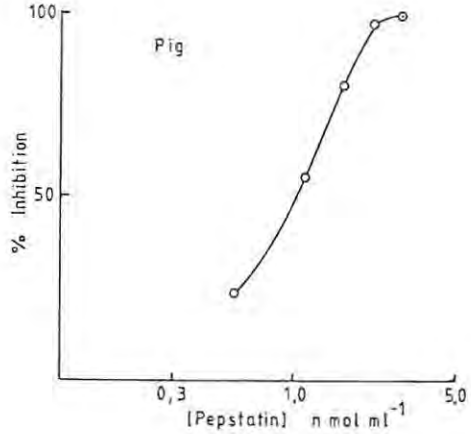

FIGURE 63

Pig pepsin: inhibition by pepstatin.

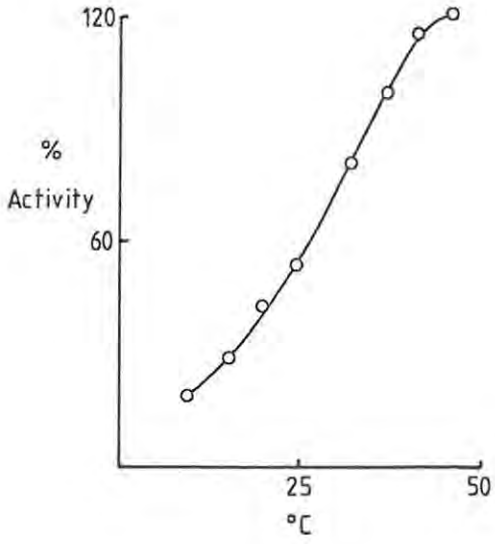

FIGURE 64

Cobra crude mucosal extract: temperature-activity profile.

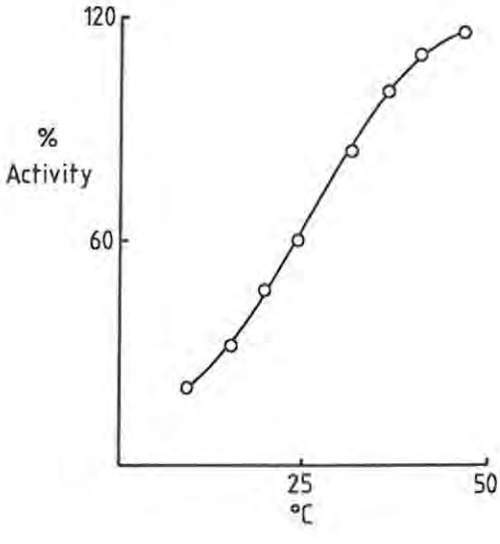

FIGURE 65

Mole snake crude mucosal extract: temperature-activity profile.

TABLE XVI

PEPSTATIN : PEPSIN MOLAR RATIOS
PRODUCING 50% INHIBITION

ENZYME		mol PEPSTATIN PER mol PEPSIN
Pig		0,35
Cobra	PI	0,56
	PII	(>3)
Mole snake	PI	0,28
	PII	0,84
Puff adder	PI	0,25
	PII	0,51

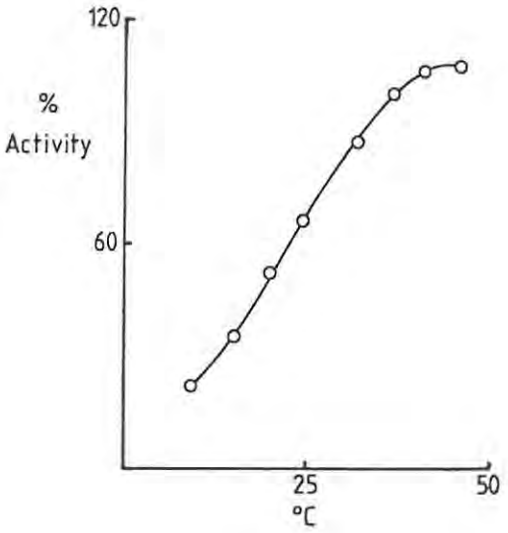

FIGURE 66

Puff adder crude mucosal extract: temperature-activity profile.

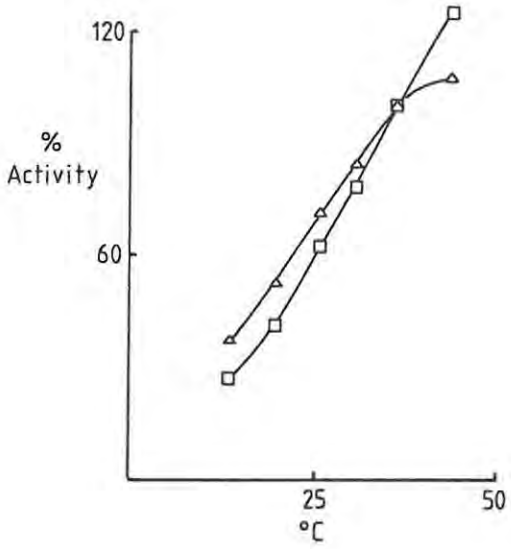

FIGURE 67

Cobra P I and P II pepsins: temperature-activity profiles. △—△: P I; □—□: P II.

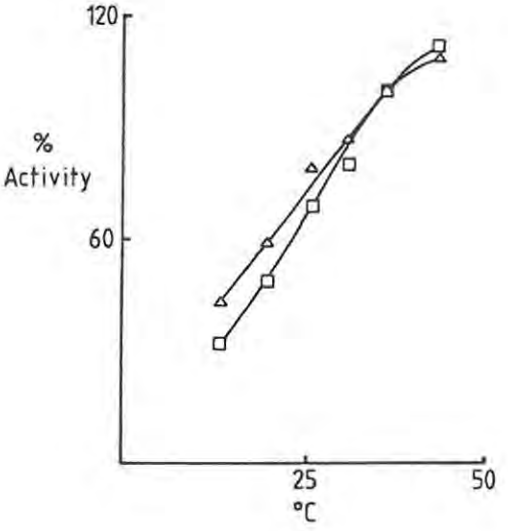

FIGURE 68

Mole snake P I and P II pepsins: temperature-activity profiles. △—△: P I; □—□: P II.

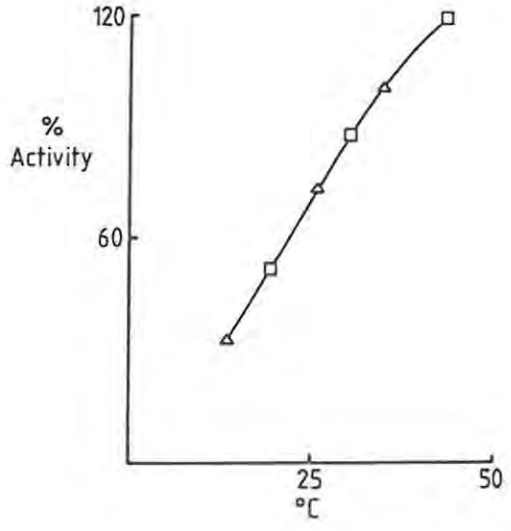

FIGURE 69

Puff adder P I and P II pepsins: temperature-activity profiles. △—△: P I; □—□: P II.

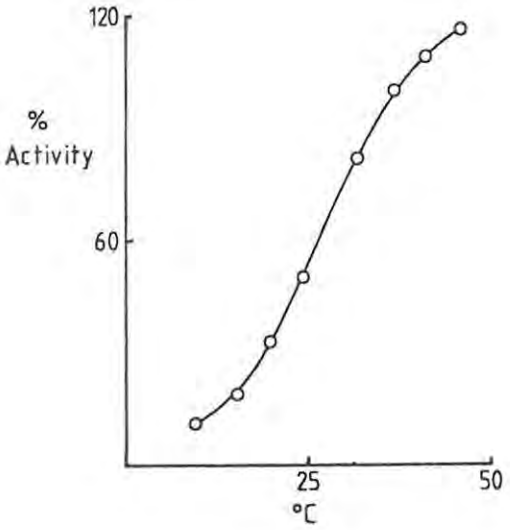

FIGURE 70

Pig pepsin: temperature-activity profile.

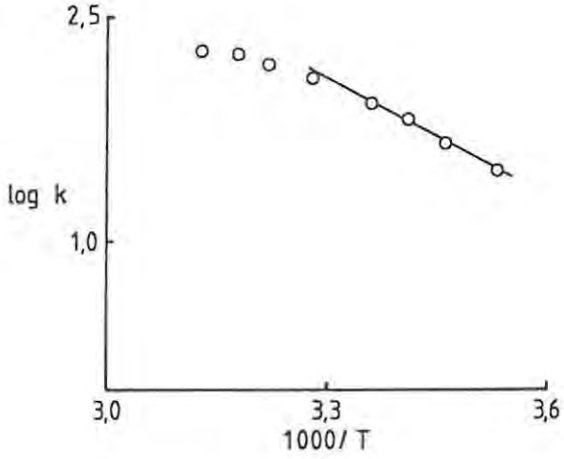

FIGURE 71

Cobra crude mucosal extract:

log reaction rate vs $1000_{/\text{Temperature (K)}}$

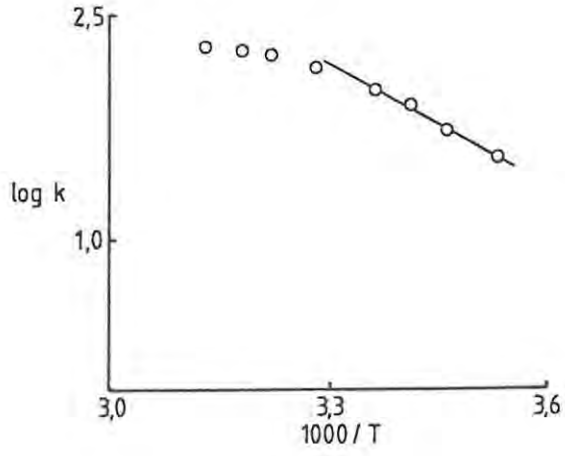

FIGURE 72

Mole snake crude mucosal extract:

log reaction rate vs $1000_{/\text{Temperature (K)}}$

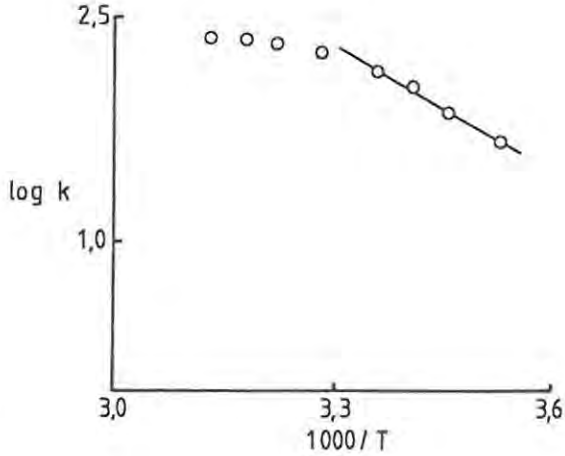

FIGURE 73

Puff adder crude mucosal extract:

log reaction $rate_{/\text{Temperature(K)}}$

TABLE XVII

ARRHENIUS ACTIVATION ENERGY DATA DERIVED FROM THE FIRST FOUR POINTS (CRUDE MATERIAL AND PIG PEPSIN) OR THREE POINTS (PURE SNAKE PEPSINS) ON RELEVANT GRAPHS

SOURCE		E_a (Kcal mol^{-1}) (15 - 25°C)
CRUDE MATERIAL		
Cobra		13,39
Mole snake		12,32
Puff adder		13,28
PURE PEPSINS		
Pig		17,29
Cobra	PI	8,46
	PII	10,36
Mole snake	PI	8,14
	PII	11,08
Puff adder	PI	10,29
	PII	11,07

Note: Each E_a was derived from the slope of an Arrhenius plot of the rate constant, k(= A_{280} X 10^2 hr^{-1} $protein^{-1}$) against temperature, up to 25°C. Reported values were determined from graphs with full-scale y-axes, i.e. not directly from the graphs as given. The y-axes have been reduced 5-fold for convenience of illustration.

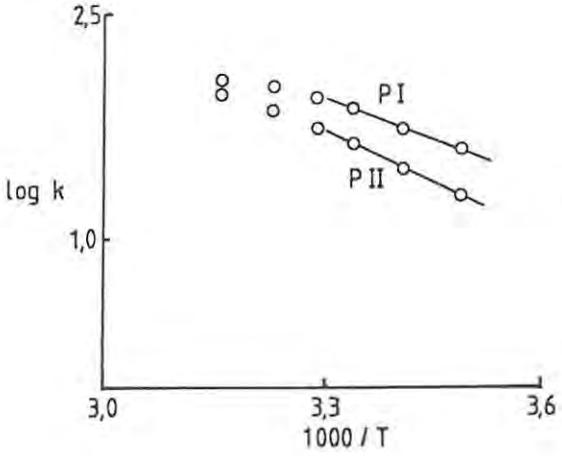

FIGURE 74

Cobra P I and P II pepsins:
log reaction rate/Temperature(K)

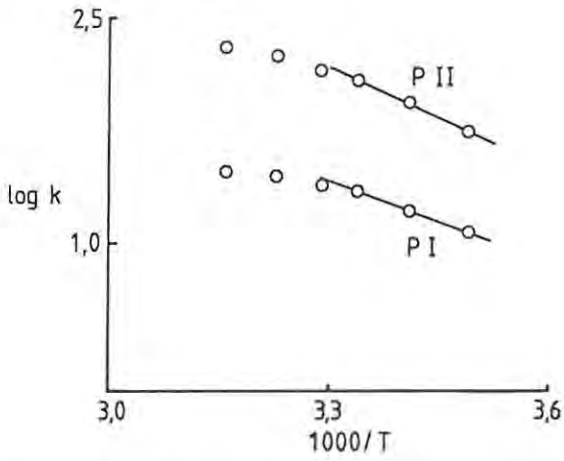

FIGURE 75

Mole snake P I and P II pepsins:
log reaction rate/Temperature(K)

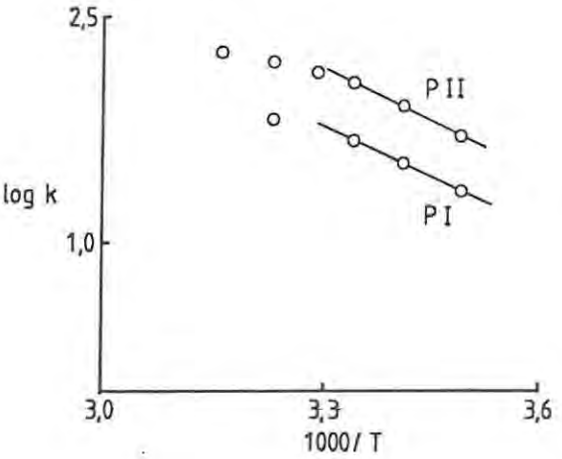

FIGURE 76

Puff adder P I and P II pepsins:
log reaction rate/Temperature (K)

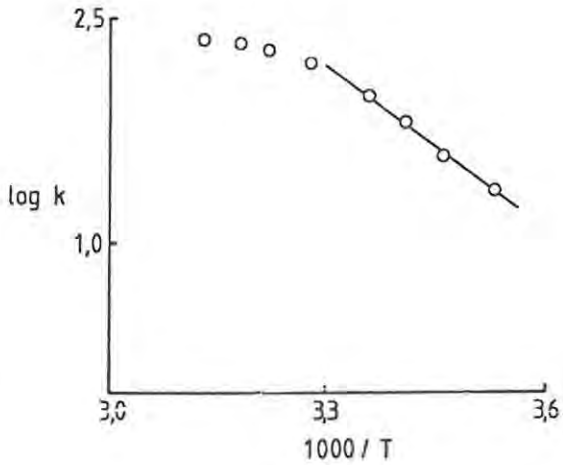

FIGURE 77

Pig (pure commercial) pepsin:
log reaction rate/Temperature (K)

6.7. Discussion

6.7.1. pH Optima

Studies on the pH optima of pepsins have been impeded by the unusually low pHs involved, and the consequent difficulty of finding suitable native substrates.

Reviewing data on peptic hydrolysis of a wide variety of synthetic substrates, Clement (1973) notes that "the details of the pH dependency of pepsin action are far from being firmly established".

Unlike several of the synthetic substrates, many proteins tend to denature, to a greater or lesser extent, in the low pH range.

Tanford (1957) notes that haemoglobin undergoes "a drastic configurational change below pH 4", and that at pH 3,5, 36 protons per mole are bound. Schlamowitz and Peterson (1959) have noted the effect on pepsin pH optima of transition from native to denatured states of BSA and hameoglobin.

Field and O'Brien (1955), quoting work by Moore and Reiner (1944, not seen by the present writer) note that (human) haemoglobin separates partly at pH 4, completely at pH 2,5, into two electrophoretically separate components. This latter pH is very close to that of maximal catalytic activity by the enzyme. A further complicating factor is that one of the acid-dissociation products - globin (Gralén, 1939) - is a rather better substrate for pepsin than is haemoglobin.

Ryle and Porter (1959) have shown that the observed pH optimum for pig gastricsin varies according to the state of digestion of the haemoglobin.

Foltmann (1981) summarised as follows:

> "The conclusion is that it is difficult to characterise these proteinases by a well-defined optimum pH activity. The observed optima depend on experimental conditions, such as denaturation of substrates, ionic strength of the solutions, the duration of the experiment, and the conditions for precipitation of undigested substrate."

In the present results, the puff adder mucosal extract exhibits a "shouldered" pH-activity profile, while those of the other two species are distinctly bimodal. In all three there is an optimum at c pH 1,8, while in cobra and mole snake, second optima appear to exist at c pH 3,0 and 2,7 respectively.

Such data would ordinarily suggest the presence, in all three mucosae, of enzymes with different pH optima. This interpretation may not be correct here.

Taylor (1962) suggested that in certain circumstances a single pepsin may exhibit a double pH optimum. In a later review publication, Taylor (1968), quoting other workers, notes that double pH-activity maxima are not unique to pepsin.

Kageyama and Takahashi (1976a) present graphic data indicating the presence of shoulders in pH optimum curves of Japanese Monkey pepsins I and II (each reportedly homogeneous). Similarly, a shoulder approaching bimodality was obtained in the pH-activity profile of (homogeneous) Crab-eating Monkey pepsin Ia (Kageyama and Takahashi, 1980). Kageyama et al (1983b) also found a shouldered profile for Asiatic Black Bear pepsin III. (In all cases here cited, other pepsins from the same mucosae exhibited smooth, though generally somewhat asymmetric pH-activity

profiles.) Twining et al (1983), studying a homogeneous pepsin from rainbow trout, found a marked shoulder in the pH-activity profile, against haemoglobin, at c pH 2,5.

Even with the dipeptide substrate APDT, Fox et al (1976), working on pure pepsins from pig, ox and sheep, found that pig and sheep pepsins showed "distinct shoulders on the curve at pH 2,2 - 2,5". (They do not present graphic data.)*

Another reason for questioning the prima facie assumption of the presence of catalytic species with different pH optima in the crude (or purified) samples is the lack of any bimodality or shoulders in the pH-activity profiles of crude mucosae against BSA substrate.

It appears indisputable, from the results, that the pH-activity profiles of cobra and mole snake pepsins are broader than those of the puff adder. This may be estimated, as noted, by expressing activity at pH 4 as a fraction of the maximum.

6.7.2. APDTase activity

APDTase activity is not routinely determined in investigation of pepsins, and general statements are difficult to make. It seems that the principal pig pepsin (A) is unusually active against this substrate. Thus Kageyama and Takahashi (1976a) report Japanese Monkey pepsins III_2 and III_3 to

---

* This report is fully consistent with pilot assays on the pH optimum for pig pepsin, using APDT substrate, in the present study. The graph exhibits a distinct shoulder at pH 2,5.

have approximately the same activity towards APDT as does pig pepsin, while pepsins I, II and III_1 have activities some 40% lower. Pepsin C, later identified in gastric mucosa of the same species, "could scarcely hydrolyse APDT" (Kageyama and Takahashi, 1976b). They report $\underline{c}$ 0,07 of the activity of pig pepsin. The pepsin designated "C" (synonymous with gastricsin : Foltmann, 1981) is partly characterised by its inability to hydrolyse APDT. Thus Tang (1970) reports that human and pig gastricsins lack APDTase activity.

Kassell and Meitner (1970) report that all bovine pepsins hydrolyse APDT, but that "bovine pepsin" is about one third as active as pig pepsin. Abuharfeel and Abuereish (1984) report k_m values for two camel pepsins of 0,10 and 0,09 m mol/ℓ (cf. Jackson et al, 1965 - pig pepsin on APDT : 0,075 mM), but do not present other comparative data.

APDTase activity is very low (1% of pig) in the main pepsin of Japanese Quail (Esumi et al, 1980).

6.7.3. Alkaline Stability

The purified pepsins of all three snakes exhibit the general property of instability under mildly alkaline conditions. It appears that certain bird pepsins are exceptions to this generalisation. Thus Levchuck and Orekhovich (1963) found three chicken pepsins to have full activity after incubation for 30 minutes at pH 8,05 (temperature not reported), and Bohak (1969) found chicken pepsin to be stable at room temperature after 20 minutes at pH 8,0. Esumi et al, (1980) found similar stability in Japanese Quail pepsin. While it cannot be assumed, on the basis of

isolated cases, that this stability is a general property of avian pepsins, it is interesting to note that the reptile pepsins examined here show no comparable stability.

Pepsin from the fish Merluccius gayi exhibits stability intermediate between those of chicken and pig pepsins (Sanchez-Chiang, 1982).

Gildberg and Raa (1983) note that pepsins of the arctic fish capelin "were irreversibly inactivated at alkaline conditions, just as other pepsins", but do not report details. Twining et al (1983) showed that a pepsin from rainbow trout is at least 90% inactivated at c pH 6,5 (temperature not reported), suggesting that its stability is no greater than that of pig pepsin.

The alkaline stability studies in the present work are not entirely satisfactory in terms of methodology. They were performed under conditions and at pH values which make detailed comparison difficult. Early assays (mole snake PII and PIII, pig pepsin and crude mucosae) were performed with TRIS/HCl buffer, providing a buffering range from pH 7,2 to 9,0.* Results showed that the enzymes are destroyed so rapidly even at pH 7,6, that the buffering capacity in the higher pH range was superfluous. Phosphate buffer was thus adopted as that routinely used for extractions and general work, and also as having a smaller pH variation with temperature than TRIS/HCl (0,003 unit per °C, cf. TRIS/HCl : 0,02 unit per °C. Documenta Geigy, 1970). pH values were routinely measured at or near 37°C - the temperature used for pre-incubation (and assay) in these studies.

---

* Documenta Geigy, Scientific Tables, 7th edition, 1970. J.R. Geigy, S.A., Basle, Switzerland.

The results for mole snake PI differ from the others in showing significant residual activity (c 20%) for the full duration of the alkaline incubation period. (A similar effect may have been observed with cobra PII pepsin had the incubation period been longer.) Aside from experimental error*, there are two possible explanations for the mole snake PI result. First, the material may contain a component pepsin stable at pH 7,6. Tentative evidence for heterogeneity of the PI zymogen was noted in Chapter 4, Section 4.4.2. Second, a homogeneous pepsin may undergo an ionic strength- and/or pH-dependent change in tertiary configuration depriving it of only part of its catalytic efficiency. Data provided by Twining et al (1983) indicate that rainbow trout pepsin, reportedly homogeneous, retains some 10% of its activity in the pH range c 6,5 to 7,5. Studies by Walker and Taylor (1979) on human pepsins show that at least two (pepsins 1 and 3) retain some 10% of their activity after 10 - 50 minutes pre-incubation at pH 7,2.

Data for mole snake PII pepsins suggest that the enzymes' response to comparable conditions of pH and ionic strength differ from that of the PI enzyme(s). Such difference presumably reflects differences in tertiary structure.

Cobra PI pepsin shows a denaturation curve similar to that of mole snake PI. The greatest intraspecific difference between pepsins PI and PII is shown by that of the cobra.

---

*Error would best be attributed to an unusually low value for Blanks. In fact the blanks - routinely performed in duplicate and recorded individually against 5% TCA, - were not lower than those generally recorded in the other assays. The likelihood of such an explanation for the results thus appears to be very small.

6.7.4. Pepstatin inhibition

Pepstatin has been widely used, generally with synthetic substrates, to provide kinetic data and to gain insight into the nature if its inhibitory mechanism. (Marciniszyn et al, 1976a; Kunimoto et al, 1974.)

This in turn is motivated partly by interest in the design of oligopeptide pepstatin analogues as specific inhibitors of renin and cathepsin D. Selective inhibition of these (carboxyl) proteases has potential applications in medical pathophysiology (Umezawa et al, 1970; Rich and Sun, 1980).

Inhibition of native protein substrates - commonly haemoglobin, but casein has also been used (Umezawa, et al, op. cit.) - is conventionally determined over a range of inhibitor concentrations, and a semilogarithmic plot of percent inhibition against pepstatin concentration shows the inhibition to occur by mixed-order kinetics, with a first-order component in the range covering 50% inhibition. The general form of such plots is sigmoidal (Kageyama and Takahashi, 1976b and 1980; Kageyama et al, 1983b). Since experimental conditions of enzyme and inhibitor concentration vary quite widely amongst workers, graphic data do not always lend themselves to easy comparison. A conventional index of susceptibility to inhibition appears to be the enzyme-inhibitor molar ratio at which 50% inhibition of activity occurs. This form of graphic treatment was used by Gildberg and Raa (1983), and has been adopted, in tabular form, in the present study (TABLE XVI, page 186).

Reporting the results of their inhibition study on two pepsins from

the fish Mallotus villosus, Gildberg and Raa (op. cit.) note that "The results were unexpected since the activity of both enzymes was reduced to about 50% at a molar concentration of inhibitor which was 20% of the molar enzyme concentration." They do not comment further on this finding. Although not reported in such terms, and not commented on by them, the results of Kageyama and Takahashi (1976b and 1980) for Japanese Monkey pepsin III_3 and Crab-Eating Monkey pepsin I-a may be calculated to reveal closely comparable molar ratios resulting in 50% inhibition. Umezawa et al (op. cit.) report data indicating that the pepsin used by them (presumably from pig : source not recorded) also showed 50% inhibition at similar ratios. Such high sensitivities are also shown by several of the snake pepsins (and pig pepsin) studied here.

Notwithstanding the comment by Gildberg and Raa, quoted above, these low proportions of inhibitor yielding 50% inhibition are perhaps neither unexpected nor surprising, in view of the work by Marciniszyn et al (1976a) on the mode of inhibition of acid proteases by pepstatin. These workers produced evidence that the inhibitory potency is due to the presence of the unusual amino acid statine (4-amino-3-hydroxyl-6-methylheptanoic acid). This they propose, on extensive evidence, to be an analogue of the transition state of peptic catalysis. Two such residues occur in the pepstatin molecule. Further, these workers present experimental data suggesting that, at pH 2,1, total inhibition of (pig) pepsin occurs at an enzyme : inhibitor ratio of 1,96 : 1. As they note, this implies that each statyl residue may bind to, and inactivate, a pepsin molecule, hence accounting for the inhibition

of (very nearly) two pepsin molecules by each pepstatin molecule, as derived by extrapolation of their graphic data. It is difficult to know the extent to which such a postulated mechanism might be expected to translate into empirical data, since Marciniszyn's value of 1,96 pepsin : 1 pepstatin was derived from extrapolation, and may therefore be seen as theoretical. Certainly, some experimental cases, as cited, (and as obtained here) have yielded values closely comparable to those of Gildberg and Raa (op. cit.).

Henderson (1972), citing a treatise by Goldstein (1944 : not seen by the writer) notes that all added inhibitor may be regarded as becoming bound to enzyme if:

$$\frac{E_t}{K_i} \geq 100$$

where

E_t = total concentration of enzyme

K_i = dissociation constant for enzyme-inhibitor complex.

The K_i value for pepstatin inhibition of pepsin is unusually low. Using haemoglobin as substrate, Marciniszyn et al (op. cit.) determined a value of $\underline{c}\, 10^{-9}$M. Later work, reported by Rich and Sun (op. cit.) suggests a value of $4{,}56 \times 10^{-11}$ (Workman and Burkett, 1979). In view of this low value, the ratio $\frac{E_t}{K_i}$ exceeds 100 by a wide margin in the assay systems mentioned. The proportion of free inhibitor, after equilibration (see below), may thus be considered vanishingly

small.

An important point, in the realisation of experimental I_{50} data* yielding such low proportions of pepstatin to pepsin, is the need for pre-incubation of enzyme and inhibitor. This is because, as noted by Kunimoto et al (op. cit.), pepstatin binds slowly to pepsin, and if (uninhibited) enzyme is added to a substrate-pepstatin mixture, the observed inhibition is much lower.

Discussing in a wider context the association of "tight binding inhibitors"** with enzymes, Cha confirms that "both the dissociation and association reactions are very slow under the conditions commonly used for enzyme assays ... ". Hence, "In the presence of a tight binding inhibitor the initial velocity of an enzyme reaction depends on the order of addition of the components," (Cha, op. cit.).

In view of this it is surprising that Marciniszyn et al (op. cit.) report that in their assays they "did not observe any difference in the extent of inhibition when the enzyme was either pre-incubated or directly mixed with pepstatin". Evaluating this aspect of their results, they suggest that the rate of equilibrium between the inhibitor and the enzyme was "sufficiently rapid for the kinetic measurements in the inhibition experiments". It may be significant

---

* I_{50} is here used to denote the mole fraction of inhibitor, per mole of enzyme, producing 50% inhibition.

** Cha (1975) uses this term for those inhibitors with K_i values of less than 10^{-9}M.

in this context that the K_i value determined by these workers is c10-fold greater than the value reported earlier by Kunimoto et al (1974), and c20-fold greater than the value subsequently obtained by Workman and Burkett (1979).

A ratio of 1 pepstatin molecule to 2 pepsins giving 100% inhibition, and the mechanism as proposed by Marciniszyn et al (1976a) would account satisfactorily for 50% inhibition of pepsin at a pepsin : inhibitor ration of 4 : 1. It is true that the enzyme inhibitor ratio obtained by Gildberg and Raa (1983), and by other workers mentioned above, is approximately 5 : 1. The extent of the discrepancies, however, compared with what may possibly be a theoretical limiting value of 4 : 1, do not appear of themselves to invalidate the findings.

Notwithstanding the foregoing, such high molar ratios of enzyme to inhibitor as are found here for the PI pepsins, and as have been reported by other workers, as noted, should be regarded as suspect. Workman and Burkitt (1979) reported 100% inhibition of (pig) pepsin by pepstatin at approximately 1 : 1 molar concentration. As pointed out by Ryle (pers. comm.) a simpler explanation of these observed high ratios may be partial inactivation of the enzyme. This possibility has not been specifically cited by those workers, noted above, whose inhibition data indicate very high enzyme : inhibitor ratios. In the present work, insufficient material was available for a formal check on specific activities in the course of the inhibition studies, but the results for zero inhibitor concentration did indeed indicate 40 to 50% loss of activity in the PI samples, as compared with earlier activity assays. (In comparison, the PII pepsins showed far smaller loss of activity. This provides some

further indication of difference between the PI and PII pepsins, since preparation and storage of all the pepsin samples was closely comparable.

6.7.5. Temperature-Activity studies

a. General considerations

It is unfortunate that the Arrhenius plot in all cases departs from linearity (or at least changes its slope : see below) at the higher temperatures.

In the cases of several of the enzyme samples (cobra PI, mole snake PII, puff adder PII, pig; and of all three crude extracts), this might conventionally be attributed to excessive A_{280} values (>1,000) in the higher temperature range, and consequent breakdown of the linear relationship between concentration and optical density. In fact, however, the incubation samples for cobra PII, mole snake PI and puff adder PI yielded maximal optical densities of less than 1,000 unit, and Arrhenius plots for these too show a comparable change in slope at $\underline{c}$ 27°C. Deviations thus appear to be a function of increasing temperature rather than of increasing optical density of the supernates. It may therefore be suggested that the observed change in the slope of the Arrhenius plots is due to a change in the configuration of the haemoglobin substrate. In general support of this, Low et al (1973) note that temperature is known to affect the higher orders of protein structure.

Further, it has been known for some time that the affinity of haemoglobin

for oxygen is temperature-dependent (Prosser and Brown, 1961; Maclean, 1978). This oxygen affinity is itself determined by the quaternary structure of haemoglobin (Ganong, 1979).

It is interesting in this context to refer to the graphic data supplied by Arunchalam and Haard (1985) for polar cod pepsin. These workers present a plot of relative activity (against haemoglobin) vs temperature, in the range 2 to 59°C. Although the authors do not refer to an Arrhenius plot of these results, nor do they describe the manner of their derivation of E_a values, it is possible, at the inevitable expense of some accuracy, to derive Arrhenius plots for each of the two purified pepsins, up to 37°C, from their graphs. Such plots give a strong indication of two slopes, with deviations at $\underline{c}$ 24°C. Although the authors do not make it entirely clear, it appears that the reported E_a values for the pepsins are derived from the temperature range 0 to 20°C.

In contradiction to the above, Twining et al (1983) present Arrhenius plots for dog, pig and rainbow trout pepsins, against haemoglobin, which appear to be linear throughout the temperature range 0 to 37°C. Their reported E_a datum for pig pepsin, at least, is surprisingly low in comparison with the value obtained here, and surprising also in that their data indicate it to be lower than the E_a for the poikilothermic rainbow trout pepsin. They note, in discussing their results, that Casey and Laidler (1950, not seen by the writer) reported an activation energy of 20 Kcal mol^{-1} for pig pepsin assayed, at pH 4, against the dipeptide carbobenzoxyl-L-glutamyl-L-tyrosine.

b. Activity data from the higher temperature range

In view of the deviation from linearity of the Arrhenius plots for all pepsin samples above $\underline{c}$ 25°C, no Q_{10} or E_a data were derived from such values. Although some of the experimental systems gave evidence of rectilinear plots above this temperature, the situation is complicated by the fact that optical density values for several assays were very high, and the overall form of the activity curves suggests some thermal damage to the enzymes at the highest temperatures used (purified snake pepsins : 43,3°C; crude samples and pig pepsin : 45,8°C).

Samples showing high A_{280} values were quantitatively diluted with 5% TCA, but the resultant solutions yielded disproportionately low optical densities. The reason for this was not apparent.

c. Possible significance of the E_a data : 15 to 25°C

It is tempting indeed to assess the E_a values in the context of the rationale which fathered this study - as outlined in Chapter One. At first sight, these data provide some grounds for believing that highly proteolytic venom, in the puff adder, has produced an adaptive response in its gastric proteases.

That many of the enzymes of poikilotherms are adapted, as catalysts, to generally lower temperatures than the homologous enzymes of homeotherms appears to be well documented (Low et al, 1973). In

thermodynamic terms, this adaptation is expressed in differences in the free energy of activation ($\Delta G^{\ddagger}$), of such enzymes. In certain cases (see below), differences in $\Delta G^{\ddagger}$ are reflected in proportionate differences in the Arrhenius activation energy, E_a. Where this is so, E_a values themselves provide comparative indices of adaptation to lower temperatures.

The finding that snake pepsins have E_a values rather lower than pig pepsin is thus consistent with other findings from poikilotherm/homeotherm homologous enzymes, and worthy of mention en passant.

What is of potentially greater interest, in the present context, are the apparent differences in E_a between the PI and PII pepsins here studied. This difference appears very small - and perhaps non-existent - in the case of the puff adder PI and PII enzymes, and appreciably greater between the PI and PII pepsins in mole snake and cobra. It is of further interest that the E_a values of both puff adder PI and PII pepsins agree more closely with the higher (PII) of the cobra and mole snake E_a values than with the lower (PI) values.

This suggests that the puff adder pepsins, jointly, will respond more strongly to increasing temperature than will those of either of the other two snakes, since the PI pepsins of both cobra and mole snake have low activation energies. Conversely, under conditions of low or falling temperature, the PI pepsins of the latter two species will show a slower fall-off in activity than either the PI or PII pepsins of the puff adder.

These considerations invite comparison with the suggestion, advanced

by Thomas and Pough (op. cit.), as noted earlier, that viperid venom is particularly important as a digestive aid at low temperatures.

It may be assumed on general grounds that, where two or more genetically distinct pepsins exist in a poikilotherm, selection might act to separate them in terms of their activation energies. Adaptation to low temperature, by producing enzymes of low Q10 and $\Delta G^{\ddagger}$ (or E_a) values, will necessarily limit the animal's capacity to respond to the potential advantages of periods of relatively high temperature. This limitation would be offset by the development of isozymes with high and low $\Delta G^{\ddagger}$ values. Such a development might in turn lead to differential secretion of the two enzymes, in response to temperature, or to the direction of temperature change. This aspect of the question will be considered in the General Discussion (Chapter Nine).

There is some evidence that pepsins from other poikilotherms have diverged on these grounds. Although they do not discuss the possible implications, Gildberg and Raa (op. cit.) note, in passing, that, of two pepsins in the arctic fish capelin, "Both enzymes, and particularly Pepsin I, had relatively high activity at low temperatures."

If we provisionally accept the suggestion by Thomas and Pough (op. cit.) that viperid venom is of particular benefit to prey digestion in the low temperature range, it may reasonably be argued that the possession of such venom would allow selection to produce pepsin isoenzymes of uniformly high activation energies. It is relevant in this context to remark that snakes in general are endowed with behavioural responses prompting them to seek warmth, up to a preferred maximum body temperature of c 32°C (Fitzsimons, op. cit.).

These arguments derive some support from the data presented, but more experimental evidence is needed before they can confidently be seen as reflecting adaptations of the sort proposed. In particular, the activation energies of the purified pepsins should be investigated with substrates yielding Arrhenius plots which are linear over a wider range of "physiological" temperatures. The derivation, in this study, of E_a values from three points, even though such points are themselves derived from duplicate assays, is certainly less than ideal. Apart from various synthetic substrates, the proteins fibrinogen, BSA and urea-denatured haemoglobin may be useful.

Another point requiring investigation is the assumption, made here, that the observed E_a values reflect proportionate differences in the free energy of activation ($\Delta G^{\ddagger}$) for the enzyme-substrate reactions. As pointed out by Low et al (op. cit.), $\Delta G^{\ddagger}$ represents the true height of the energy barrier to a reaction. Since $\Delta G^{\ddagger} = E_a - RT - T\Delta S^{\ddagger}$*, E_a values may be directly compared only where the relevant values of $\Delta S^{\ddagger}$ (and T) are the same. Determination of $\Delta S^{\ddagger}$ requires accurate knowledge of V_{max} and molecular mass for the enzyme(s) concerned. The specific activities and molecular masses of the pepsins studied here appear to be closely similar, but are not known with sufficient precision to justify the derivation of $\Delta S^{\ddagger}$, and hence $\Delta G^{\ddagger}$ values.

As noted earlier in this work, the PII pepsins in each of the three species comprise more than one enzyme. Similar heterogeneity may exist

---

* R = Universal Gas Constant
T = Absolute temperature
$\Delta S^{\ddagger}$ = Entropy of activation

within the PI pepsins. Full resolution of the question of differing activation energies will require that these components be separated and individually examined.

CHAPTER SEVEN

GENERAL DISCUSSION AND CONCLUSIONS

CHAPTER SEVEN

GENERAL DISCUSSION AND CONCLUSIONS

7.1. Mole Snake PII and PIII Zymogens

As has been noted, the possibility exists that the mole snake PII and PIII zymogens are in fact the same entities. Support for this rests largely on the comigration of the two samples on SDS-PAGE, and the apparently identical proportions of the a and b moieties in the PII and PIII electrophoretograms. A further indicator is that on one ion-exchange separation (Figure 17b, page 89) there is no peak equivalent to the PIII of Figure 17a, yet peak PII (Figure 17b) includes material separated into PII and PIII components in Figure 17a. It is not clear why these differences exist between the two elution profiles : both were run from the same stock of mucosa, on the same batch of DE52 exchanger, and under nominally identical conditions of pH, temperature, protein concentration, ionic strength and gradient, and flow rate. Lack of agreement between the profiles limits the confidence which may be placed in either. Ryle (pers. comm.) has agreed that the implied distinction, in Figure 17a, between PII and PIII may be artefactual.

The amino acid composition analyses do not support the suggestion of identity, but, as noted, it is reasonable to suggest that the observed differences in composition may result from small differences in amount and/or type of contaminant present in the PII and PIII samples. The elution profiles of effluent from G150 columns (Figures 23 and 24, Pages 97 - 98) indicate that some residual contamination remains in both samples, and

that such contaminants differ between the two. The G150 elution volumes of PII and PIII are identical (TABLE V, page 101).

While it is true in a quite general way that proof of identity can derive only from consistent failure to demonstrate differential behaviour in analytical systems, whereas one "solid proof of difference" (Ryle, pers. comm.) is entirely adequate to prove non-identity, such solid proof of difference may require extensive work to be reliably established. This is particularly true where the entities concerned are at least closely similar in several of their physical and catalytic characteristics. The present work, while certainly not proving identity, gives strong evidence of close similarity, at least, between mole snake PII and PIII pepsinogens.

Some of the work carried out might have provided solid proof of difference were such difference to exist. In this context it must also be noted that the alkaline instability studies (Figures 55 and 56, page 179) on PII and PIII pepsins do not clearly support the suggestion of identity. The (slender) indication of difference provided in Figures 55 and 56 could perhaps be attributed to association with other proteins, although the pepsins in each case were subjected to further purification on G50 Sephadex after activation.

An obvious means of further testing the relative status of the PII and III material is by tryptic peptide mapping. This was not done in the present study because after much preparative work is was clear that, as used here, the technique had not been developed to its full analytical potential, and as such it was unlikely to settle the matter. With many demands on the Department's supplies of $Na^{125}I$, it was decided not to pursue the investigation.

7.2. Purity of the various zymogen samples

(To avoid confusion it should be noted that the term 'heterogeneity', or 'microheterogeneity' is applied, in this work, to zymogens (or pepsins) and is intended to denote the existence, within a sample, of two or more pepsin(ogen) types having very similar physico-chemical and kinetic properties. 'Microheterogeneity' has been used by Foltmann (1981) in this context. 'Contaminant', on the other hand, is used for protein(s) unrelated to pepsin(ogen)s and thus, by implication, differing markedly from such enzymes or proenzymes. In the context of this terminology, the work has shown PII (and mole snake PIII) zymogen fractions to be (micro) heterogeneous but not obviously contaminated. The PI zymogens (See Plates I to III, pp. 106/108) are clearly (though not in all cases heavily) contaminated, yet show rather less evidence of heterogeneity. The PI material has been less fully examined, however.)

The various zymogens have been purified from crude mucosal extracts by single, sequential steps of anion exchange- and molecular exclusion-chromatography. Although these techniques are highly effective, and resolve components on different criteria, it would nonetheless be surprising, on general grounds, if the zymogens were entirely devoid of contaminants after such relatively simple preparation. Indeed, as noted, SDS-PAGE shows some contamination of the PI materials, while the elution profiles of PII (and mole snake PIII) samples from G150 columns indicate that residual contamination remains.

The presence, in SDS-PAG-electrophoretograms, of more than one band in the PII and mole snake PIII material has been interpretted as revealing 'heterogeneity' in the sense defined. It is possible that this is

incorrect, and that at least one of the component bands is a contaminant. That this is unlikely - indeed highly unlikely - is suggested by two independent sets of data. Firstly, the amino acid composition analysis of puff adder PII material indicates by the correspondence of best-fit mass with the PAGE-derived mass, and by the obvious general correspondence with reported composition of other pepsinogens, that gross contamination is very improbable. In the case of the cobra PII material, the mass correspondence is not as good but, as noted, Haylett reports that an analysis of the composition data suggests the presence of two closely related proteins. In the case of the mole snake PII and PIII material the mass correspondences are again not very close, and some detectable contamination here has been conceded. The levels implied by the data presumably should not be regarded as gross contamination.

Secondly, the activation studies, based on SDS-PAGE and some limited kinetic data, as discussed, give no evidence that any of the visible bands are contaminants. It would be difficult to account for the character of the sequential electrophoretograms if one of the visible bands were indeed a contaminant.

It may also be relevant to note, more generally, that microheterogeneity, as claimed for the snake pepsinogens, appears to be the rule rather than the exception for pepsinogens from most species (Foltmann, 1981).

Notwithstanding the foregoing, and as has been noted, the claim of total purity is not made for any of the samples. Final purification will probably require large-scale preparative PAGE and/or affinity chromatography to separate the individual zymogens from each other,

and from residual contaminants.

The pepsins used in kinetic studies have been subjected to further purification by chromatography on G50 Sephadex as described, but are themselves clearly heterogeneous at least in the cases of the PII and mole snake PIII samples.

It appears that the small levels of contamination existing in the materials studied here do not in themselves impugn those data cited as supporting the notion of venom-pepsin adaptation.

7.3. Phylogenetic implications of the data

Any enquiry into the evolutionary relationships between the higher taxa of snakes is bedevilled by the paucity of fossil material (Minton and Minton, 1969) - most of which is restricted to vertebral segments (Underwood, 1967) - and the fact that extant forms have radiated widely.

The development of venom occurred early in the evolution of the group, and venom-injecting mechanisms, and the venoms themselves, have undergone extensive diversification. Whether venom developed only once in early snake stock is a moot point. Underwood (op. cit.) appears to suggest that it did, but the matter is not clear - nor, perhaps, particularly important in the present context. While some 'non-venomous' snakes with undifferentiated oral glands may have mildly toxic saliva (Schmidt, 1950), the glands responsible for secreting true venom fall into two different types : Duvernoy's gland, and the venom gland per se. The former is characteristic of the back-

fanged snakes. The latter is possessed by both families Elapidae and Viperidae, and they appear, in spite of minor differences in histology and associated musculature, to be homologous (Minton and Minton, op. cit.; Underwood, op. cit.).

It has been common to regard the Elapidae, or early pre-elapid stock, as phylogenetic antecedents of the Viperidae (Bogert, 1943; Johnson, 1955, 1956, not seen by the writer, quoted by Underwood, op. cit.). Underwood notes that this is not supported by certain studies on jaw musculature. Mebs (1970) appears implicitly to view the viperid group as derivative of the elapids.

The mole snake is viewed as a development of early (non-venomous) stock, placed, by Underwood (op. cit.) in the Family Natricidae, but by Fitzsimons (1962) in the Family Colubridae. All three families are placed by Underwood in the infraorder Caenophidia. Although all families within this infraorder have equivalent taxonomic 'rank', this of course does not imply that all are at similar phylogenetic 'distance' from each other. Although the evidence of affinities as provided by the serological tests and the tryptic peptide maps is indeed slender, the data perhaps attain some greater significance in that they are mutually consistent, and both point in a general direction also consistent with the orthodox - albeit vague - picture of relationships among the higher ophidian taxa.

In this context particularly it is unfortunate that the doubt about the relative status of the mole snake PII and PIII zymogens, the likelihood of some contamination in both, and the associated variation in observed amino acid content, makes it impossible to draw even tentative conclusions

from the composition data concerning relatedness of the three snake species.

It may of course be postulated that, since both cobra and puff adder possess venom - albeit of differing types - the greater similarity of their gastric proteases reflects convergence rather than proximity of their overall genomes. This suggestion, while consistent with the rationale of this project, is entirely speculative.

7.4. Relationship between snake pepsinogens and those of other species

Insofar as a classification of pepsinogens may aim to be 'natural' in the sense of revealing degrees of homology amongst members of the group, the problems involved in its compilation are great. As noted by Kageyama and Takahashi (1980), "the relationship of each pepsinogen and pepsin among these animals (monkey, pig, cow, chicken, dogfish, rat, dog and man) is so complicated that their mutual correlation is rather unclear at present". It is generally accepted that all members of the protease group EC 3.4.23 arose from a common ancestor, and are thus homologous (Tang, 1979; Foltmann, 1981). As noted by Foltmann (op. cit.), "our knowledge is not sufficient to make up a complete phylogenetic tree for these proteinases ...".

Merrett et al (1969), in discussing dogfish pepsinogens, use the term 'analogous' in comparing dogfish and pig pepsinogens. Presumably this implies that no homology is intended by equivalent designation.

Attempts at classification of pepsinogens have preceded any adequate

knowledge of underlying genetic codes and/or amino acid sequences. Full sequence analyses are only recently beginning to appear, and it will presumably be possible, in due course, to include the whole vertebrate group in a 'family tree' of carboxyl proteases, as has tentatively been done by Tang (1979) for the ruminants and other high mammals. As 'natural' groupings emerge from the rather arbitrary classification commonly used, some revision and extension will probably be necessary.

Without fundamental sequence data, classifications neccessarily rely on other experimentally accessible variables - electrophoretic mobility, mass, antigenic similarity, substrate specificities, inhibition characteristics, presence or absence of phosphate and/or carbohydrate moieties, etc.

Certain of these parameters have an extremely indirect correspondence with the underlying sequences reflecting homology. It is known, for example, that although several pepsin variants within a species may be phyogenetic in origin, further heterogeneity is conferred by post-translational modification. This appears to apply particularly to phosphorylation and glycosylation (Foltmann, 1981).

Kageyama and Takahashi (1980) have included presence or absence of carbohydrate as a component of their classification of Crab-eating Monkey pepsins. Similarly, pig pepsin D appears to be distinguished from the main pig pepsin (A) by the lack of a phosphate group in the former (Lee and Ryle, 1967).

Clearly, none of these methods of citing difference or similarity may be criticised as a means of drawing distinctions between enzymes or

their zymogens. Efforts to reduce - or promote - such catalogues to 'natural groupings' expressive of homology are best avoided. This course is widely adopted by workers in the field, and will conscientiously be followed here.

The main component of the mammalian pepsin group is designated Pepsin(ogen) A (EC 3.4.23.1) (Foltmann and Pedersen, 1977).

Since one of the reported characteristics of this group is high activity towards APDT, it is inappropriate as a reference category for any of the snake pepsinogens or their pepsins.

For similar reasons the snake pepsins cannot be seen as equivalent to pig pepsin D.

The pepsin derived from pepsinogen B is distinguished by its inability to hydrolyse haemoglobin, and its high activity against APDT (Ryle, 1970).

The assays for APDTase activity used in scanning column effluents in this study indicate that none of the snake pepsins belong in this group. (This diagnosis of pepsin(ogen) B was used by Merrett et al (1969), who found the zymogen to be present in dogfish stomachs.)

Pig pepsin C is active against haemoglobin but not APDT (Ryle, 1970). These considerations, and the Glu : Asp and Leu : Ile ratios of the zymogen are in agreement with the Japanese Monkey and Crab-eating Monkey pepsin(ogen) C of Kageyama and Takahashi (1976a and 1980).

The amino acid ratios of the snake PII zymogens, and the pepstatin sensitivities of their pepsins, suggest that they cannot be called

pepsin(ogen) C. It remains possible that the PI snake zymogens will reveal amino acid ratios consonant with the 'C' designation.

While the tentative groupings suggested by Foltmann (1981) for mammalian pepsinogens may well be natural, they are of course not encompassing in the broad vertebrate context, and it is not surprising that snake pepsinogens should not be accomodated within them.

7.5. Evidence of a possible adaptive relationship between pepsins and proteolytic venom

The temperature-activity data give grounds for believing that an adaptation, of the type suggested in the Introduction to this work, may indeed exist. The point was also made, in the Introduction, that the specific parameters which may reflect such putative relationship are problematic. In addition, therefore, to providing some support for the suggestion, the present work has indicated an area in which further evidence may profitably be sought. In this context an important consideration is the prevalence, within the order Ophidia, of many species which occupy the same relative positions, vis-á-vis their oral secretions, as do the three species studied here.

7.6. Possibilities for further work

In addition to the possibilities outlined above, the data on mucosal

masses, as a fraction of total body mass, suggest another avenue of possible enquiry.

Radioimmunoassay of blood pepsinogen may give useful information in the context of the hypothesis, besides offering the advantage of providing data without requiring the animal's demise in pursuit of an academic question.

Another question deserving attention in the general context of the problem is the possibility of differential secretion of PI and PII zymogens in relation to temperature stimuli. A relevant technique here is the use of tissue culture to maintain gastric mucosal tissue in an active state. This has been done for guinea pig chief cells (Raufman et al, 1984), and for human and rabbit mucosae (Defize et al, 1984).

Generally, of course, this technique carries the appealing possibility of accumulating large quantities of relatively pure zymogens without heavy predation on natural and unstable animal populations.

7.7. CONCLUSIONS

1. The gastric mucosae of all three species are a good source of pepsinogens.

2. The pepsinogens in each species may be divided into two groups in terms of their masses, and charge characteristics as reflected in their responses to anion exchange chromatography.

3. In the cases of the cobra and mole snake, there are strong indications that pepsins of groups I and II differ in their activation energies. Such differences are smaller, or perhaps absent, between the PI and PII pepsins of the puff adder.

4. The group PII pepsinogens in all three species are microheterogeneous. At least in terms of molecular mass, the mole snake PII pepsinogens have diverged more widely than those of the cobra and puff adder.

5. The PI pepsinogens in all three groups show less evidence of heterogeneity. Some limited evidence of heterogeneity exists in the case of the mole snake PI zymogen(s).

6. While the reported data carry no direct implications for taxonomy as such, some

evidence suggests that the cobra and puff adder are phylogenetically closer to each other than to the mole snake.

7. Some of the data support the suggestion that puff adder pepsins reflect adaptation to the presence, in this species, of highly proteolytic venom. This support derives essentially from the studies on Arrhenius activation energies, and suggests that this area may be the most promising, of those explored here, for further investigation.

APPENDIX

APPENDIX

1. ABBREVIATIONS

AGPA	a-amino-β-guanidinopropionic acid
APDT	N-acetyl-L-phenylalanyl-3,5-diiodo-L-tyrosine
BSA	Bovine Serum Albumin
BIS	N,N^1-methylene-bis-acrylamide
DIT	3,5-diiodotyrosine
EDTA	Ethylenediamine tetra acetic acid
IgG	Immunoglobulin Gamma
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate-buffered saline
PEG	Polyethylene glycol
SDS	Sodium dodecyl sulphate (= sodium lauryl sulphate)
TCA	Trichloracetic acid
TEMED	N,N,N',N',-tetramethylethylenediamine
TNBS	2,4,6-trinitrobenzenesulfonic acid
TRIS	Tris(hydroxymethyl) aminomethane

2. COMMERCIAL PIG PEPSINOGEN : MOISTURE CONTENT

Water content of the material (Sigma P 7250, Grade III, lot 29C - 0155) was not stated by the supplier. In view of the cost of the protein, water content was not determined in the course of this study. Approached on the matter, a representative of Sigma Chemicals (P.O. Box 14508, St. Louis, Missouri, 63178) reported that, "We have not assayed specifically for water content, but would expect it to be no more

than 5%," (C. DiCamelli, pers. comm.)

3. NINHYDRIN-APDT ASSAY

Ninhydrin	0,532 g
Methyl cellosolve	20,000 mℓ
Hydrindantin	0,080 g
Sodium acetate buffer, pH 5,5, 4,0M	6,600 mℓ

Store in dark. Use within two hours.

Above solution added (1,0 mℓ) to NaOH-adjusted incubation mixture, as described.

4. GOA'S MICROBIURET ASSAY

Benedict's Reagent

A.	Trisodium citrate	17,3 g
	Sodium carbonate	10,0 g
	Distilled water	c 80 mℓ
B.	Copper sulphate	1,73 g
	Distilled water	c 10 mℓ

Mix A) and B) slowly, with stirring, and make to 100 mℓ.

Method:

To 0,20 mℓ protein solution add 3,8 mℓ 0,75M (3% m/v) NaOH, followed by 0,20 mℓ Benedict's reagent. After 15 minutes read absorbance at 550 nm in 1,0 cm cells.

5. HARTREE/LOWRY PROTEIN ASSAY

A)	Sodium potassium tartrate	0,20 g
	Sodium carbonate	10,00 g
	Dissolve in sodium hydroxide, 1,0M,	50 mℓ
	Distilled water to make	100 mℓ
B)	Sodium-potassium tartrate	0,20 g
	Copper sulphate, $5H_2O$	0,10 g
	Distilled water	9,00 mℓ
	Sodium hydroxide, 1,0M,	1,00 mℓ

C) Folin-Ciocalteu reagent, diluted with 15 volumes of distilled water.

Method:

Add 1,0 mℓ protein samples (10 - 80 µg) to test tubes.

Add to each 0,9 mℓ solution A).

Place all tubes in waterbath at 50°C for 10 minutes.

Cool.

Add 1,0 mℓ solution B).

Leave at least 10 minutes.

Add 3,0 mℓ solution C) rapidly, with immediate stirring.

Incubate at 50°C for 10 minutes.

Cool.

Read at 650 nm against protein-free blank.

6. PRE-TREATMENT OF DIALYSIS MEMBRANES

Solution : 0,5% SDS in 0,01M EDTA.

Method:

1. Boil in above solution for 5 minutes.
2. Rinse.
3. Boil again in above solution.
4. Rinse thoroughly.
5. Store in 0,005M EDTA. Autoclave.

7. PHOSPHATE-BUFFERED SALINE (PBS)

Sodium dihydrogen phosphate, $2H_2O$	2,62 g
Disodium hydrogen phosphate (Anhyd.)	11,50 g
Distilled water to make	1 ℓ

(0,1M, pH 7,4)

Dilute to 0,02M and add sodium chloride 9g/ℓ.

8. POLYACRYLAMIDE GEL ELECTROPHORESIS : LAEMMLI TECHNIQUE

A) Acrylamide stock:

Acrylamide	30,0 g
Bis acrylamide	0,8 g
Water to make	100 mℓ

(Acrylamide : bis ratio = 37,5 : 1)

B) Resolving gel (10% acrylamide), for two slabs:

Acrylamide stock	13,5 mℓ
TRIS/HCl pH 8,8, (1,0M)	15,0 mℓ
Water	9,2 mℓ
---------- degas ----------	
SDS (10%)	0,4 mℓ
Ammonium persulphate (1,5%, fresh)	2,0 mℓ
TEMED	10 µℓ

C) Stacking gel (4% acrylamide), for two slabs:

Acrylamide stock	2,00 mℓ
TRIS/HCl pH 6,8, (1,0M)	1,90 mℓ
Water	9,25 mℓ
Glycerol (80%)	1,00 mℓ
SDS (10%)	0,15 mℓ
Ammonium persulphate (1,5%, fresh)	0,70 mℓ
TEMED	20 µℓ

D) Dissociation buffer:

SDS	5,0 g
2 mercapto-ethanol	5,0 mℓ
TRIS/HCl, pH 6,8, (1,0M)	35,0 mℓ

(Bromophenol blue and sucrose are added to the prepared samples before boiling.)

E) Bath buffer:

TRIS	15,15 g
Glycine	71,05 g
SDS	5,00 g

Water to make 500 mℓ

(Dilute 10 x for use).

F) Coomassie Blue Stain:

Methanol	45 mℓ
Acetic acid (glacial)	10 mℓ
Coomassie Brilliant Blue	0,2 g
Water to make	100 mℓ

(The dye is first dissolved in some of the methanol and filtered.)

G) Destaining solution:

Methanol	450 mℓ
Acetic acid (glacial)	70 mℓ
Water to	1000 mℓ

9. TRYPTIC PEPTIDE MAPPING : REAGENTS AND SOLUTIONS

1. Laemmli destain (see Appendix 8).
2. 10% and 50% methanol.
3. Sodium phosphate buffer, pH 7,5, 0,5M .
4. $Na^{125}I$.
5. Chloramine T.
6. Sodium bisulphite, 1 mg/mℓ.
7. Trypsin solution, 1 mg/mℓ.
8. Ammonium bicarbonate, 5mM.
9. Toluene.

10. BUFFERS FOR TRYPTIC PEPTIDE MAPPING

A) Buffer I:

Acetic acid : formic acid : water

15 : 5 : 80

(450 mℓ per plate, made fresh daily.)

B) Buffer II:

n-butanol : pyridine : acetic acid : water

32,5 : 25 : 5 : 20

REFERENCES

REFERENCES:

Abuharfeel, N.M. & G.M. Abuereish, 1984, "Isolation and Characterisation of Camel pepsins, in Comp. Biochem. Physiol., 77A : 175 - 182.

Al-Janabi, J., J.A. Hartsuck & Jordan Tang, 1972, "Kinetics and Mechanism of Pepsinogen Activation", J. Biol. Chem., 247 : 4628 - 4632.

Andreeva, N.S. & A.E. Gustchina, 1979, "On the supersecondary structure of acid proteases", in Biochem. Biophys. Res. Commun., 87 : 32 - 42.

Andreeva, N.S., A.E. Gustchina, A.A. Fedorov, N.E. Shutzkever & T.V. Volnova, 1977, "X-ray Crystallographic Studies of Pepsin", in Tang, J., (ed), Adv. Exp. Med. and Biol., 95 : 23 - 32.

Anson, M.L., 1938, "The Estimation of Pepsin, Trypsin, Papain and Cathepsin with haemoglobin", J. Gen. Physiol., 22 : 79.

Aoyagi, T., S. Kunimoto, H. Morishima, T. Takeuchi & H. Umezawa, 1971, "Effect of pepstatin on acid proteases", J. Antiobiotics, 14 : 687 - 694.

Arai, K.M., N. Muto, S. Tani & K. Akahane, 1984, "The N-terminal sequence of rat pepsinogen", in Biochem. Biophys. Acta, 788 : 256 - 261.

Arunchalam, K. & N.F. Haard, 1985, "Isolation and Characterisation of Pepsin from Polar Cod (Boreogadus saida)", in Comp. Biochem. Physiol., 80B : 467 - 473.

Baker, L. 1951, "New Synthetic substrates for Pepsin", in J. Biol. Chem., 193 : 809 - 819.

Bar-Eli, E. & T. Merrett, 1970, "Dogfish pepsinogens", in G.E. Perlmann & L. Lorand (eds.), Methods Enzymol., 19 : 364 - 372.

Barrett, A.J., 1969, "Properties of lysosomal enzymes", in Frontiers in Biology, 14B. Editors : A. Neuberger & E.L. Tatum, North Holland, Amsterdam.

Barrington, E.J.W., 1975, "The Alimentary Canal and Digestion", in Brown, M.E., (ed.), The Physiology of Fishes, 1, Academic Press, N.Y.

Bateson, G., 1979, Mind and Nature - a necessary unity, Wildwood House, (Great Britain).

Baudys, N & V. Kostka, 1983, "Covalent Structure of Chicken Pepsinogen", in Eur. J. Biochem., 136 : 89 - 99.

Berstad, A. 1970, "A Modified Haemoglobin Substrate method for the estimation of Pepsin in Gastric Juice", in Scand. J. Gastroent., 5 : 343 - 348.

Black, L.W. & D.S. Hogness, 1969, "The Lysozyme of Bacteriophage ," in J. Biol. Chem., 244 : 1976 - 1981.

Blain, A.W. & K.N. Campbell, 1942, "A Study of Digestive Phenomena in Snakes with the aid of Roentgen Kay", in Am. J. Roentgenol., 48 : 229 - 239.

Bohak, Z., 1969, "Purification and Characterisation of Chicken Pepsinogen and Chicken Pepsin", in J. Biol. Chem., 244 : 4638 - 4648.

Bohak, Z., 1970, "Chicken Pepsinogen and Chicken Pepsin", in Perlmann, G.E. & L. Lorand, (eds) Methods Enzymol., 19, Academic Press.

Bradford, M.M., 1976, "A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding", in Anal. Biochem., 72 : 248 - 254.

Brust, J. & H. Fasold, 1981, "Microfingerprints of Proteins through Labeling Acetylation of their Proteolytic Peptides", in Anal. Biochem., 116 : 268 - 272.

Bustin, M. & A. Conway-Jacobs, 1971, "Intramolecular Activation of Procine Pepsin", in J. Biol. Chem., 246 : 615 - 620.

Castellino, F.J. & R Barker, 1968, "Examination of the Dissociation of Multichain Proteins in Guanidine Hydrochloride by Membrana Osmometry", in Biochem., 7 : 2207 - 2217.

Cavadore, J-C, M. Cataldi, R. Steffens & D.M. Glick, 1979, "On the activation of the canine pepsinogens", in Biochimie, 61 : 355 - 360.

Cha, S., 1975, "Tight-Binding Inhibitors - I Kinetic Behaviour", in Biochem. Pharmacol., 24 : 2177 - 2185.

Chiang, L., L. Sanchez-Chiang, J.N. Mills & Jordan Tang, 1967, "Purification and Properties of Porcine Gastricsin", in J. Biol. Chem., 242 : 3098 - 3102.

Chow, R.B. & B. Kassell, 1968, "Bovine Pepsinogen and Pepsin", in J. Biol. Chem., 243 : 1718 - 1724.

Christensen, P.A., 1955, South African Snake Venoms and Antivenoms, Institute for Medical research, Johannesburg, South Africa.

Clement, G.E., 1973, "Catalytic activity of pepsin", in Kaiser, E.T. & Kezdy, (eds), Progress in Bio organic Chemistry, 2 : 177 - 238, John Wiley & Sons, N.Y.

Daniel, W.W., 1983, Biostatistics : A Foundation for Analysis in the Health Sciences, John Wiley & Sons, 3rd edition.

Davies, G. & G. Stark, 1970, "Use of Dimethyl Suberimidate, a Cross-Linking reagent, in Studying the Subunit Structure of Oligomeric Proteins", in Proc. Nat. Acad. Sci. U.S.A., 66 : 651 - 656.

Davies, P., 1983, God and the New Physics, Pelican Books.

Defize, J., G. Pals, R.R. Frants, B.D. Westerveld, S.G.M. Meuwissen & A.W. Erkisson, 1984, "Pepsinogen Synthesis and Secretion in isolated gastric glands", in J. Clin. Pathol (Lond.), 37 : 531 - 536.

Delpierre, G.R. & Joseph S. Fruton, 1965, "Inactivation of Pepsin by Diphenyldiazomethane", in Proc. Nat. Acad. Sci. U.S.A., 54 : 1161 - 116

Delpierre, G.R., 1968a, "Studies on African snakes venoms - II. Differentiation between proteinase and amino acid esterase activities of some African Viperidae venoms", Toxicon, 6 : 103.

Delpierre, G.R., 1968b, "Studies on African Snake Venoms - I. The Proteolytic Activities of some African Viperidae venoms", Toxicon, 5 : 233.

Diefenbach, C.O.da C., 1975, "Gastric Function in Caiman crocodilus (Crocodylia Reptilia) - I. Rate of Gastric Digestion and gastric motility as a function of temperature", Comp. Biochem. Physiol., 51 A : 259 - 265.

Dixon, M. & E.C. Webb, 1979, Enzymes, 3rd edit., Longmans.

Dubois, M., K. Gilles, J.K. Hamilton, P.A. Rebers & F. Smith, 1951, "A Colorimetric Method for the Determination of Sugars", Nature (London), 168 : 167.

Dunn, B.M., C. Deyrup, W.G. Moesching, W.A. Gilbert, R.J. Nolan & M.L. Trach, 1978, "Inhibition of Pepsin by Zymogen Activation Fragments", J. Biol. Chem., 253 : 7269 - 7275.

Dykes, C.W. & J. Kay, 1976, "Conversion of Pepsinogen into Pepsin is Not a One-Step Process", Biochem. J., 153 : 141 - 144.

Eagle, H., 1937, "The coagulation of blood by snake venoms and its physiological significance", J. Exp. Med., 65 : 613 - 639.

Elder, J.H., R.A. Pickett II, J. Hampton & R.A. Lerner, 1977, "Radioiodination of Proteins in Single Polyacrylamide Gel Slices", J. Biol. Chem., 252 : 6510 - 6515.

Estoe, J.E. & A. Courts, 1963, Practical Analytical Methods for Connective Tissue Proteins, E. & F.N. Spon Ltd.

Esumi, H., S. Yasugi, T. Mizuno & H. Fujiki, 1980, "Purification and Characterisation of a Pepsinogen and its Pepsin from Proventriculus of the Japanese Quail", Biochem. Biophys. Acta, 611 : 363 - 370.

Etherington, D.J. & W.H. Taylor, 1967, "Nomenclature of the Pepsins", Nature (London), 216 : 279 - 280.

Etherington, D.J. & W.H. Taylor, 1969, "The Pepsins of Normal Human Gastric Juice", Biochem. J., 113 : 663 - 668.

Field, E.O. & J.R.P. O'Brien, 1955, "Dissociation of Human Haemoglobin", Biochem. J., 60 : 656 - 661.

Fields, R., 1972, "The Rapid Determination of Amino Groups with TNBS", Methods Enzymol., 15 : 464 - 468.

Fitzsimons, V.F.M., 1962, Snakes of Southern Africa, Purnell & Sons, Johannesburg.

Foltmann, B., 1981, "Gastric Proteinases - Structure, Function, Evolution and Mechanism of Action", in Campbell, P.N. & R.D. Marshall, (eds), Essays in Biochemistry, 17 : 52 - 84, Academic Press.

Foltmann, B. & V.B. Pedersen, 1977, "Comparison of the primary structures of acidic proteinases and their zymogens", Adv. Exp. Med. and Biol., 95 : 3 - 22.

Fourie, J. R.S. Arnot, J. Carter, R. Hickman & J. Terblanche, 1974, "Measurement of Pepsin in Porcine Gastric Juice", S. Afr. Med. J., 48 : 1873 - 1875.

Fox, P.F., J.R. Whitaker & P.A. O'Leary, 1977, "Isolation and Characterisation of Sheep Pepsin", Biochem. J., 161 : 389 - 398.

Fruton, J.S., 1977, "Specificity and Mechanism of Pepsin Action on Synthetic Substrates", Adv. Exp. Med. and Biol., 95 : 131 - 140.

Ganong, W.F., 1979, Review of Medical Physiology, Lange Medical Publications, 9th ed., California.

Gans, C. & W.B. Elliott, 1968, "Snake venoms : Production, Injection, Action", in Advances in Oral Biology, Volume 3, Academic Press, N.Y.

Garvey, J.S., N.E. Cremer & D.H. Sussdorf, 1977, Methods in Immunology. A Laboratory Text for Instruction and Research, 3rd edit., W.A. Benjamin, Inc.

Gildberg, A. & J. Raa, 1983, "Purification and Characterisation of Pepsins from the Arctic Fish Capelin (Mallotus villosus)", Comp. Biochem. Physiol., 75 A : 337 - 342.

Goa, J., 1953, "A microbiuret method for protein determination. Determination of total protein in cerebro-spinal fluid", Scand. J. Clin. Lab. Invest., 5 : 218.

Gralén, N., 1939, "The splitting of haemoglobin by acids", Biochem. J., 33 : 1907 - 1912.

Green, M.L. & J.M. Llewellin, 1973, "The Purification and Properties of a single Chicken Pepsinogen Fraction and the Pepsin derived from it", Biochem. J., 133 : 105 - 115.

Haggstrom, G.D. & B.I. Hirschowitz, 1984, "Histamine H_1 and H_2 effects on Gastric Acid and Pepsin, Heart rate and Bloood Pressure in Humans", J. Pharmacol. Exp. Ther., 231 : 120 - 123.

Hally, A.D., S.M. Lloyd & T. Scratcherd, 1976, "Gastrointestinal System", in Passmore, R. & J.S. Robson (eds), A Companion to Medical Studies : 1 : Anatomy, Biochemistry, Physiology and related subjects, 2nd edit., Blackwell Scientific Publications.

Harboe, M., P.M. Andersen & B. Foltmann, 1974, "The activation of bovine pepsinogen", J. Biol. Chem., 249 : 4487 - 4494.

Hartree, E.F., 1972, "Determination of protein. A modification of the Lowry Method that gives a linear photometric response", Anal. Biochem., 48 : 422 - 427.

Hartsuck, J.A. & J. Tang, 1972, "The carboxylate ion in the active centre of pepsin", J. Biol. Chem., 247 : 2575 - 2580.

Henderson, P.J.F., 1972, "A linear equation that describes the steady-state kinetics of enzymes and subcellular particles interacting with tightly bound inhibitors", Biochem. J., 127 : 321 - 333.

Herriott, R.M., 1939, "Kinetics of the formation of pepsin from swine pepsinogen and identification of an intermediate compound", J. Gen. Physiol., 22 : 65 - 78.

Herriott, R.M., 1955, "Swine pepsin and pepsinogen", in Colowick, S.P. & N.O. Kaplan, (eds), Methods Enzymol., 2 : 3 - 7, Academic Press.

Herriott, R.M., 1962, "Pepsinogen and pepsin", J. Gen. Physiol., 45 : 57 - 76.

Hirschowitz, B.I., 1955, "Pepsinogen in the blood", J. Lab. Clin. Med., 46 : 568 - 579.

Hirschowitz, B.I., 1967, "Secretion of pepsinogen", in Heidel, W., (ed.), Handbook of Physiology. Section 6, Alimentary Canal, Vol. II., Secretion : 889 - 918, American Physiological Society, Washinton.

Hodge, J.E. & B.T. Hofreiter, 1962, "Phenol-sulphuric colometric method", in Whistler, R.L. & M.L. Wolfrom, (eds), Methods in carbohydrate chemistry, I : 388 - 389, Academic Press.

Holladay, M.W., F.G. Salituro, P.G. Schmidt & D.H. Rich, 1985, "Pepsin-catalysed addition of water to a Retomethylene peptide isostere : observation of the tetrahedral species by carbon-13 nuclear magnetic resonance spectroscopy", Biochem. Soc. Trans., 13 : 1046 - 1048.

Inoue, M., J. Fong, G. Shah & B.I. Hirschowitz, 1985, "Mediation of muscarinic stimulation of pepsinogen secretion in the frog", Am. J. Physiol., 248 : G79 - G86.

Itzhaki, R. & D.M. Gill, 1964, "A micro-biuret method for estimating proteins", Anal. Biochem., 9 : 401- 410.

Jackson, W.T., M. Schlamowitz & A. Shaw, 1965, "Kinetics of the pepsin-catalysed hydrolysis of N-acetyl-L-phenyl-alanyl-L-diiodotyronine", Biochem., 4 : 1537 - 1543.

James, M.N.G., I-Nan Hsu & L.T.J. Delbaere, 1977, "Mechanism of acid protease catalysis based on the crystal structure of penicillo-pepsin", Nature (London), 267 : 808 - 813.

Jiménez-Porras, J.M., 1964, "Intraspecific variations in composition of venom of the jumping viper Bothrops numinifera", Toxicon, 2 : 187 - 195.

Kageyama, T. & K. Takahashi, 1976a, "Pepsinogens and pepsins from gastric mucosa of Japanese Monkey", J. Biochem., (Tokyo), 79 : 455 - 468.

Kageyama, T. & K. Takahashi, 1976b, "Pepsinogen C and Pepsin C from gastric mucosa of Japanese Monkey. Purification and Characterisation", J. Biochem., (Tokyo), 80 : 983 - 992.

Kageyama, T. and K. Takahashi, 1980, "Monkey pepsinogens and pepsins. Purification, characterisation and amino-terminal sequence determination of Crab-Eating Monkey pepsinogens and pepsins", J. Biochem. (Tokyo), 88 : 635 - 645.

Kageyama, T. & K. Takahashi, 1982, "Monkey pepsinogens and pepsins. VI. One-step activation of Japanese Monkey pepsinogen to pepsin", J. Biochem. (Tokyo), 92 : 1179 - 1188.

Kageyama, T. & K. Takahashi, 1983a, "Occurrence of two different pathways in the activation of porcine pepsinogen to pepsin", J. Biochem., Tokyo), 93 : 743 - 754.

Kageyama, T. & K. Takahashi, 1983b, "Purification and characterisation of pepsinogens and pepsins from Asiatic Black Bear, and amino acid sequence determination of the NH_2-terminal 60 residues of the major pepsinogen", J. Biochem., (Tokyo), 94 : 1557 - 1568.

Kageyama, T. & K. Takahashi, 1984, "Rabbit pepsinogens. Purification, characterisation, analysis of the conversion process to pepsin and determination of the NH_2-terminal amino-acid sequences", Eur. J. Biochem., 141 : 261 - 269.

Kageyama, T. & K. Takahashi, 1985, "Monkey pepsinogens and Pepsins. VII. Analysis of the activation process and determination of the NH_2-terminal 60-residue sequence of Japanese Monkey Progastricsin, and molecular evolution of pepsinogens", J. Biochem., (Tokyo), 97 : 1235 - 1246.

Kay, J., 1980, "Zymogen activation systems", in Freedman, R.B. & H.C. Hawkins, (eds), The Enzymology of Post-translational modification of proteins : 1, Academic Press.

Kassell, B. & P.A. Meitner, 1970, "Bovine pepsinogen and pepsin", Methods Enzymol., 19 : 337 - 347.

Kew, O.M., M.A. Pallansch, D.R. Omilianowski & R.R. Rueckert, 1980, "Changes in three of the four coat proteins of oral polio vaccine strain derived from Type I POliovirus", J. Virol., 33 : 256 - 263.

Kunimoto, S., T. Aoyagi, R. Nishizawa, T. Komai, T. Takeuchi & H. Umezawa, 1974, "Mechanism of inhibition of pepsin by pepstatin. II", J. Antibiotics, 27 : 413 - 418.

Laemmli, U.K., 1970, "Cleavage of structural proteins during the assembly of the head of Bacteriophage T_4", Nature, (London), 227 : 680 - 685.

Laurent, T.C. & J. Killander, 1964, "A theory of gel filtration and its experimental verification", J. Chromatog., 14 : 317 - 330.

Lechago, J., N.C.J. Sun & W.M. Weinstein, 1979, "Simultaneous visualization of two antigens in the same tissue section by combining immunoperoxidase with immunofluorescence techniques", J. Histochem. Cytochem., 27(a) : 1221 - 1225.

Lee, D. & A.P. Ryle, 1967, "Pepsin A minor component of commercial pepsin preparations", Biochem. J., 104 : 742 - 748.

Levchuck, T.P. & V.N. Orekhovich, 1963, "Production and some properties of chick pepsin", Biochemistry (Russian) (Eng. translation), 28 : 1004 - 1010.

Levine, D.F. & M. Beer, 1984, "Measurement of plasma group I pepsinogens", Postgrad. Med. J., 60 : 582 - 585.

Lin, Y., G.E. Means & R.E. Feeney, 1969, "The action of proteolytic enzymes on N,N-dimethyl proteins", J. Biol. Chem., 244 : 789 - 793.

Low, P.S., J.L. Bada & G.N. Somero, 1973, "Temperature adaptation enzymes : role of the free energy, the enthalpy and the entropy of activation", Proc. Nat. Acad. Sci., U.S.A., 70 : 430 - 432.

Lowry, O.H., N.J. Rosebrough, A.L. Farr & R.J. Randall, 1951, "Protein measurement with the Folin phenol reagent", J. Biol. Chem., 193 : 265 - 275.

Maclean, N., 1978, Haemoglobin , Institute of Biology's Studies in Biology, 93, Edward Arnold.

Magee, D.F. & S. Naruse, 1983, "Neural control of periodic secretion of the pancreas and the stomach of fasting dogs", J. Physiol., 344 : 153 - 160.

Mahler, H.R. & E.H. Cordes, 1966, Biological Chemistry, Harper & Row, New York & London.

Marciniszyn, J. J.A. Hartsuck & J. Tang, 1976a, "Mode of inhibition of acid proteases by pepstatin", J. Biol. Chem., 251 : 7088 - 7094.

Marciniszyn, J., J.S. Huang, J.A. Hartsuck & J. Tang, 1976b, "Mechanism of intramolecular activation of pepsinogen", J. Biol. Chem., 251 : 7095 - 7102.

McPhie, P., 1977, "On the Apparent Inhibition of Intramolecular Activation Pepsinogen by Pepsin Substrates", J. Biol. Chem., 252 : 4438 - 4439.

Means, J. & R,E. Feeney, 1968, "Reductive alkylation of amino groups in proteins", Biochemistry, 7 : 2192 - 2201.

Mebs, D., 1970, "A comparative study of enzyme activities in snake venoms", Int. J. Biochem., 1 : 335 - 342.

Merrett, T.G., E. Bar-Eli & H. Van Vunakis, 1969, "Pepsinogens A, C and D from the smooth dogfish", Biochem., 8 : 3696 - 3702.

Minton, S.A. & M.R. Minton, 1969, Venomous Reptiles, Allen & Unwin, London.

Mirsky, A., P. Futterman, S. Kaplan & R.H. Broh-Kahn, 1952, "Blood plasma pepsinogen 1. The source, properties, and assay of the proteolytic activity of plasma at acid reactions", J. Lab. Clin. Med., 40 : 17 - 26.

Moore, S. 1972, "The precision and sensitivity of amino acid analysis", in Meienhofer, J., (ed.), Chemistry and Biology of Peptides, pp. 629 - 653, Ann Arbor Science Publishers.

Moore, S. & W.H. Stein, 1954, "A modified Ninhydrin reagent for the photometric determination of amino acids and related compounds", J. Biol. Chem., 211 : 907 - 913.

Murata, Y., M. Satake & T. Suzuki, 1963, "Studies on snake venom. XII. Distribution of proteinase activities among Japanese and Formosan snake venoms", J. Biochem., 53 : 431 - 437.

Narasinga Rao, D., S.N. Koselak & J.A. Hartsuck, 1977, "Crystallization and preliminary crystal data of porcine pepsinogen", J. Biol. Chem., 252 : 8728 - 8730.

Netter, H., 1969, Theoretical Biochemistry, 2nd edit., Oliver & Boyd, Edinburgh.

Neumann, H. & U. Zehavi, 1969, "Acid labile sugar in pepsinogen", Biochem. Biophys. Res. Comm., 36 : 151 - 155.

Nomura, A.M.Y., G.N. Stemmermann & I.M. Samloff, 1980, "Serum pepsinogen I as a predictor of stomach cancer", Ann. Int. Med., 93 : 537 - 540.

Northrop, J.H., 1930, "Crystalline pepsin. 1. Isolation and tests of purity", J. Gen. Physiol., 13 : 739 - 780.

Nyman, M.M 1959, Supplement 39, Scand. J. Clin. Lab. Invest., 11 : 43.

Okonogi, T. M. Homma & S. Hoshi, 1964, "Pathological studies on Habu-snake bite", Gumma J. Med. Sci., 13 : 101.

Ouchterlony, Ö., 1958, "Diffusion-in-gel methods for immunological analysis", in Kallos, P., (ed.) Progress in Allergy, 5 : 1 - 78, Basel, Karger.

Pickford, L.M. & A.T. Lambie, 1976, "Kidney" in Passmore, R. & Robson, J.S., A companion to medical studies, 1. Anatomy, Biochemistry, Physiology and related subjects, 2nd edit., Blackwell Scientific Publications.

Piskiewicz, D., 1977, Kinetics of Chemical and Enzyme-Catalysed Reactions, Oxford University Press, N.Y.

Plummer, D.T., 1978, Introduction to Practical Biochemistry, 2nd edit., McGraw Hill (UK).

Powers, J.C., A.D. Harley & D.V. Myers, 1977, "Subsite specificity of porcine pepsin", Adv. Exp. Med. and Biol., 95 : 141 - 157.

Press, E.M., R.R. Porter & J. Cebra, 1960, "The Isolation and Properties of a Proteolytic Enzyme, Cathepsin D, from Bovine Spleen", Biochem. J., 74 : 501 - 514.

Prosser, C.L. & F.A. Brown, 1961, Comparative Animal Physiology, 2nd edit., W.B. Saunders Company.

Purves, L.R., M. Purves, G.G. Lindsey & N.J. Linton, 1986, "Specific cleavage of fibrin-derived D-dimer by a metalloproteinase isolated from venom of the puff adder (Bitis arietans)", S. Afr. J. Sci., 82 : 30 - 33.

Rajagopalan, T.G., W.H. Stein & S. Moore, 1966a, "The inactivation of pepsin by diazoacetylnorleucine methyl ester", J. Biol. Chem., 241 : 4295 - 4297.

Rajagopalan, T.G., S. Moore & W.H. Stein, 1966b, "Pepsin from pepsinogen. Preparation and properties", J. Biol. Chem., 241 : 4940 - 4950.

Raufman, J.P., V.E. Sutcliff, D.K. Kasbekar, R.T. Jensen & J D. Gardner, 1984, "Pepsinogen secretion from dispersed chief cells from guinea pig stomach", Am. J. Physiol., 247 : G95 - G104.

Ravenscroft, N. E.H. Merrifield & A.M. Stephen, 1985, "Depolymerisation of the exopolysaccharide of Klebsiella K_{64} by means of bacteriophage Ø64 suspensions", S. Afr. J. Sci., 81 : 380.

Rich, D.H., E.T.O. Sun & E. Ulm, 1980. "Synthesis of analogues of the carboxyl protease inhibitor pepstatin. Effect of structure on inhibition of pepsin and renin", J. Med. Chem., 23 : 27 - 33.

Righetti, P.G., B.M. Molinari & G. Molinari, 1977, "Isoelectric focusing of milk-clotting enzymes", J. Dairy. Res., 44 : 69 - 72.

Robertson, S., 1979, "A preliminary report on gastric proteases in the puff adder (Bitis arietans, a. Merrein), Abstract, 4th Congress, South African Biochemical Society.

Robertson, S.S.D., K. Steyn & G.R. Delpierre, 1969, "Studies on African snake venoms - III. The caseinase activity of some African Elapidae venoms", Toxicon, 6 : 243 - 245.

Robertson, S.S.D., 1984, "Studies on gastric proteases in three South African snake species", (Abstract), S. Afr. J. Sci., 80 : 145.

Ryle, A.P., 1965, "Pepsinogen B : the zymogen of pepsin B", Biochem. J., 96 : 6 - 16.

Ryle, A.P., 1965b, "Behaviour of Polyethylene Glycol on Dialysis and Gel-filtration", Nature, (London), 206 : 1256.

Ryle, A.P. & M. Pauline Hamilton, 1966, "Pepsinogen C and Pepsin C. Further purification and amino acid composition", Biochem. J., 101 : 176 - 182.

Ryle, A.P., 1970, "The porcine pepsins and pepsinogens", in Perlmann, G.E. & L. Lorand (eds), Methods Enzymol., 19 : 316 - 336, Academic Press.

Ryle, A.P., 1984, "Pepsins, gastricsins and their zymogens", in Bergmeyer, H.U., (ed.), Methods of enzymatic analysis, V. Enzymes 3, 3rd edit., Verlag Chemie, Weinheim.

Ryle, A.P. & R.R. Porter, 1959, "Parapepsins : Two proteolytic enzymes associated with porcine pepsin", Biochem. J., 73 : 75 - 86.

Sanchez-Chiang, L. & P. y Oscar Ponce, 1982, "Purification and properties of Merluccius gayi pepsinogen and pepsin", (Spanish, with English abstract, Bol. Soc. Biol. de Concepción, Chile, 53 : 129 - 143.

Sanny, C.G., J.A. Hartsuck & J. Tang, 1975, "Conversion of pepsinogen to pepsin", J. Biol. Chem., 250 : 2635 - 2639.

Schlamowitz, M. & L.U. Peterson, 1959, "Studies on the optimum pH for the action of pepsin on "native" and denatured bovine serum albumin and bovine haemoglobin", J. Biol. Chem., 234 : 3137 - 3145.

Schlamowitz, M. & L.U. Peterson, 1961, "The effect of sodium chloride on peptic digestion of bovine serum albumin", Biochem. Biophys Acta, 46 : 381 - 383.

Schlamowitz, M. & R. Trujillo, 1968, "A new solubilizing group for synthetic pepsin substrates", Biochem. Biophys. Res. Commun., 33 : 156.

Schmidt, K.P., 1950, "Modes of evolution discernible in the taxonomy of snakes", Evolution, 4 : 79 - 86.

Schmidt, P.G., M.W. Holladay, F.G. Salituro & D.H. Rich, 1985, "Identification of oxygen nucleophiles in the tetrahedral intermediates : deuterium and oxygen-18 induced isotope shifts in carbon-13 NMR spectra of pepsin-bound ketone pseudosubstrates", Biochem. Biophys. Res. Commun., 129 : 597 - 602.

Schroeder, W.A., 1968, The Primary Structure of Proteins, Harper & Row, N.Y.

Seijffers, M.J., H.L. Segal & L.L. Miller, 1963, "Separation of pepsin I, pepsin II A, pepsin II B, and pepsin III from human gastric mucosa", Amer. J. Physiol., 205 : 1099 - 1105.

Sepulveda, P., J. Marciniszyn Jr., D. Liu & J. Tang, 1975, "Primary structure of porcine pepsin. III. Amino acid sequence of a cyanogen bromide fragment CB 2A, and the ocmplete structure of porcine pepsin", J. Biol. Chem., 250 : 5082 - 5088.

Shapiro, A.L., E. Vinuela & J.V. Maizel, 1967, "Molecular weight estimation of polypeptide chains by electrophoresis in SDS polyacrylamide gels", Biochem. Biophys. Res., 28 : 815 - 820.

Shewale, J.G. & J. Tang, 1984, "Amino acid sequence of porcine spleen cathepsin D", Proc. Nat. Acad. Sci. U.S.A., 81 : 3703 - 3707.

Skoczylas, R., 1970a, "Influence of temperature on gastric digestion in the grass snake, Natrix natrix L", Comp. Biochem. Physiol., 33 : 793 - 804.

Skoczylas, R., 1970b, "Salivary and gastric juice secretion in the grass snake, Natrix natrix L", Comp. Biochem. Physiol., 35 : 885 - 903.

Stemmermann, G.N., I.M. Samloff, A. Nomura & J.H. Walsh, 1980, "Serum pepsinogen I and gastrin in relation to extent and location of intestinal metaplasia in the surgically resected stomach", Dig. Dis. Sci., 25 : 680 - 687.

Subramanian, E., M. Liu, I.D.A. Swan & D.R. Davies, 1977, "The crystal structure of an acid protease from Rhizopus Chinensis (sic) at 2,5 A resolution", Adv. Exp. Med. Biol., 95 : 33 - 42.

Taggart, R.T., R.C. Karn, A.D. Merritt, P.L. Yu & P.M. Conneally, 1979, "Urinary pepsinogen isozymes : a highly polymorphic locus in man", Hum. Genet., 52 : 227 - 238.

Tanford, C., 1957, "The acid denaturation of ferrihemoglobin", J. Am. Chem. Soc., 79 : 3931 - 3932.

Tang, J., S. Wolf, R. Caputto & R.E. Trucco, 1959, "Isolation and crystallization of gastricsin from human gastric juice", J. Biol. Chem., 234 : 1174 - 1178.

Tang, J., 1965, "Competitive inhibition of pepsin by aliphatic alcohols", J. Biol. Chem., 240 : 3810 - 3815.

Tang, J., 1970, "Gastricsin and pepsin", in Perlmann, G.E. & L. Lorand, (eds), Methods Enzymol., 29 : 406 - 421.

Tang, J., 1971, "Specific and irreversible inactivation of pepsin by substrate-like epoxides", J. Biol. Chem., 246 : 4510 - 4517.

Tang, J., 1979, "Evolution in the structure and function of carboxyl proteases", Mol. Cell. Biochem., 26 : 93 - 109.

Taylor, W.H., 1962, "Proteinases of the stomach in health and disease", Physiol. Rev., 42 : 519 - 553.

Taylor, W.H., 1968, "Biochemistry of pepsins", in Heidel, W., (ed), Handbook of Physiology, Section 6, 5 : 2567 - 2578, American Physiological Society, Washington.

Thomas, R.G. & F.H. Pough, 1979, "The effect of Rattlesnake venom on digestion of prey", Toxicon, 17 : 221 - 228.

Twining, S.S., P.A. Alexander, K. Huibregtse & D.M. Glick, 1983, "A pepsinogen from Rainbow Trout", Comp. Biochem. Physiol., 75 B (1) : 109 - 112.

Umezawa, H., T. Aoyagi, H. Morishima, M. Hamada & T. Takeuchi, 1970, Pepstatin, a new pepsin inhibitor produced by actinomycetes", J. Antibiotics, 23 : 259 - 262.

Underwood, G., 1967, "A Contribution to the Classification of Snakes", Publication No. 653, Trustees of the British Museum (Natural History), London.

Van Vunakis, H. & R.M. Herriott, 1956, "Structural changes associated with the conversion of pepsinogen to pepsin. 1. The N-terminal amino residue and amino acid composition of the pepsin inhibitor", Biochim. Biophys. Acta, 22 : 537 - 543.

Visser, J. & D.S. Chapman, 1978, Snakes and Snakebite, Purnell & Sons, Cape Town.

Waldum, H.L. & P.G. Burhol, 1980, "The effect of Somatostatin on serum Group I pepsinogens (PGI), serum gastin, and gastric H^+ and pepsin secretion in Man", Scand. J.Gastroent., 15 : 425 - 431.

Waldum, H.L., P.G. Burhol & B.K. Straume, 1979, "Serum Group I pepisnogens during prolonged infusion of pentagastrin and secretin in Man", Scand. J. Gastroent., 14 : 761 - 768.

Walker, V. & W.H. Taylor, 1979, "Pepsin 5 in gastric juice : determination and relationship to the alkali-stable peptic activity", Gut, 20 : 977 - 982.

Webber, D.E. & S.M. Morrissey, 1979, "Separate hypoglycaemic thresholds for gastric acid and pepsin secretion following insulin or posterior hypothalamic stimulation", Can. J. Physiol. Pharmacol., 57 : 1283 - 1288.

Weber, K. & M. Osborn, 1969, "The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis", J. Biol. Chem., 244 : 4406 - 4412.

Weber, K. & M. Osborn, 1975, "Proteins and sodium dodecyl sulfate : molecular weight determination on polyacrylamide gels and related procedures", in Neurath, H., R.L. Hill & C-L Boeder, (eds), The Proteins, 1, 3rd edit., Academic Press, N.Y.

Weiner, A.M., T. Platt & K. Weber, 1972, "Amino-terminal sequence analysis of proteins purified on a nanomole scale by gel electrophoresis", J. Biol. Chem., 247 : 3242 - 3251.

Whitaker, J.R., 1963, "Determination of molecular weights of proteins by gel filtration on Sephadex", Anal. Chem., 35 : 1950 - 1953.

White, T.J. & I.M. Ibrahimi, 1978, "Evolutionary substitutions and the antigenic structure of globular proteins", Nature, (London), 274 : 92 - 94.

Williams, B.L. & K. Wilson, (eds), 1975, A Biologist's Guide to Principles and Techniques of Practical Biochemistry, Edward Arnold, London.

Wolfenden, R., 1976, "Transition state analog inhibitors and enzyme catalysis", Ann. Rev. Biophys. Bioeng., 5 : 271 - 306.

Workman, R.J. & D.W. Burkitt, 1979, "Pepsin Inhibition by a High Specific Activity Radioiodinated Derivative of Pepstatin", Arch. Biochem. Biophys., 194 : 157 - 164.

Wright, R.D., H.W. Florey & A.G. Saunders, 1957, "Observations on the gastric mucosa of reptilia", Quart. J. Exptl. Physiol., 42 : 1 - 14.

Zeller, E.A., 1948, "Enzymes of snake venoms and their biological significance", Adv. Enzymol., 8 : 459 - 495.