X

PROTEOLYTIC ACTIVITY IN ADULT HAEMONCHUS CONTORTOS

BY

SAMMY T. SACKEY

A Thesis submitted to the Department iBf Biochemistry, University of Ghana for the Award of a Master *of* Science Degree in BIOCHEMISTRY

DEPARTMENT OP BIOCHEMISTRY
UNIVERSITY OF GHANA
L E G 0 N.

1982

sfT ^ bq - H-4- S.a.

IT.ai Cl U_co'>v\

DECLARATION

The experimental work described in this Thesis was carried out by me at the Department of Biochemistry, University of Ghana, Logon, under the supervision of Dr. K.K. Odure. I certify that this work has not previously been accepted for any degree and is not being concurrently submitted in candidature for any other degree.

Signed

(SAMMY T. SACKEY) CANDIBATE

(DR. K.K. ODURO) SUPERVISOR

1982

Dedication

to my family

for standing by mo

all this whilo

ACKNOWLEDGEMENTS

I should like te express my deepest gratitude te Dr. Kvabena Kenadu Odiare fer his steadfast guidance throughout the peried this project was undertaken.

I am grateful also te Prof. R.K.G. Asseku of the Faculty of Agriculture, University of Ghana, Logon* who told me about Haomenchus ggntortus. Dr. Amanfre and his staff at the Veterinary Services Department who shoved mo the verm* and Messrs Mensah and Laryea and their staff at the Jamestesn Slaughterhouse vho gave them te me. My gratitude also goes te the senior members* Biochemistry Department* particularly Dr. Fred Gyang vho provided me vith seme of the synthetic substrates used* Bob Acquah vho offered advice and encouragement* and te the Technicians in the department fer all the assistance.

Finally J should like to say thanks te Mr. Oferi— Anyinam vho drew the figures* and my friends Dan Mahama and Judy Quarshie vho put my scribble into neat type.

While acknowledging the assistance ef these good people fer the credit* I accept full responsibility fer any shortcomings in form and content.

TAPT.IC OF CONTENTS

LIST OP TABLES

LIST OF FIGURES

PROTEOLYTIC ACTIVITY IN ADULT HAEMONCHUS CONTORTUS

ABSTRACT,

Proteolytic activity of theJQ.qOOxgsupernate of homegenates ef adult Haemonchus contortus has been studied with respect to substrates susceptible to its proteolytic activity, and the effects of factors such as temperature, pH, substrate concentration, extract concentration, dibasic metal ions, detergents, heat and some specific inhibitors of proteolytic activity.

The supernate had two pH optima, 5.6 with haemoglobii as substrate, and 7.8 using L-alanyl - p-nitroanilide. It showed optimum activity at 36°C, was heat labile and in general was not significantly affected by the presence of metal ions in the hydrolysis of haemoglobin and L-alanyl p-nitroanilide. In screening for substrates susceptible to its hydrolytic action the most suitable were found to be those p-nitroanilides and jp-naphthylamides which have a single amino acid group (small R-group), unprotected at the free functional group end.

The kinetic studies indicated that haemoglobin was more rfeadily hydrolysed than the p-nitroanilides and p-naphthylamides.

C H A P T E R O N E

INTRODUCTION AND LITERATURE REVIEW

The sheep stomach worm, Ha.eaioB.chuB heritortus. is a member of the nematode family trichostrongylidae. The adult worm is recognizable by a finely drawn out head with a well developed bursa without a large buccal capsule, (1)

Haemonchosis is an important disease of sheep* goats and cattle. The disease exerts its greater economic effect in sheep in temperate and tropical countries especially where there is a good summer rainfall* While it is not uncommon for serious outbreaks to occur in colder climates such as those of the Northern United States and Canada when humdity is high in summer,the disease is rare in arid regions. (2)

1.2 Economic Importance of Haemonchosis

Haemonchosis of ruminants has always been considered of major economic importance throughout the world history of livestock production. The great fecundity of Haemonchus. greater than the fecundity of other trichostrongylid nematodes, and the continued use of pastures for the production of the world* s supply of meat and fibres ensures the continued prevalence of the parasite (3). In Ghana, livestock production assumes an added importance because of the inadequate protein in the diet of the majority of the inhabitants. The 1939 report of the Committee on Nutrition for the Colonial Empire reported as a result of a survey in Ghana that "the diet is deficient in those foodstuffs which

previde fat, g««d protein, vitamins and mineral matter..... the protein content la generally lew" (4). Teday the situation has net significantly altered; if anything, it has worsened. Dae te the presence ef tsetsafly ever large areas ef the country and the cerrespending incidence ef trypanosomiasis, livestock preductien has been limited mainly te the relatively fly free areas in the northern regien ef the ceuntry. As te the diseases that affect livesteck preductien, Jackson's 1965 assessment ef haomonchus as ene ef the three most important parasites ef ruminants (5) has net changed.

1,3 Life Cycle and Epidemiology

Studies on the life cycle ef the nematode (1,6-9) have shewn that haemenchns centortns has two larval stages present in an obligate free living existence. Bggs passed in the faeces hatch into the first larval stage which feeds actively, grows, moults and enters the second larval stage in which the feeding and growth continue. At the end ef the second larval stage its free life is ever, and from then on it is sustained on nutrient stored within its cells nntil it enters a host and starts parasitic life. This development from egg te infective larva occurs in a period ef two weeks or less.

The infective larvae are ingested with the vegetation

»BH i_n the intestines bury themselves between the villi in the glwads fer a brief period until the third raeult is completed. The third stage larvae then acquire provisional mouth capsules with which they adhere to the mucosa and then moult fer the fourth and last time with the acquisition of definitive mouth and reproductive organs. The adult worm lives in the abemassum of the definitive host. The males are 10-20mm long, and the females 18-30mm long. White uteri and ovaries wound around the bleed filled intestines give them the twisted appearance (6).

1,*k* Pathegonosi s

Haemenchosis is, for the most part, a primary parasitosis* predisposing causes fer infestation including overcrowding, lush pastures and hot humid climatic conditions. Development of clinical illness is however, favoured by a fall in the plane ef nutrition. Though the importance of diet as a predisposing cause is debatable, there is good evidence that a ration very lew (less than 3?0 in protein makes rtunina&ts much mere susceptible to heavy infestation than a normal diet; the worms become established mere readily and persist fer longer periods (2).

The attachment ef the parasite has a number ef effects on the host. Firstly, the membrane is deprived ef the living epthollnm and shows defects in the superficial layers of the propria mucosa. The physical damage favours

the entrance of bacteria and the absorption of toxins (8,9). Besides these primary effects the presence of the helminths resalts in a reduction in the uptake and digestibility of food (10) the utilisation of digested energy and a reduction in calcium deposition in the host animal (11). The villi are damaged leading to a reduced absorption of amino acids, fats and minerals. Sykes and Coop (11) have suggested that reduction in Nitrogen digestibility due to abomassal parasitism could result not only from reduced digestion of exogenous protein but also from increased endogenous losses into the alimentary tract, such as leakage of serum proteins, digestive secretions or cell sloughings. The reduction in protein digestion, they further suggested, could also result from the loss of differentiation and function of acid secreting parietal cells and pepsinogen secreting ehief eells. Hyperglobuliaaemia and hypoalbumeaaemia associated with subclinical helminth infection results (12). Heavy infections are also characterised by a high pH and low pepsin concentrations in the abomassum suggesting that gastric function is significantly reduced in heavy infection (13).

The parasites* persistence in the host animal to cause these destructive effects is made possible by its possession of a number of protective mechanisms against expulsion from

or destruction by the host. Protection from the host digestive enzymes such as trypsin is explained by the fact that those enzymes are specific to proteins different in structure from that constituting the sheath encasing the parasite (l4). Besides this* ttaemonchus contortus is thought to produce resistance stimulating antigens during the moulting of the infective stage (15).

1.5 Clinical Findings

Since the late 1950's Increased emphasis has been placed on the study of the physiological and biochemical changes which occur in animals and man during the course of nematode infection. Some of this attention has been directed at the mechanism of the anaemias which develop in lambs infested by the members of the family Trlchostrongvlidae.

Fourie {16) in 1931 and Andrews (17) in 19^2 showed that when members of the genus Haemonchus are the predominant parasites, the anaemia produced is of a haemorrhagic nature in which blood losses of up to 140ml per day have been estimated (18$_y$19). Whitlock (20) has suggested that the peripheral blood picture may be quite varied depending on whether the disease is complicated by a deficiency of iron or some other nutrient essential for the haemopoietic system. Shalm (21) demonstrated indications of decreased erythrepoiesis and Baker and Douglas (22) noted a reduced survival time of

red bleed cells within the vascular system. Baker and coworkers (19) associated serum iron binding and serum iron binding capacity with the anaemia caused by tfaomPM<?fo^{u}S and shewed that iron deficiency is the most important sign of haemonchosis. This phenomenon, it was suggested, was due either to interference with intestinal absorption or to the blood sucking propensity of the worm. Examination of blood and abomassal contents by Ross and Todd (12) showed that heavy Infection resulted in serum albumin levels falling, a situation which was reversed by heavy concentrate indoor feeding. The packed cell volume (PCV) estimations supported the view that hemoconcentration is associated with a fall in the albumin level in early infection and haemodilution occurs as albumfn level rises after treatment. Downey (23) has shewn that theu^i there is no growth response to cobalt administration, liver cobalt levels of non supplemented lambs were in the range associated with cobalt deficiency. Ihe cobalt deficient animals were found to be less susceptible to the effects of infestation with Haemonchus contortus. Similar studies have been conducted to determine the relationship between various levels of other metal ions and infection by Haemonchus contortus (19, 2*k*),

h

t.6. Disease Control

The use of anthelmintics has been the most widespread method of combating gastrointestinal parasitism. Control methods have been thus largely dependent upon periodic treatment of animals, with the net result that animals are often allowed to remain untreated due to the logistics or the economics of the individual situations, or both (3)* Where chemicals are routinely used in a parasitic control programme, the livestock owner is often unaware that continuous administration of a drug has the property of screening for resistant strains within the species under attack (25),

In recent years, increasing emphasis has been placed on studies into control ef infestation by management procedure (26 - 30). There has been little agreement din the results of these studies especially because of the multiplicity of factors that affect infection and host responses. One useful result, however, has been the production of Bioclima-tographs for different geographic; areas for the estimation of possible levels of infestation at different times of the year (2).

1.7 Immunological Flndlngt

It was not till Stoll (7) and then Miller, (31) reported their work that the concept of immunologic

resistance against met&zoal parasites was recognised* The report by Stoll that sheep became resistant to infection with toaMaehns established the sheep-haemonchus system as a satisfactory model for the study of immunity against gastrointestinal nematodes. Haemonchus is fortunately a prolific egg producer* yielding large numbers of infective larvae essential for the production of antigens (32).

An experimental investigation of the nature and mechanism of the "self-cure phenomenon" in helminthiasis in sheep showed that as a result of the self-cure there was no release of heterologous antibodies into the blood stream of hosts (33). Further, the study showed that the administration of anti—histamine drugs prevented the fall in the egg count characteristic of the self-cure, but did not affect the subsequent rise in titre of the specific or homologous antibody. (Christie (34) has suggested that abrupt changes in the abomassal pH were partly responsible for the self-cure)

The introduction of a vaccine against helminths was first made by Jarrett and coworkers (35) iu studies in which infective larvae of cattle lung worm (dictvocaulus viviparous) attenuated by ionising radiation were given orally to calves. Irradiated vaccines were also made with Haemonchus contortus and Trichostrongvlus colubriformis (36)

Subsequent studies used naturally attenuated larvae (36). Scott his ©©workers (32) showed that resistance of young rapidly growing lambs against initial inoculations of haemonchus larvae depended on the capacity of the haeraopoietlc system to compensate for blood loss and on the ability of the immune mechanisms of the hosts to reduce the severity of the infection. Wilson and Samson (37) have also shown that immunity to haemonchus was increased by the use of a longer immunizing period and by the use of multiple immunizing inoculations. Their studies also showed that a slight immunity may have been produced by subcutaneous inoculations of exsheathed larvae, but that no immunity was produced by intraperitoneal or intravenous inoculations. These studies into the immune reactions of host to helminth infection are considered important in the light of a continued need for the development of purified and stable antigen capable of being standardized and injected in controlled dosages.

1.8 ProteolyticEnzymes

The proteases are enzymes which are capable of hydrolysing the peptide bonds of simple peptides or proteins. The study of these enzymes has always maintained a prominent place in enzymology partly because of their usefulness in classifying problems in protein structure. Advances in the

knowledge of the proteases have contributed immensely to the knowledge of the understanding of many phases of the chemistry of proteins. In parallel with these developments the proteases, like other enzymes, have been studied with a view to gaining insights into their mode of action. The preteases have thus yielded a great deal of information concerning specificity, largely because of the availability *ok* excellent methods for the synthesis of peptides and peptide derivatives developed first by Emil Fisher and collaborators (38) and then by Max Bergmann and his coworkers (39)•

1.9 Proteolytic Activity In Parasites

In much the same way that the study of the proteins of protozoan parasites, their structure, synthesis activity and function is very much in its infancy *(b0)*, the information on helminthic proteins is scanty. This is, however, not because the enzymology of helminthic parasites is not important. It is a result of the difficulty in obtaining helminthic material for study, a problem accentuated by the complexity and number of developmental stages of these parasites. Besides the important uses of the information derived from studies of proteolytic enzymes mentioned above, the study of proteases of parasitic protozoa and helminths has additional uses. Whenever chemotherapy has been applied, the targets attacked have usually been important metabolic pathways like energy

production membrane function, cofactor, nucleic acid, protein and cell wall synthesis (4-0), all involving enzyme activity of one form or another. The effect of the drug may thus be due to its inhibition of one or more of these pathways. Besides this, it has been suggested that protective immunity against nematode infection is an enzyme—antienzyme mechanism in which the enzymes used by the parasite for invasion and nutrition serve as antigens, and anti-bodies formed against them in the host inhibit their activity and create an unfavourable environment for the parasite (4J)«

Proteases can be discharged by parasites into the environment either to initiate extracoporeal digestion in the ease of those with alimentary canals, or to assist during penetration into and migration through the host, functions which are frequently difficult to distinguish between (42). Tissue penetrating protozoa and helminths thus often contain enzymes active against the acellular components of the dermal connective tissues. There is histochemical evidence that some intestinal nematodes produce lessions in the host by extracoporeal digestion of the latter's Intestinal mucosa (8).

Antienzymes, including enzymes inhibiting the digestive enzymes of the host have been shown in nematodes and cestodes (43 - 45). De Waele (46) has shown that

intestinal nematodes and cestodes withstand trypsin, digestion in vitro for as long as they are alive and intact, bat are digested when dead or when the cuticle is damaged. Ascaria has been shown to possess at least one trypsin inhibitor and up to three chymotrypsin inhibitors. According to Rhodes and coworkers (4?), these inhibitors are low molecular weight proteins.

Various species of parasites utilize protein to a different degree for energy production. It is not known definitely whether, or to what extent endoparasitic stages of helminths utilize protein as energy source. Whilst the nature of the nitrogenous 4$ad products could be indicative of some such use, the evidence is not conclusive since the products in question could be derived from various other processes, e.g. syntheses and intereonversions (48).

1.10 Classification of Proteolytic Enzymes

On the basis of the manner in which proteases hydrolyse proteins and peptides, they have been classified as endopeptidases (proteinazes) or exopeptidases (peptidases) (3^). Exopeptidases also known as peptidyl hydrolases cleave consecutive carboxyl (COOH) or amino (NH^ or NH-) terminal amino acids from the end of the chain. They can thus be classified further into aminopeptidases and carboxy peptidases.

These enzymes usually require only two points of attachment on the substrate for their activity, the sensitive peptide bond, and a free or COOH group. A third group of exopeptidases is composed of peptidases which attack: dipoptides and tripeptides, the dipeptidases (49 — 51) and tripeptidases (52 - 55) respectively. These require three points of attachment, a sensitive peptide bond (or bonds), and both terminal amino acids.

The endo-peptidases hydrolyse internal peptide bonds. This is because the specificity sites will not accept substrates with free amino or carboxyl termini, unlike those of the exopeptidases which require one or both of these.

One concept for the classification of proteases, found no longer practicable, was based on the specificity towards synthetic substrates. This impracticability stems from the fact that not only do these enzymes show preference for certain amino acids in the bonds to be hydrolysed but they also exhibit some predilection for the type of bond.

Hartley (56) put forward the idea of classifying proteinases on the basis of catalytic mechanism rather than origin, substrate specificity or physiological

function. On this basis, Hartley grouped these enzymes into 'acid', 'metal*, thiol and serine proteinases. Barret (57) has since suggested modifications to this classification, giving the terms serine, cysteine, aspartic and metallic proteinases, considered as being more informative and accurate in description. The classification of proteinases into these groups has been facilitated by the recognition of various groups of specific inhibitors of proteolytic activity of the different groups of proteinases, e.g. diisopropyl-fluoro-phosphate as a specific inhibitor of serine proteinase activity (57).

1.11 Proteases of Helminthic Parasites

What information is available on the proteases of helminthic parasites reflects the diversity in the nature and form of these enzymes of parasites. True digestive trypsin-like enzymes have been found in the intestinal tract of Cordvlobia. (58) Gastrophilus larvae (59* 60) Ascari* lumbricoides, Strongvlus edentatus (61) and Lendvnemia appendiculata (62). Pepsin-like enzymes have not yet been found in the intestines of any parasites. Poly-and dipeptidases occur in some of them, sometimes in the form of multiple enzymes in the intestines of larval gastrophilus (60). Rhodes and coworkers (63) have

purified and characterised an intestinal amino peptidase of Ascarls- Leucine amino-peptidase shown, by Lee (64) to occur in the intestines, the anterior part of the excretory canals and the hypodermis of adult Ascarl3, has been detected in Porrocaecum decioiens £65)/ ^ Tricho strongylus colubriformis (66,67) end Haemonchus contortus» Xts role in the latter as an exsheatbment agent has been controversial. (68 - 70).

Besides the problem of availability of these helminth parasites for study, there is often encountered the problem of a suitable range of substrates for their study. Haemoglobin, because of the apparent dependence of Haemonchus contortus on the host blood, is a natural substrate for the study of its proteases.

1.12 Subject of Dissertation

This work has been designed to screen a number of available proteolytic substrates to determine their suitability for farther studies on the nature and types of proteases present in the adult worm. Kinetic studies as well as the relationship between enzyme activity and various experimental conditions were also determined to facilitate future and more detailed studies of these proteases. 'Those parameters studied include the effect of heat on enzyme integrity, the effect of hydrogen ion

concentration and the nature of the buffering ion, the effect of a amber of divalent ions and specific proteolytic inhibitors.

The effect of heat on enzyme integrity may be reversible te a point. It generally involves a denaturing of the protein unit, and thus depending on the extent of denaturing the protein molecule may revert to its original structure and functional ability once the degradative force has been removed. Different proteins have characteristic limitations on the extent to which they can revert to their original state. With respect to temperature effects, this phenomenon leads to the characterization of proteins (hence enzymes) as being heat labile or otherwise. Heat lability studies thus give an additional parameter for the characterization of the enzyme protein.

Enzymes as proteins have the ability to exist in different states of ionization. The enzymatic activity is, however, associated with a particular state (or states) of ionization. This functional state of ionization may be confined to the active site of the enzymes. Thus changes in the state of ionization of the active site may result in different levels of significance

of enzymatic activity. This parameter is thus important in their characterization into acid, neutral and basic enzygies corresponding to the pH ranges within which they exhibit optimum activity. The effect of different buffering ions may be due to the manner in which these combine with the substrate or enzyme ion. Phosphate ions for example are known to form metal salts which are not completely dissociated (71)* Thus in a situation where the metal ion is involved one way or the other in the enzymatic reaction, its removal by the phosphate ion could lead to an inhibition or activation of the enzyme. The effect of the buffering ion could also be purely one of steric hindrance of the enzyme-substrata binding, (71).

There is no absolute specificity in metal ion activ&tion; in all investigated cases more them one metal ion has pronounced effect on the catalytic activity of the protein. Malmstrong and Rosenburg (71) have suggested that there are distinct differences in the efficiency with which different metal ions act as activator for a given enzyme. They further suggested that meaningful conclusions can be drawn on these metal

ion effects only when these studies are conducted on relatively pure enzyme preparations using sensitive assay methods and varying concentrations of the metal ions. Indirect evidence of the participation of metal ions in the reaction mechanisms of aminopeptidases comes from the work of Smith (72) who concluded that almost all the exopeptidases were dependent for their activity on metal ions. The factors involved in metal ion activation include the effect of the metal ion on the stability of the enzyme complex (71)» stereochemical factors basically restricted to the size and charge of the metal ions (73) and the electronic properties of the activating metal ions (74). A wealth of information can thus be derived from detailed studies on the metal ion effects on enzymatic activity. In the particular ease of Haemonchus contortus. a number of metal ions have been shown to be implicated one way or the other in the aetiology of the disease, making them of special interest in enzymological studies.

2.1 Adult Haemonchus Contortus Collection and Preparation;

The contents of the abomassum of sheep and goats slaughtered and skinned at the Accra slaughterhouse, Jamestown, were squeezed out and transported to the laboratory. Using a snail mesh seive, the gat content was washed under running tap water to remove the fine particles and leave a clear wash. The debris containing the worms was transferred into a petri dish of water. The worms* recognizable by structure, pink-coloration and vigorous movement, were picked out and placed in a second petri dish containing physiological saline. The worms were kept frozen in physiological saline until required. Homogenates of 20mg worms/ml concentration ware prepared in physiological saline using a teflon peitle tissue homogenizer. The horaogenate was then centrifuged in the cold at 30,00Q}&pfor 30 minutes using an MSE MB' centrifuge. The supematent containing the 'enzyme' was kept frozen until required.

2.2a Assay For Enzyme Activity

2*2.0 (a) Proteolytic activity using Haemoglobin as substrate t

The method used is based on the measurement of the colour formed with Folin-Lowry reagent (75) by the tyrosine equivalent released in the enzymatic breakdown of denatured haemoglobin (76).

2.2i1 Preparation of Haemoglobin Snbstrate:

The haemoglobin substrate was prepared as described by Wirnt Rick (76) in the procedure attributed to Anson (77). Haemoglobin powder (2.00gm) was suspended in about 50ml of distilled water in a beaker and 36gms of urea and 8ml of 1.0M NaOH added. The mixture was stirred with a magnetic stirrer for an hour at room temperature within which time period the haemoglobin would be denatured. Ten ml of 6.2$ boric acid solution made 0.3$ with respect to NaCl was then added, and after further stirring 4.4ml of 5tf> Calcium chloride added. The substrate pH was adjusted to 7.5 using 0.5M HCJL and the solution diluted to a total volume of 100ml with distilled water. It was then filtered through Whatman No.4 filter paper to remove any debris. the solution was kept in a refrigerator at 8 C until required, each solution thus prepared being used within a week.

2.2.2 Method of Assay

The reaction mixtures used were of T.Oml aliquots incubated at 36°C except where otherwise stated. The substrate was buffered to the desired pH such as to give substrate and buffer concentrations of 1$ and 0.05M respectively. Except where otherwise stated 0.1ml of

haemoochus contortus 30,000)$£ soluble fraction containing 0.1 - 0.2mg of protein iras added to 0.9ml of buffered substrate equilibrated to the incubation temperature and thoroughly mixed vith a mechanical shaker. At the end of the incubation' period 2.0ml of 5$ Trichloroacetic acid (TCA) was added to stop the reaction by precipitating substrate and enzyme protein. The TCA also acted as a medium for the tyrosine equivalents released as a result of the enzymatic breakdown of the. haemoglobin. After thoroughly mixing, the mixture was left to stand for half an hour at room temperature, and then filtered through a Whatman No. *k* filter paper. All enzymatic reactions were done in dupliaate. Controls in which the TCA was added to the substrate at the end of the incubation period, before the enzyme was added were also ran in duplicate.

*2*2*3* Colour Reaction:

The method used is that developed by Lowry, yt al (75) as modified by Tach and Newburger {78). The tyrosine equivalent produced enzymatically was estimated from a tyrosine standard curve.

2,»2*.4 Effect of Triton X-100 On Homogenate

Aliquots of 0.10ml of the crude homogenate of Haemonchus contortus were treated with different concentrations of Triton X-100 detergent for one hour. Proteolytic activity and protein concentration were determined for each treatment.

The time effect of the detergent was determined by

treating 0# 1 ml aliquots of the homogenate with 0.20^ Triton X—100 and incubating for various time intervals. The proteolytic activity protein concentrations were subsequently determined for each treatment.

The above tests were also performed on the *50,000%%* supernatant.

2.2.5 Effect Of pH Of Reaction Medium On ProteolyticActivity

Aliquots of stock 2$ Haemoglobin substrate vere placed in boiling tubes and buffered to various pHs such as to give substrate and buffer concentrations of *1$* and 0.05M respectively* using distilled water where necessary to achieve the concentration requirement. The buffered substrates were used in the enzyme assays as described above.

2•2„6 Time Course Of Proteolytic Activity

The assay system described above was used* at optimum temperature and pH leaving the different reaction mixtures to run for different reaction times.

2,2'7 Effect Of Temperature On Proteolytic Activity

The enzyme assays were conducted at different temperatures

*2.2*38 Effect Of Enzyme Concentration On Proteolytic Activity

Reaction mixtures were prepared consisting of varying amounts of enzyme protein in a total volume of 0.5ml and 0.5ml of 1$ buffered substrate* and tyrosine equivalents measured as described.

2*9 Effect of Substrate Concentration On Enzyme Activity

Varying concentrations of buffered haemoglobin substrate (concentration determined gravimetrically and by the cyan-methaemoglbbin method(79)j were used with 0.1ml of enzyme sample. ; Oitrate-Phosphate buffer (0.05M, pH 5,6) was

used as diluent. Tyrosine equivalents were determined as described.

2*2 <*.10 Effect Of Different Buffers On Proteolytic Activity

The different buffers considered were used for the proteolytic activity determination.

2.2,!11 Effect Of Metal Ions On Proteolytic Activity

Aliquots of 0.1ml of the extract were placed in labelled test tubes and appropriate volumes of the stock metal ion solutions added so that in a final volume of 0.20ml the metal ion concentration was 1.0mM. The mixtures were left to stand at room temperature for 20 min. after which 0.80ml of haemoglobin substrate buffered to pH 5*6 and equilibrated to the reaction temperature was added. A control consisting of 0.10ml of the extract, 0.10ml distilled water and 0.80ml haemoglobin substrate was prepared.

2«2«;12 Tyrosine Standard Curve

A 1.OmM solution of L(-) Tyrosine prepared by dissolving 18.12mg of tyrosine in 2.0ml of 0.2M HC1 and making up to 100ml was used to prepare a tyrosine standard curve using the Folin-Lowry colour reaction. Hie resultant curve was used to estimate the tyrosine equivalent produced in the assays.

2.2,13 Bovine Serum Albumin (BSA) Standard Curve:

This curve was used to estimate the amount of protein in various preparations of the Haemonchus contortus extract used in the enzymatic reactions. A stock solution of BSA vas prepared by dissolving I.OOgm of BSA in sufficient 0.9$ saline *o give 100.00ml of solution.

Prom the stock solution various dilutions wore prepared for the Folin-Lowry reaction and a standard curve obtained.

2*2 £-14 Estimation Of Haemonchus fiontortus Extract Protein

For each preparation of the extract, the protein was estimated using: 0.10ml volumes extract in duplicate in the Folin-Lowry reaction. The amount of protein present was estimated from the BSA standard curve.

2.3.0 Proteolytic Activity Using Amino Acid p-Kitroanilides As Substrate:

The method used is based on the estimation at 405nm (80) of p-nitroaniline released from the enzymatic breakdown of amino-acid p-nitroanilides. The p-nitroanilides used were dissolved in 1$ formdimethylamide. In the assays the reaction mixture of total volume 4.0ml, was buffered to a final buffer concentration of 0.05M, made up as follows: 2.0ml 0.1M citrate"phosphate buffer& 1.0ml of 1.OmM substrate H 7-S, solutions except where otherwise stated, 0.9ml distilled water and 0.1ml of H*«nnnnniiTi« contortus extract. Controls were rfetn in which the substrate-buffer component was incubated, and the enzyme'added ^ ly before absorbance reading was taken. All tests and controls were run in duplicate. The p—nitroaniline produced in the enzymatic reaction was estimated from a standard curve of p-nitroaniline, after the reaction had gone on for 30 minutes or as otherwise stated.

2.3.1 **Of Amino Acid p-Nitroanilides For Suitability As Substrate For Proteolytic Activity**

One (l.O)mM solutions were used as substrates for enzymatic activity. Reaction rates with different substrates were estimated from a standard curve.

2.3.2 **Effect Of pH On Proteolytic Activity**

Citrate-phosphate (0.10M) buffers in the pH range 3-8 were prepared and used in the reaction mixtures, using L-alanyl p—nitroanilide as substrate in all tests. The p-nitroanilide produced was estimated from a standard curve.

2»3.3 **Time Course Of Enzyme Activity**

The time course of the proteolytic activity of the sample was determined using L-alanyl p-nitroanilide as substrate in a reaction mixture as outlined above.

.2,3.4 **Effect of Substrate Concentration OnProteolytic Activity** >

Reaction mixtures were prepared using different known volumes of stock 1.OmM L—alanyl p—nitroanilide in a total volume of 4.0ml.

2.3.5 **Effect of Heat On Sample Protein**:

Aliquots of 0.10ml of the sample were placed in labelled test tubes and 0.4ml volumes of distilled water added. Sets of four test tubes were each incubated at various known temperatures for ten minutes after which they were placed in ice chilled water for 5 minutes. Reaction

mixtures vara prepared by adding to each, tube buffered substrate consisting of 1.0ml stock 1mM L-alanyl p-nitroanilide, 2.0ml 0.1M citrate-phosphate buffer pH 7.5 and 0.5ml distilled water, preincubated at 36°C, The enzymatic reaction was run at 36°C for 30 minutes.

2.3,6 Effect Of Different Concentrations Of Metal Tons And Proteolytic Inhibitors On Enzyme Activity

Aliquots (0.10ml) of extract were added to such volumes of stock 2;.0mM metal ion and inhibitor solutions in a total volume of 0.50ml to give the required concentrations of the ions. The mixtures were left to stand at room temperature for 20 minutes. The enzyme reaction was started by adding preincubated mixture of 1.0ml L-alanyl p-nitroanilide >0mM stock solution, 2.0ml 0.1M citrate-phesphate buffer pH 7.5 and 0.5ml distilled water. A standard reaction mixture was prepared in which no metal ion was added to the enzyme. The p-nitroamiline released was estimated as before.

2.3.7 p-Nitroanillne Standard Curve

A standard curve was obtained by determination of the absorbance at 405nm of various known concentrations of the p-nitroaniline prepared from a stock 1.0mM solution in *ifo* formdimethylamide.

2.i4>0 Proteolytic Activity Using Amino Acid B-Nanhthvlamides As Substrates

The method used is based on the measurement of the colour produced by the diazonium salt coupling of enzymatically

released uaphthylamine $_{f}$ (81), The amino acid 0-naphthylamid® substrates were dissolved in 50$ methanol* Reaction mixtures consisted of 2.0ml of 0.10M citrate-phosphate buffer pH 7.5, 1.0ml of 1.0mM substrate (except where otherwise stated), 0.90ml of distilled water and 0.10ml of extract added to start reaction. The addition of 2.0ml of reagent consisting of 0.1$ w/v diazonium salt Fast Garnet GBC in 1.0M acetic acid buffer pH 4.2 made 10$ with respect to polyajtyethylene sorbitan mono-eleate (Tween 80) stopped the reaction as well as formed the mauve colour which was measured at 525nm 15 minutes after the colour reagent was added. The product formed was estimated from a 2-naphthylaraine standard curve. All tests and controls were run in duplicate. For the controls, the colour reagent was added before the extract was added. The absorbance readings were taken against a blank consisting of 2.0ml each of buffer, distilled water and dteonium salt reagent.

2 .4 Screening Of Amino Acid ft-naphthvlamides for Suitability As Substrates For Proteolytic Activity

One (1.O)mM solutions of the available amino acid p-napthylaaides were used as detailed above.

2.4,2 Effect Of L-alanyl fe-naphthvlanide Concentration On Proteolytic Activity

Various known volumes of stock 1.OmM solution of L-alanyl jMnaphthylanide were used in total reaction volumes

of 4.0ml and the 2-naphthylamine released was estimated as detailed above.

2 ..4*3 *2* naphthylamine Standard Curve

From an 0.1OmM stock solution of 2 naphthylamine in 50$ methanol various amounts were used in reaction mixtures containing 2.0ml buffer and distilled water to give 4.0ml and 2.0ml of diazonium salt reagent. The absorbance at 525nm$_N$was plotted against the concentration of the 2 -naphthylamine.

C H A P T E R T H R E E

RESULTS

3»1 Hawaonchtts Contortua Extract Preparation

The 3ppQ0g(| soluble fraction obtained had a pale yellow colour. Twenty mg worms/ml homogenates were prepared. Protein estimations for these ranged between 0.10 - 0.20rag protein per rag. of worms. The presence of host blood in the parasite may have accounted for both the colour of the extract, and some of the protein estimated.

3.2 AssaysFor Proteolytic Activity

Effect of Triton X-100 On Homogenate;

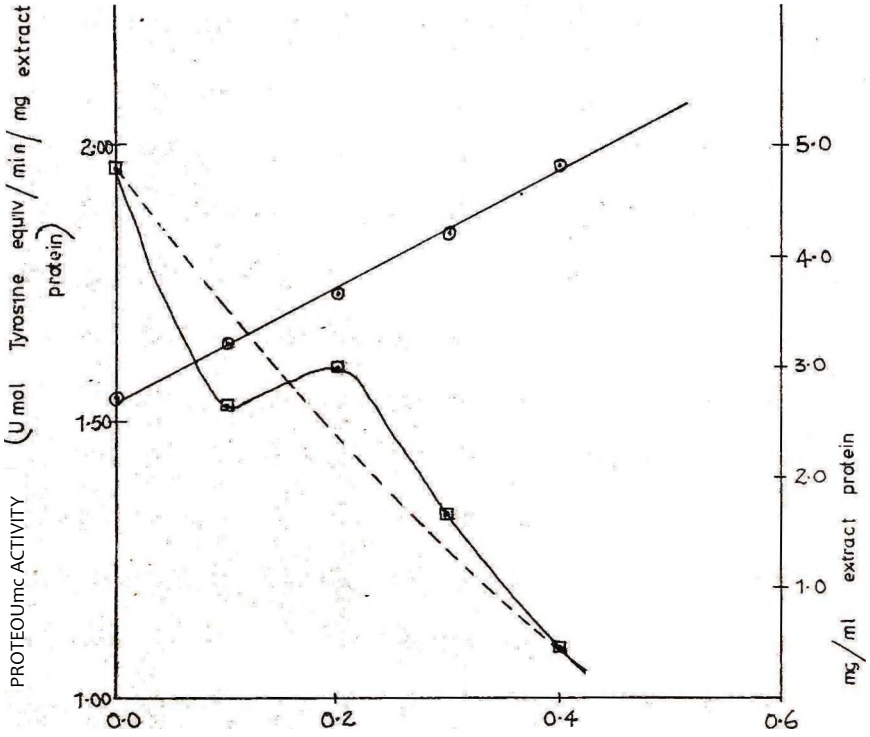
Figures X and XI show the concentration and time effects of Triton X-100 on the protein concentration and proteolytic activity of the homogenate. Proteolytic activity decreased with increasing concentration of Triton X-100 and time of incubation. Protein concentration however increased with increasing concentration of and time of incubation with Triton X-100.

With the 30, OOOysgsupernatent, there was no increase in protein concentration with either treatment but a decrease in proteolytic activity was observed for both treatments.

3.3 Effect Of pH Of Reaction Mixture On Proteolytic Activity

Figure III gives the proteolytic activity relative to different pHo of enzyme reaction mixtures for both haemoglobin and L-alanyl - p-nitroanilide substrates. Two pH optima, 5.6 and 7.8 were respectively recorded with the haemoglobin and amino acid p-nitroanilide substrates.

EFFECT OF CONCENTRATION OF TRYTON — X.100 DETERGENT ON PROTEIN (©) AND PROTEOLYTIC ACTIVITY. O* CRUDE HOMOGENATE

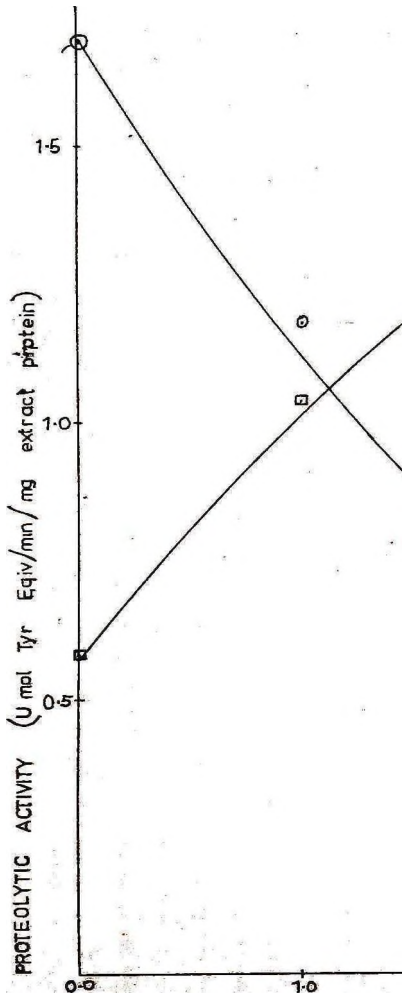


per cent concafttrtion of Triton - X 100

FT (5 I

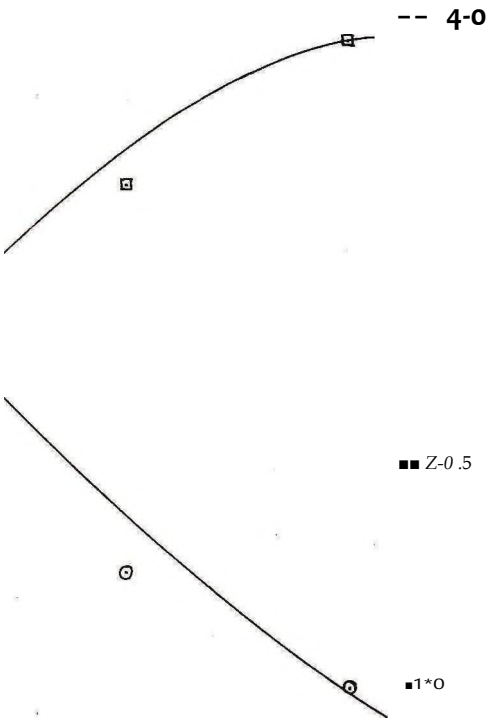
TIME EFFECT OF 0 - 2 %

AND ENZYME ACTIVITY(o) OF



PROTEIN CONCENTRATION (s)

HOMOGENATE



S ' O 0-0

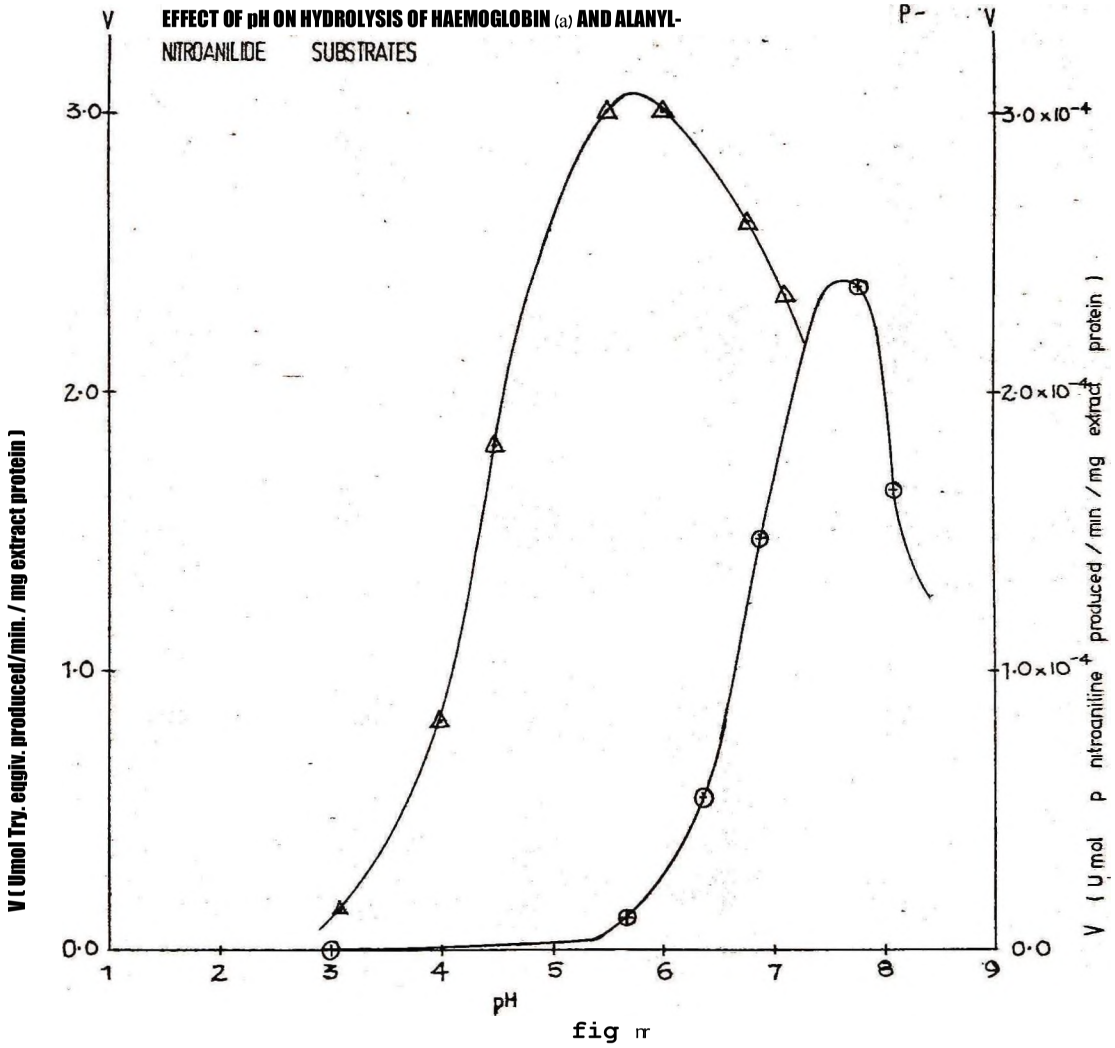

EFFECT OF pH ON HYDROLYSIS OF HAEMOGLOBIN (a) AND ALANYL-
NITROANILIDE SUBSTRATES
V (Umol Try. equiv. produced/min. / mg extract protein)
V (U mol p nitroaniline produced / min / mg extract protein)
pH
3·0
2·0
1·0
0·0
3·0 x 10⁻⁴
2·0 x 10⁻⁴
1·0 x 10⁻⁴
1
2
3
4
5
6
7
8
9

fig m

3.^ Time Coarse Of Enzymatic Activity

Figure IV shows the time course of proteolytic activity of the extract with two substrates, L-alanyl p—nitroanilide and haemoglobin. The amino acid p—nitroanilide showed a linear relationship between rate of product formation and time, for the 90 minutes the reaction was ran. In the case of haemoglobin substrate on the other hand, a linear relationship was observed only in the first 20 minutes of the reaction.

3.5 Optimum Temperature For Proteolytic Activity

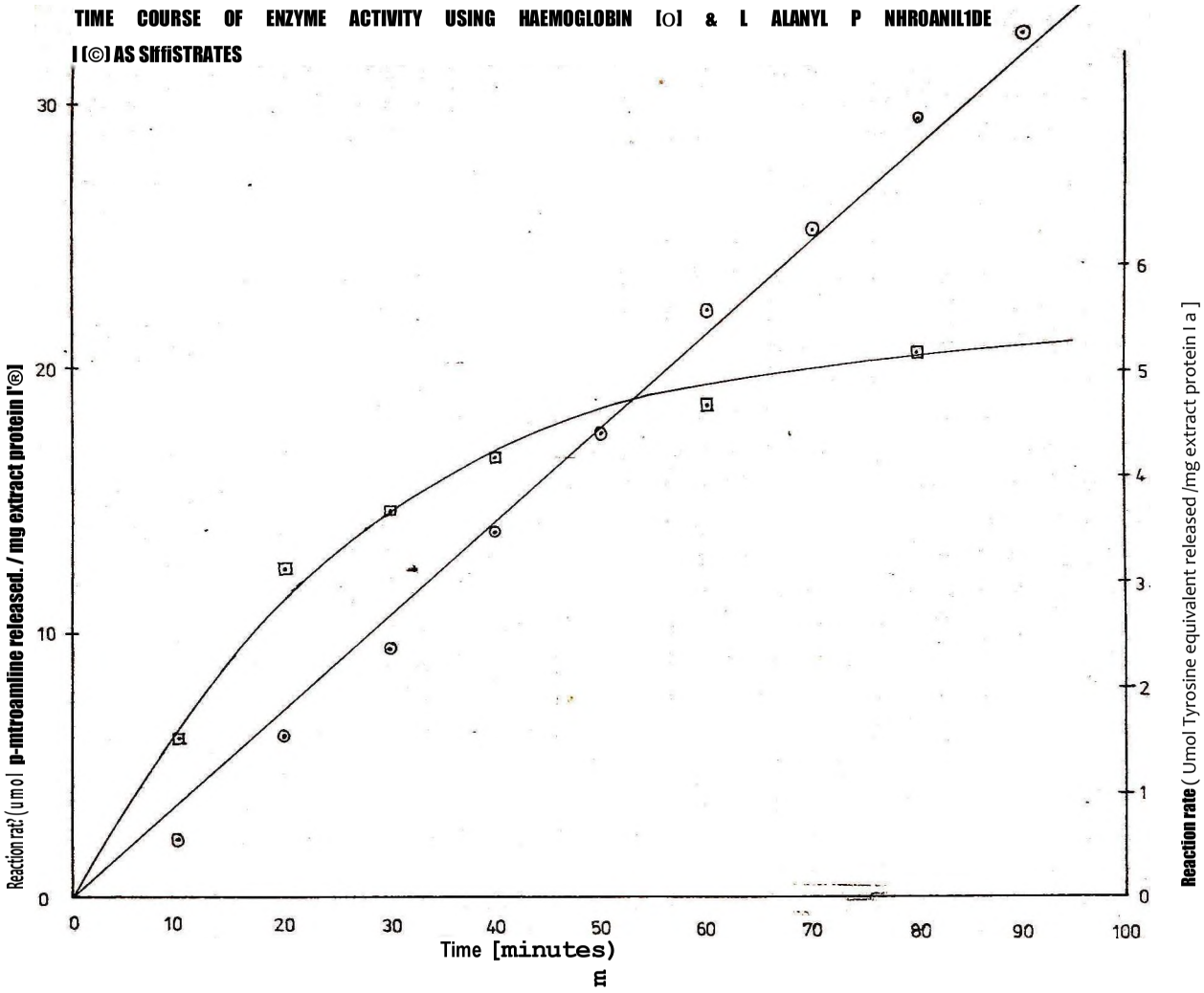
Figure V shows the effect of the temperature of incubation on the rate of hydrolysis. An optimum temperature of 36°C was observed for proteolytic activity at pH 5.6 using haemoglobin as substrate.

3.6 Effect Of Heat On Proteolytic Activity

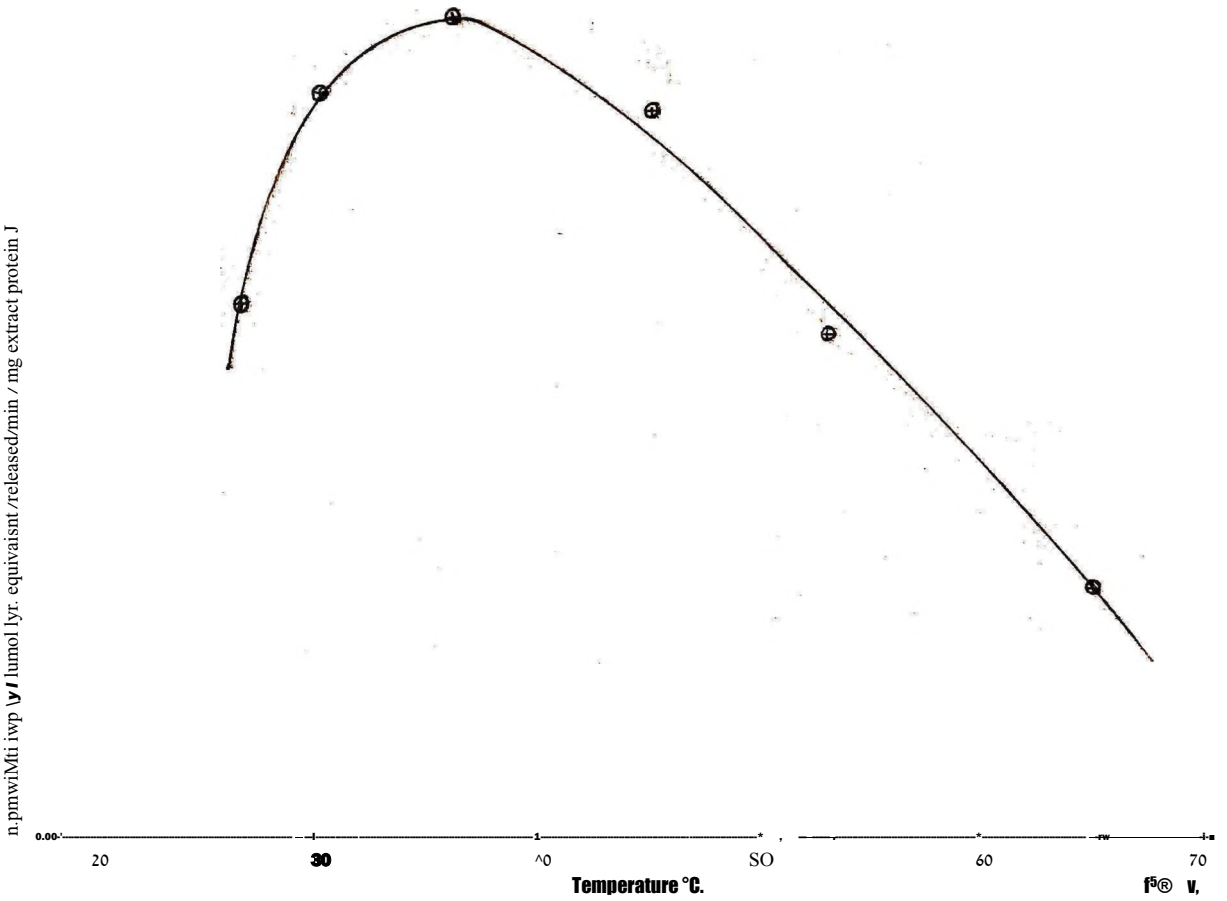
Table I gives relative activities of the extract after pretreatment at various temperatures before assay for activity using L-alanyl p-nitroanftlide as substrate. The results indicate that the arylamidase involved is heat labile, and loses all activity after exposure to a temperature of 66°C for 10 minutes.

3.7 Effect Of 'Enzyme* Concentration On Proteolytic Activity

Using haemoglobin as substrates the extract showed a linear relationship between the rate of substrate hydrolysis and the concentration of extract protein in the reaction mixture. The linear relationship occured for protein con-centrations up to 0.21mg per ml (Fig. Vi). Above this concentration there was no such linear relationship.


TIME COURSE OF ENZYME ACTIVITY USING HAEMOGLOBIN [o] & L ALANYL P NHROANIL1DE
I [©] AS SIffiSTRATES
Reaction rat? (umol p-nitroaniline released. / mg extract protein l'®]
Reaction rate (Umol Tyrosine equivalent released /mg extract protein l a]
Time [minutes)
0
10
20
30
40
50
60
70
80
90
100
0
10
20
30
0
1
2
3
4
5
6

EFFECT OF TEMPERATURE DM RATE QE JffDRQLYStS OF HAEMQGLQBW;



EFFECT OF ENZYME CONCENTRATION ON RATE OF HYDROLYSIS OF HAEMOGLOBIN SUB.STRATE

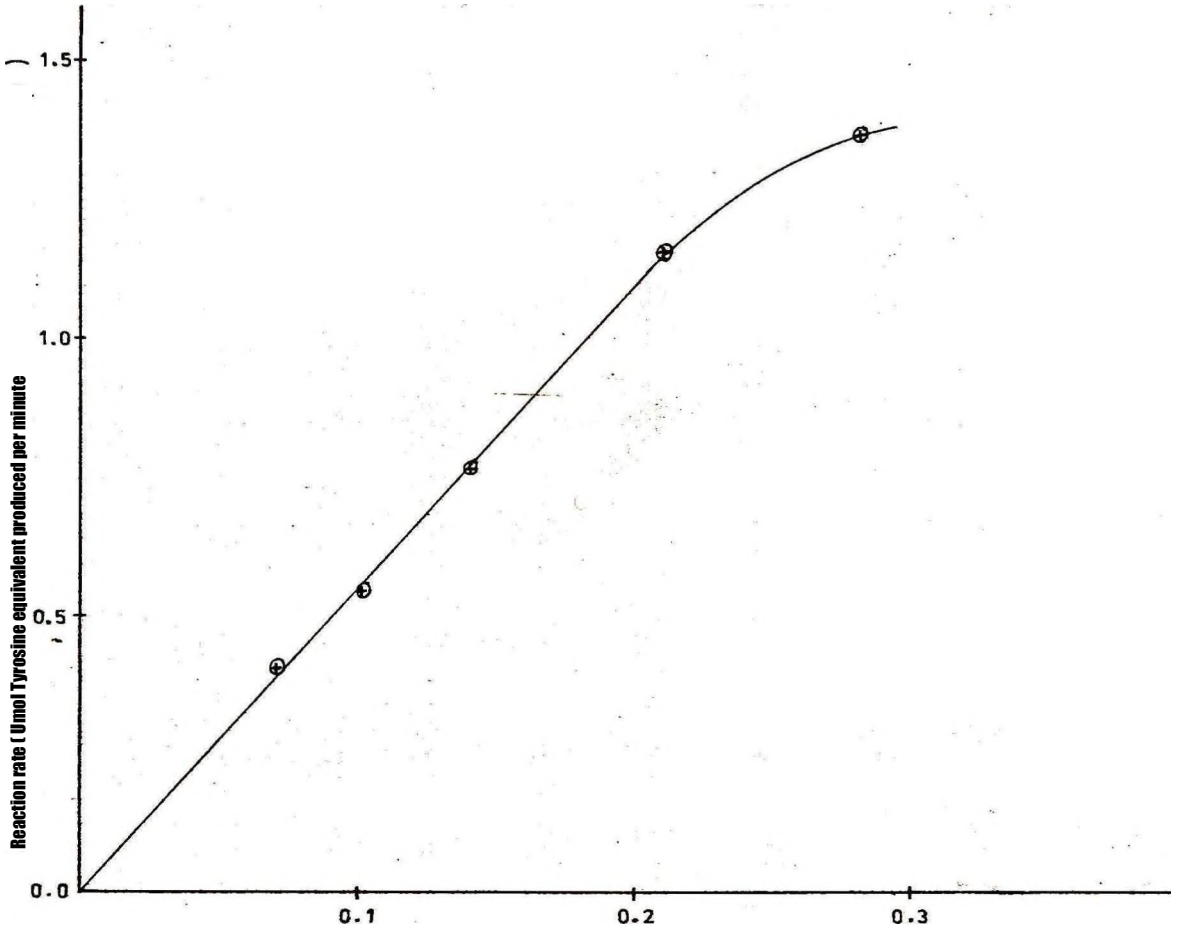


FIG VI

Table X

Effect Of Heat On Enzvme Integrity

Values are given as residual activity relative to the activity obtained after treatment at *j6°C* which was 2.0 x 10″ uM p~nitroamilin« produced/minute/mg extract protein.

Temperature	Relative Activity
26	110
36	100
45	95
54	65
66	0

3.8 Screening Of Amino Acid B-nanhthvlamides and p-nitroanalides For Susceptibility To Hydrolysis By Extract

Table II shows results of enzymatic activity using different amino acid p-nitroanHides and p-naphthylamides as substrates. The figures given are relative to activity with L-alanyl p—nitroanallde as 2.53 umol product/minute/mg extract protein for the p-nitroanilides $_f$ and with L-Alanyl naphthylamide as 1.81 umol product/minute/mg extract for the p-naphthylamides.

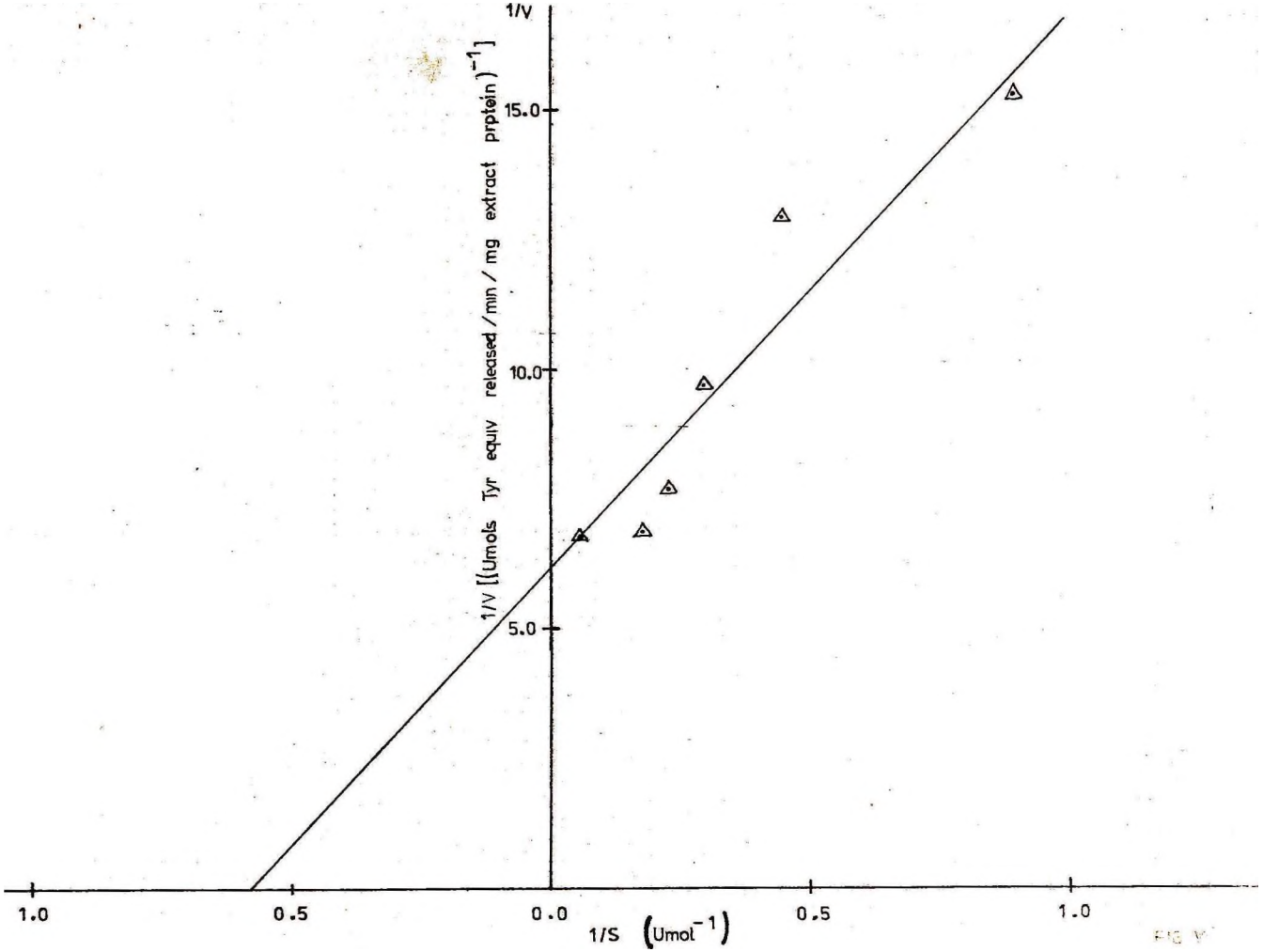
Table IX

SUBSTRATE	RELATIVE ACTIVITY
L-Alanyl-p-nitroanilide	100
Leucin p-nitroanilide	16
Acetyl alanyl p-nitroanilide	0
Succinyl-phenyl-alanyl-p-nitroanilide	0
Glycine p-nitroanilide	19
Alanyl-alanyl-alanyl-p-nitroanilide	0
L-Alanyl p-naphthylamide	100
L-Arginine p-naphthylamide	17
L-Glutamyl p-naphthylamide	3
Aspartyl p-naphthylamide	0
N (-L—Glutamyl p-naphthylamide)	0
Benzoyl-t~leucine |!-naphthylamide	0

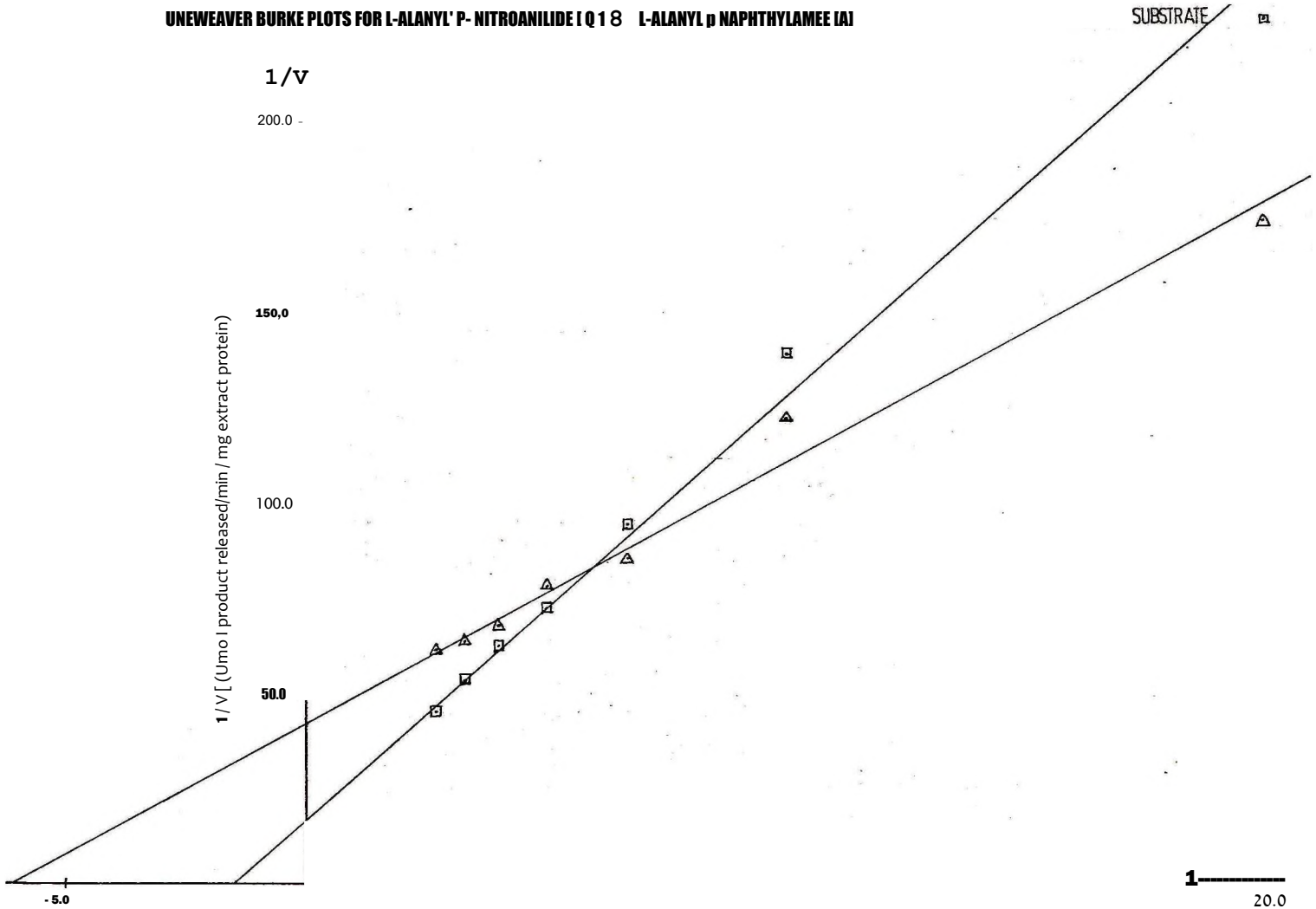
3*9 Effect Of Sabstrate Concentration On Proteolytic Activity

Figures ¥11 -IX are graphs derived from the relationship between substrate concentration and proteolytic activity. Table III gives the kinetic parameters, Em and Vmax, derived from these graphs» using, Haemoglobin, L-Alanyl p-nitroanilide and L-Alanyl B-naphthylamide as substrates.

.EWEAVER BURKE PLOT HAEMOGLOBIN SUBSTRATE CONCENTRATION EFFECT ON EMME ACTIVITY



UNEWEAVER BURKE PLOTS FOR L-ALANYL' P- NITROANILIDE [Q 1 8 L-ALANYL p NAPHTHYLAMEE [A]



- ko -

EffiIE HQFSTEE PLOT FOR ALANYL P N A (H) A N D ALANYL ONA (®) SUBSTRATE CONCENTRATION EFFECTS ON ENZYME ACTIVITY

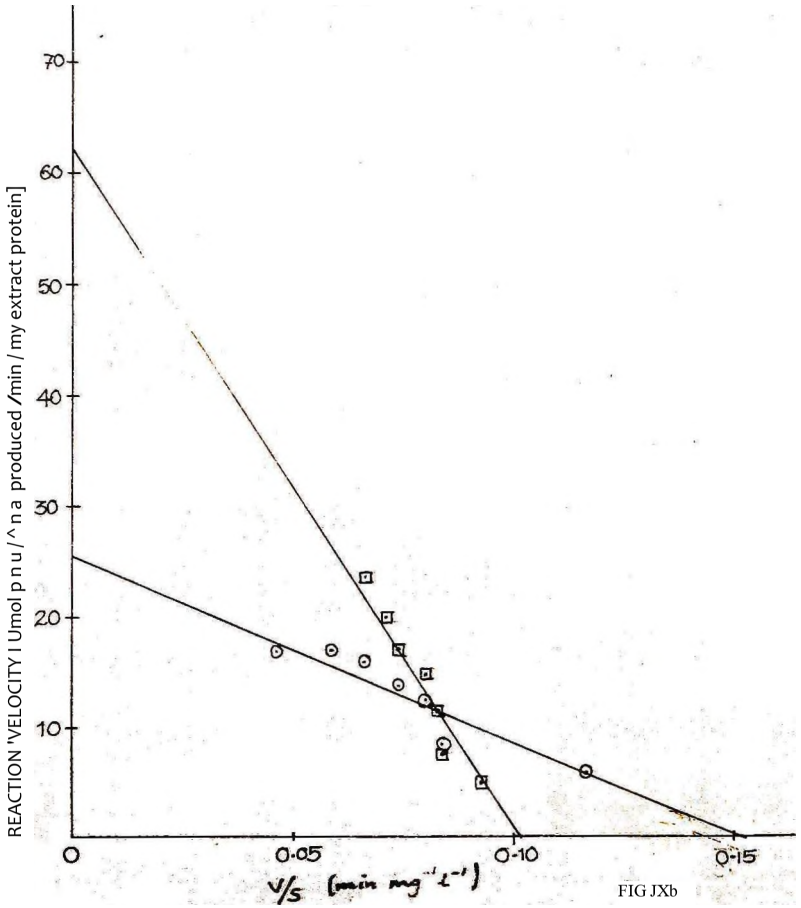


FIG JXb

EADIE HOFSTEE PLOT FOR HYDROLYSIS OF HAEMOGLOBIN SUBSTRATE

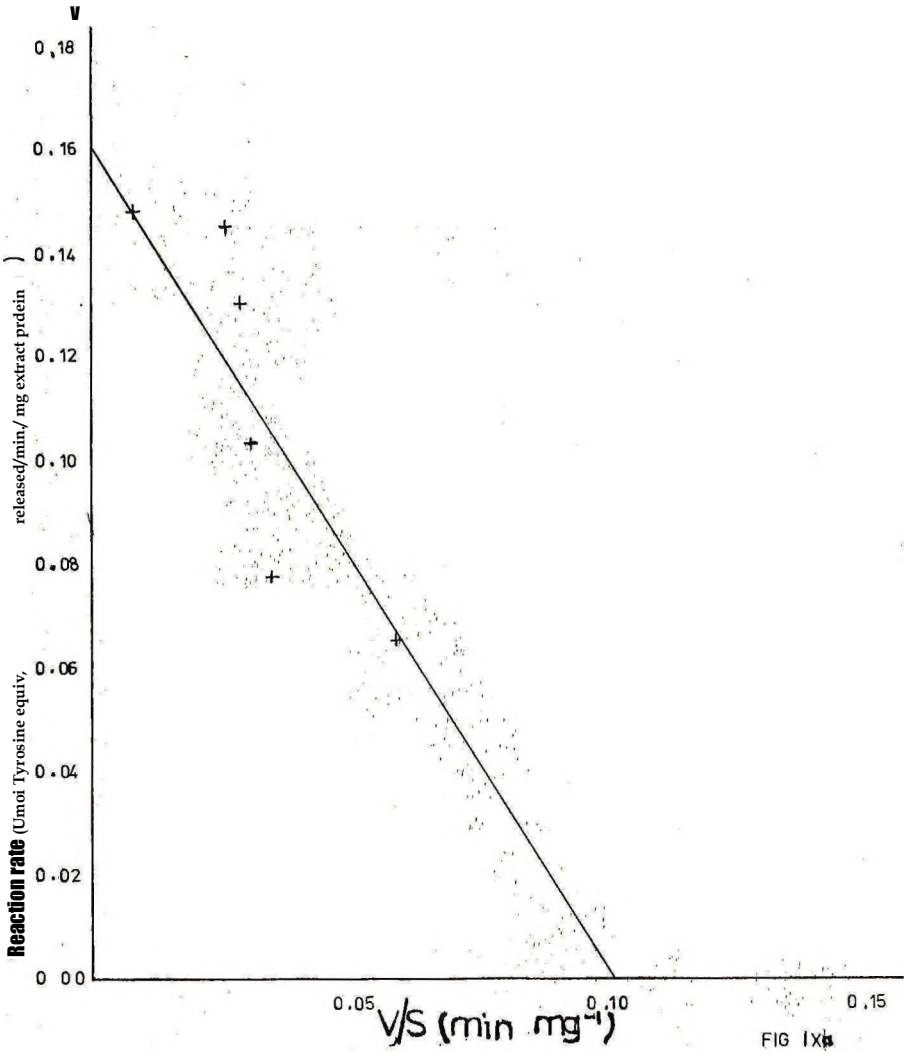


FIG IXb

Iiable XXX

Substrate	Em	Vmax/mg. Protein /
Haemoglobin	1.7*tt«	0.16 umol Tyr equiv/min
L-Alanyl p-nitroanilide	0.89mM	0.0615 umol pna/min
L-Alanyl ^-naphthylamide	0.16mM	0.0235 umol pna/min

3.10 Effect Of Metal Iona On Proteolytic Activity Of Extract

Table XV gives proteolytic activities of the sample when pretreated with different divalent metal ions* The figures are given relative to activity in a blank in which the extract was not pretreated with any metal ion$_t$ determined to be 3.1 nmol p-nitroaniline produced/minute/mg of extract protein. The substrate used was L*rA.lanyl p-nitroanilide*

Table XV

	Metal Xon Concentration (M)			
Metal Xon	0	10^{-6}	10^{-k}	$10^{r\ 3}$
	100		106	102
$^{2+}$	100		103	97
	100		106	0 (?)
	100	109	99	92
	100	100	100	0 (?)
	100		111	101
	100		101	101
Hi	100		116	90
Zn	10		99	52

3110b **Effect Of Metal Ions On. Proteolytic Activity Oil Extract Using- Haemoglobin As Substrate**

Table V gives proteolytic activities of the sample when pretreated with different divalent metal ions. Figures given are relative to a blank in which the extract was not pretreated with any metal ion$_y$ determined to be 3.06 x 10^{-2} nmol Tyrosine equiv&lent/minute/mg extract Protein.

Table V

Metal Xon	Metal Xon Concentration (M)		
		10^{-6}	10^{-k}
Ca^{2+}	100	83	102
Co^{2+}	100	89	
Cu^{2+}	100	61	*2k*
Mg^{2+}	100	*6h*	1J0
$^{2+}$	100	89	71
Ni^{2+}	100	89	52
Zn^{2+}	100	82	86

3*11 **Effect Of Some Specific. Inhibitors Of Proteolytic Activity Of Extract Of Haemoachns Contortus**

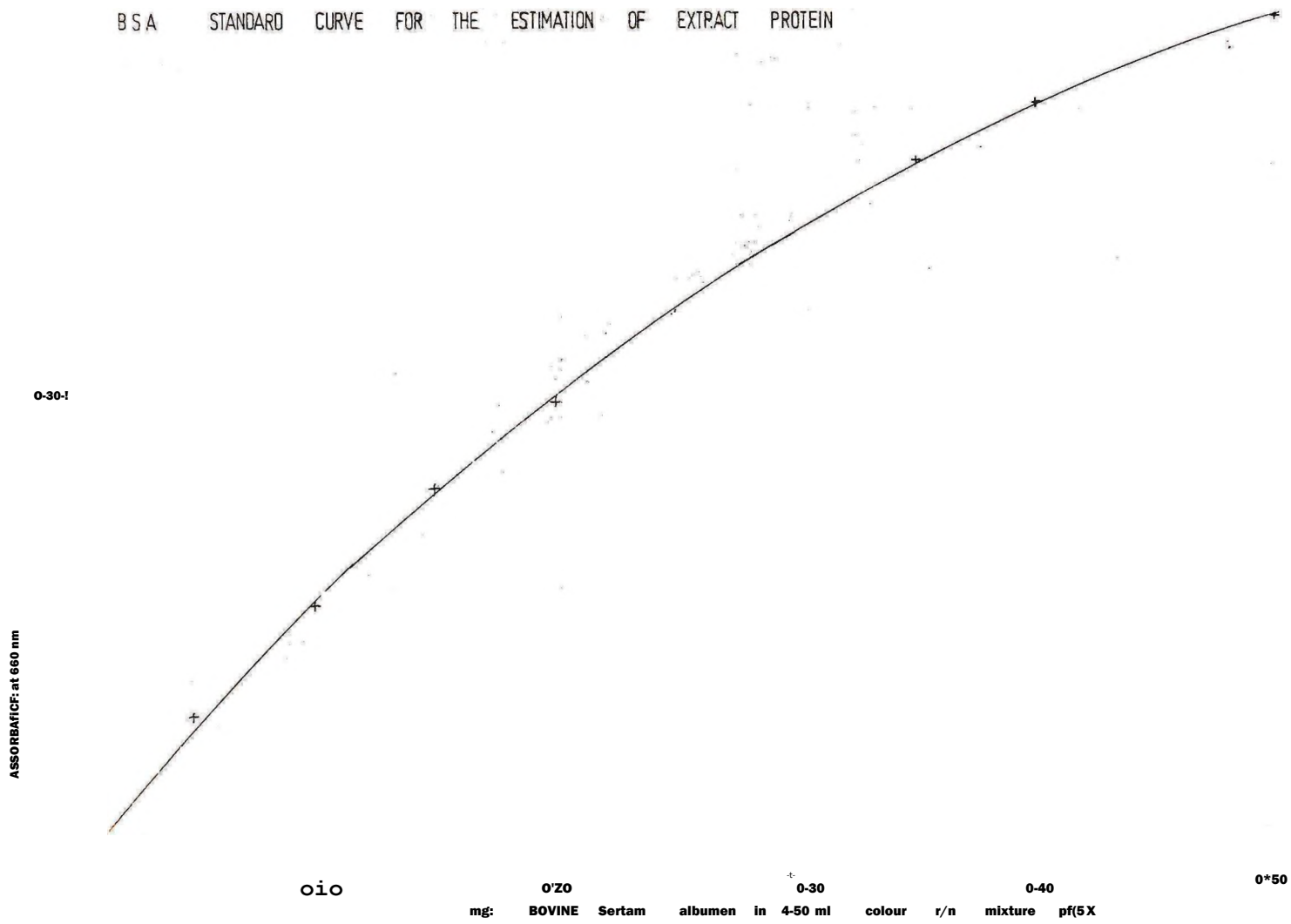
Table VI gives values of proteolytic activity of the sample after pretreatment with the inhibitors. The figures

givBa are relative to a control reaction in which in place of the inhibitor buffer solution was used. For the control the activity was 3*1 umol p-nitroaniline produced/minute/mg of extract protein, using L-Alanyl p-nitroanilide as substrate.

Table VT

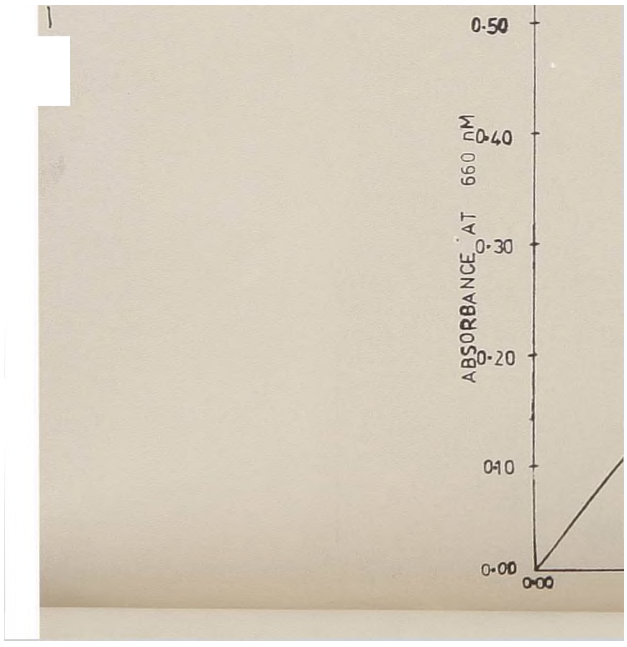
Inhibitor	Relative Activity
Cysteine - HCjjt	0
Dithiothreitol	105
Iodoacetate	70

In
4-


B S A STANDARD CURVE FOR THE ESTIMATION OF EXTRACT PROTEIN
0-30-!
ASSORBAfiCF: at 660 nm
oio
0'Z0
0-30
0-40
0*50
mg: BOVINE Sertam albumen in 4-50 ml colour r/n mixture pf(5X

0-70 ■

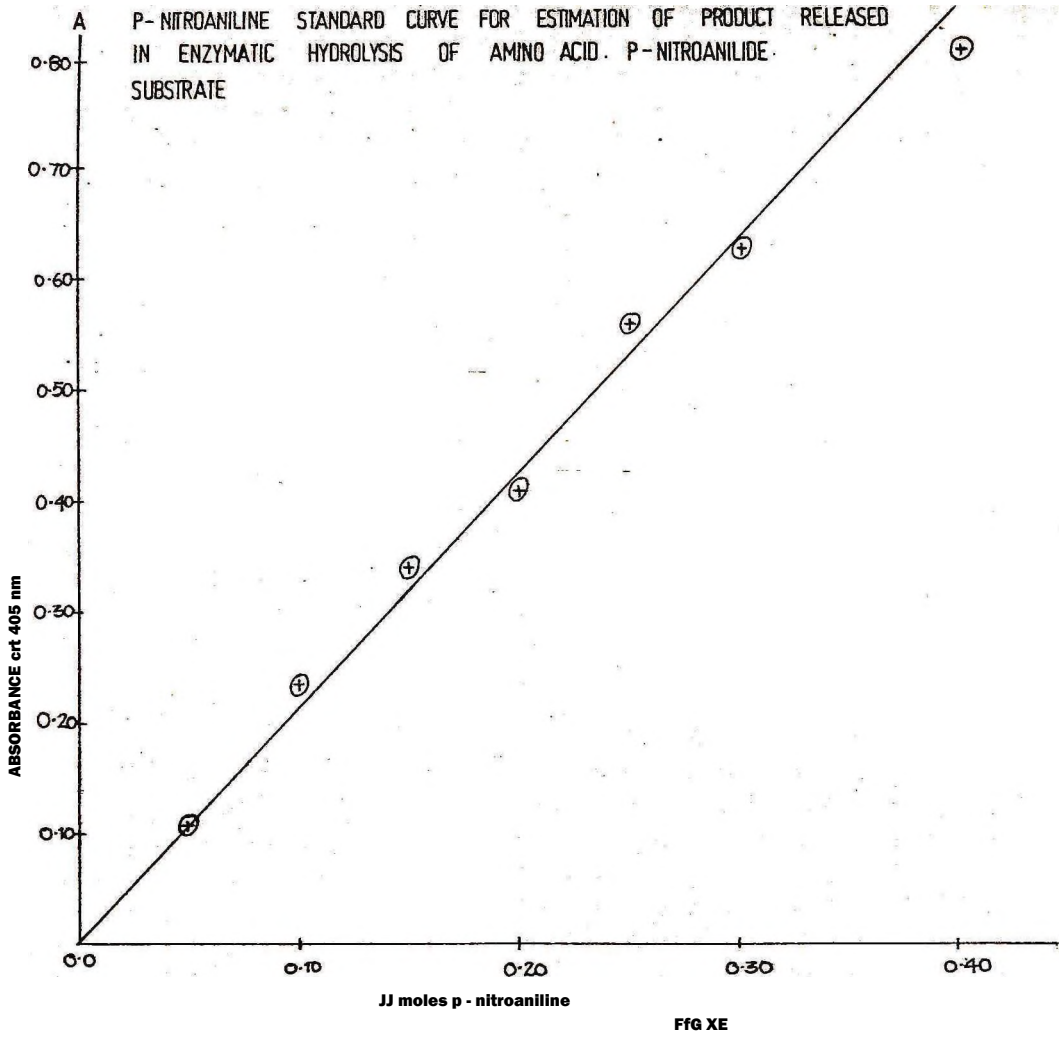
0'6c



TYROSINE STANDARD CURVE FOI

TYROSINE EQUIVALENTS RELEASED

OF HAEMOGLOBIN-

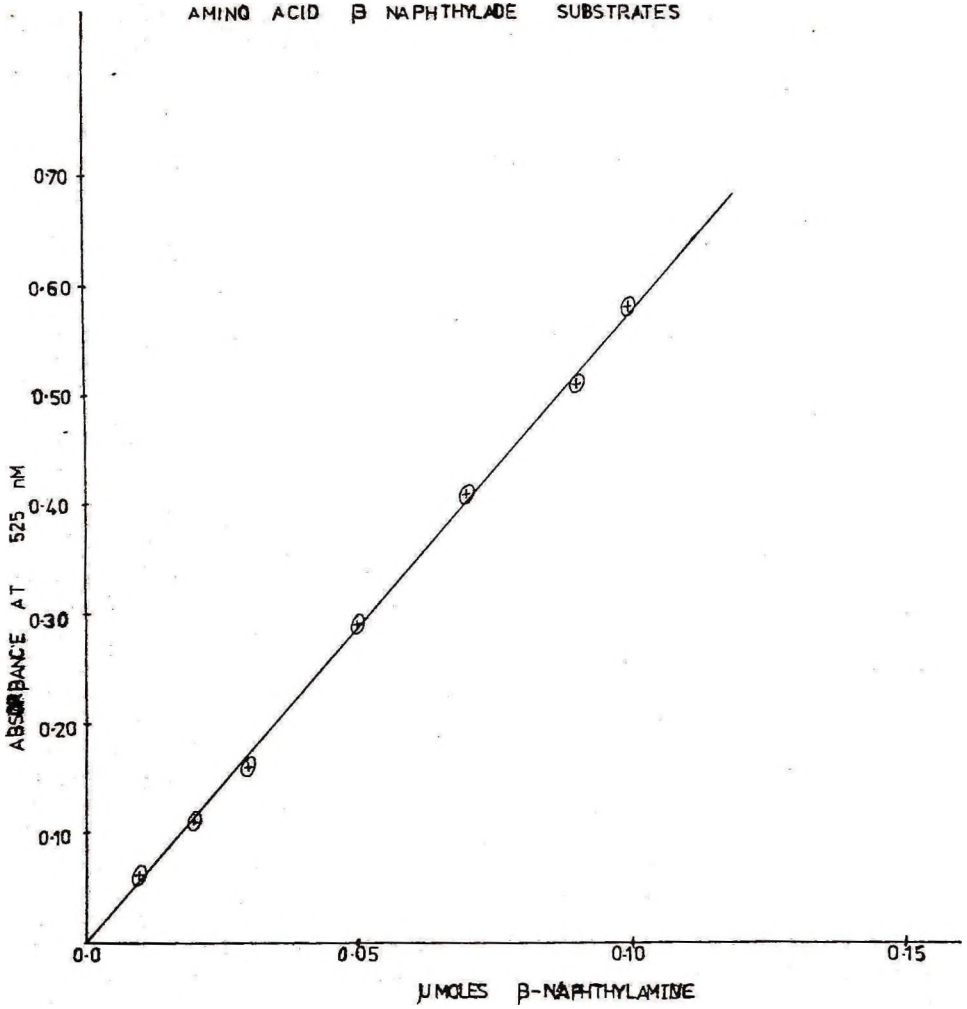

A
P-NITROANILINE STANDARD CURVE FOR ESTIMATION OF PRODUCT RELEASED IN ENZYMATIC HYDROLYSIS OF AMINO ACID. P-NITROANILIDE. SUBSTRATE
0·80
0·70
0·60
0·50
0·40
0·30
0·20
0·10
0·0
0·10
0·20
0·30
0·40
ABSORBANCE crt 405 nm
JJ moles p - nitroaniline

FfG XE

F-naphthylamine standard curve for- estimation of

PRODUCT ,'RELEASED IN THE ENZYMATIC HYDROLYSIS OF

AMINO ACID β NAPHTHYLADE SUBSTRATES



FfG xirt

V

CHAPTER FOUR

DISCUSSION AND CONCLUSION

Hie preliminary experiments that were performed in this study involved assays with not only the crude homogenate of the adult worm in various media - physiological saline and 0.1M concentration of a number of buffers - but also with various centrifuge fractions. The results of these preliminary studies indicated that physiological saline which has been used by other researchers for similar purposes* was suitable. This was considered to be of particular importance in view of the observation that when the homogenates were prepared in buffer there was a decrease in proteolytic activity with increase in the period of storage in the frozen state. The 30,000jg (30 minutes) supernatent fraction was chosen for the study because of the defined range of components in it. According to Clark and Switzer (82)* centrifugation at 30*000)0 for 30 minutes sediments most cell components leaving ribosomes* polysomes and smaller particles* as well as soluble molecules like proteins and carbohydrates.

Detergents were used on both the crude homogenates and the 30*OOOJjgsoluble fraction in an attempt t© increase the total protein in solution and thus the observed activity. The results indicated that with the crude homogenate* the protein concentration increased with both the concentration of the Triton-X100 that was used* and the length of time that the

solubilization was effected. With the 30,000^soluble fraction however, there was no significant increase in level of protein by both treatments. Xn both eases however, it was observed that the proteolytic activity expressed decreased with Increasing concentration of the detergent when activity was expressed as umoles of product per minute per milligram of extract protein. Furthermore, in the ease of the crude homogenate, the decreased activity was observed in the studies on effect of time of solubilization. From these observations, it seemed that even though Triton X-100 increased the level of protein possibly by releasing membrane bound proteins, it decreased observed proteolytic activity. This, it is suggested may have been either because of a direct inhibitory action, or because the protein released has no proteolytic activity to begin with. On the basis of the above conclusion, and in the absence of other detergents for use, the studies were done without the use of any solubilising agent.

The protein estimates for various preparations of the extract ranged between 0.10 - 0.20mg per mg of worms homogenate^. This wide range may have been due to the method of homogenisation which, needless to say, could not have produced consistent homogeaates in terms of final particle size of the homogenate. A possibly more important factor may have been due to the sex ratios of the worms homogenised at different times. The females are twice the size of the male worms and contain by far larger amounts of host blood in their guts. The contribution of host

blood proteins to the total protein estimated, it is being suggested, was significant enough to explain the observed difference* A 1967 study by Eckert (S3) gives the percent of protein in the dry weight of Haemonchns contortus at 54. The 10 - value obtained for the wet, live sample, it is considered, compares favourably with this literature value.

As indicated in the literature review, the anaemias produced by Infections of Haemonchus Contortus have become important areas of study in the search for methods of combating Haemonchosis. The use of haemoglobin as a substrate, in the light of this, is only logical. Rogers (6*,84) has indicated that helminths split haemoglobin into glebin and heme, but the details of blood digestion vary in the different species. Celam (85*86) in subsequent studies with Rhabdias bufonis and S. sphaeroceohala has shown that the greater part of the haemoglobin is digested extracellularly with the help of an endopeptida.se, probably an exopeptidase, and an acid phosphatase leading to the formation of hematin. Intracellular lysosomal digestion involving an endopeptidase and an acid phosphatase "on the other hand leads to production of haemosdftrin. In the use ef denatured haemoglobin as substrate for pepsin-like and trypsin-like enzymatic action, the products measured however, are the tyrosine equivalents and tyrosine.

The use of synthetic substrates in kinetic studies been very rewarding, especially in studies of the specific structures susceptible to hydrolysis. Hartley (£6) however, suggests that few valid generalisations can be made about the

aetion ©f protexflasss on proteins • Synthstic substrates on the Other hand are simple to use and yet give a wealth of information* and were thus convenient to use in studying some of the factors that affect the activity of the enzyme(s) under study.

The assays in general showed the classical effects on enzymes of parameters like hydrogen ion concentration* temperature* substrate and enzyme concentrations and inhibitors of proteolytic activity. These results altogether give a basis for a preliminary classification of the enzyme present in the extract studied.

tfsing haemoglobin as substrate the maximum activity was observed at pH 5.6. Subsequent assays with haemoglobin as substrate were performed at this pH. In studies by Timms and Bueding fBf) with Schistosoma tomaoai a similar pH optimum was observed. It is interesting te note that the observed pH optimum is half-way between the mean pH of the abomassum of the host animal found to be 4.2* and the pH of the host blood.

With the synthetic substrates on the other hand the optimum pH was found to be 7.8. This compares with a Plasmodium Knowlesi enzyme which showed maximum activity in the pH range 7-8 after partial purification by Cook and coworkers This enzyme was inhibited distinctly by d.i is ©propyl fluorophosphate and to a lesser extent by several chelating agents* but not by cyanide or SH inhibitors. It differs from the enzyme studied* vhich was completely inhibited by cysteine.

For both haemoglobin and synthetic substrates, the snzym©(3) shoved —t<«» activity at *j6* C. In the studies on the effects of heat using alanyl pna as substrate the extract was shown to be heat labile, losing all proteolytic activity after incubation at 66°C for ten minutes* This property is considered useful for purposes of characterisation of the arylamidase responsible for the observed activities* Where beat activation has been observed, it has been attributed te, among other factors, the destruction of a thermolabile inhibitor bound to the enzyme, or on the other hand, to the presence of a thermostable factor which stabilizes the secondary and tertiary conformations of the enzyme* Heat inactivation may be a consequence of the denaturation of the enzyme protein*

The use of metal ion activation/inactivation as a characterising tool, it has been suggested by Malmstrong and Rosenborg (?H)» is quite meaningless except with pure extracts of enzyme. Besides this purity factor, there is the need to use as wide a range of metal ion concentrations as possible, bearing in mind the faet that generally high concentrations of metal ions are inhibitory and/or interfere with the method of estimation* Finally, it is important to use an assay method sensitive enough to detect slight differences in activity consistently* Besides the fact that the extract used in these studies was impure, the method of estimation was not particularly sensitive, ~~making~~ it difficult to interprete with any level of conviction the slight activation with decreasing concentration of some of

tb# mttsil ions used, Hovevtri it could be useful to study further the effect of metal ioas on the proteolytic activity in order to understand the role of metal ions in various physiological changes of the diseased host animal.

The kinetic studies show that haemoglobin is more susceptible to hydrolysis. There is no real basis for comparison of the Km and Vmax values obtained fer haemoglobin and the synthetic substrates (Table III) because of the different assay methods used and more Important because of the different pH optima observed for the two types of substrates. This latter factor could be interpreted to mean the presence of at least two different enzymes active at the different pHs, or complex enzyme with at least two active sites activated at different pHs. *For* the synthetic substrates, the values for Em and Vmax indicate that alanyl pna is slightly more susceptible to hydrolysis by the enzyme than alanyl—J3NA.

In the screening of amino acid pnas and pHAs for susceptibility to hydrolysis, there was clear indication of a preference of the L-aAauyl derivatives to the leucyl, arginyl *OF* glutamyl derivatives (Table II). Often in the characterisation of aminopeptidases, the hydrolysis of such substrates has been attributed to leucine amino peptidase (LAP) activity *($9),* However, mammalian tissues for example have been shown to contain amino peptidases or arylamidases with a great variety of substrate specificities. Enzymes have been isolated which preferentially hydrolysA peptide bonds involving N-terminal acidic

amin© acids - glutamate or aspartate (90,91)* basic amin® acids - lysine or arginine (92), alanine (93). glycine (9*0, ^-aspartate (95) cysteine (96) ®r pyrrolidine carboxylate (97)* Behai and coworkers (82) in studies of an aryiamidase ©f Iranian liver found that only amin© acid p-naphthylamides ©f the L-configuration were susceptible to aryiamidase catalysed hydrolysis. In their studies, L-alanine pNA had the highest Vmax value. Several ether substrates in which the amino acid residues had a larger nen-polar, ©r basic R-groupings such as methionine $NA or arginine JJNA had the lower Km values. The enzyme had no dipeptidyl aryiamidase activity. These studies by Behai and coworkers (93) also showed that amino acid , substrates having straight chain or ^-branched R-groups were much more susceptible to hydrolysis than those with ^-branched or acidic R—groups on the amino acid residue. Similar observations have been made in the present study, with both amino acid pnas and $NEA.s.

The relative resistance of the ^-branched N-terminal residues to hydrolysis has been noted even in the case of non enzymatic (acid or alkaline) hydrolysis of valyjglycine and isoleucylglycine independently studied by Levene and coworkers (98) and Synge (99). This factor is probably reflected in the enzymatic hydrolysis of these amides as pointed out by Smith and coworkers (100), though it is probably not the only factor since they further reported that neither isoleucinamide nor valine amide inhibits LA.P catalysed hydrolysis of L-leucinamide when present in equal concentrations. These latter findings have been interpreted to mean that the ^-branched amino acid derivatives do not interact with the enzyme as strongly as do the ^-branched derivatives.

This group of enzymes having no LAP activity but which hydrolyso a variety of amino acid pNAs reported by Behai and coworkers (93) have been classified as arylamidases (lOl). Studies by Behai and Folds (102) described the predominance of arylamidase in certain gram negative bacteria among a representative group of gram positive and negative bacteria. This enzyme lacked the divalent cation requirement characteristic of arylamidase from animal ? fsources. The enzyme studied has shown, by the nature of substrates susceptible to its hydrolytic action, a close similarity to the enzymes in these earlier studies.

Barret in his classification of proteinases, has indicated the use to which specific inhibitors of proteolytic activity can be put to •confirm' classification of enzymes studied. The enzyme(s) studied with respect to synthetic substrates was completely inhibited by cysteine and partially by iodoacetate (Table V). Dithiothreitol of the same concentration as the above (0.10M) had no effect on activity.

It is a consequence of Hartley's (56) idea of classifying the proteinases by catalytic mechanism that inhibitors tend to be more informative in initial characterisation of a particular enzyme than substrates. The enzyme responsible for the arylamidase activity observed cannot be meaningfully characterised in this respect because some of the inhibitors used in the Barret scheme devised for characterising what seems to be an exopeptidase effect of the enzyme(s) showing the arylamidase activity wer© not available for use.

3k conclusion, the 30,000 x g supernatant fraction of hoaogenates •f adult HaS52iS£feSS ooBtortus showed maximum proteolytic activity at pi 5.6 sad 7.8 for haemoglobin and synthetic (amino acid pna and (3 HA) substrates respectively* With haemoglobin as substrate, *Km* aad Vmax were respectively found to bo 1*flm aad 0.16uko1 Tyrosine equivalent/ minute/mg of extract protein. She ensyme was inhibited by cystcine, while the effect of netal ions ia activation was not significant*

The synthetic substrates nest susceptible to the hydrolytid effect of pH 7.8 were the L-alanyl derivatives# *Tk*ere Was clear indicate ion that the sise aad nature of the amino acid residue was an important factor in determining whether or not the substrate was susceptible to hydrolysis. For the ananyl pna and alanyl $NA, Vmax was found to bo 0.065uw»l pna/minute/iag protein, end 0.0235uaol |5W«ia«t®/ag protein respectively. The aryiamidase activity was inhibited by cysteine and partially by iodoacetate, but net lay dithiothreitol. It lest all activity bfter heating at 66°C for 10 sd&utes, suggesting that it is beat labile* The results with various cations cannot be meaningfully interpreted, however, because the extract was impure and the assay aethod not sufficiently sensitive for the purpose* These possibly explain the observation that unlike most arylamidases of anisal origin, there was mo significant activation by the cations, particularly Magnesium aad manganese.

In the light of* the great economic importance of Haemonchus 'Contortus in livestock production, there is need for farther studies into the enzymology not only of the adult worm but also of the infective larval stage, especially with regard to the effect of metal ions which have been shown to feature quite prominently in the disease state of the host animal.

APPENDIX

REAGENTS AND MATERIALS

All chemicals used were of* the highest analytical grade available.

a. Assays Using Haemoglobin As Substrate

Sodium hydroxide, calaium chloride and potassium cyanide were from Fluka-Garantie. Boric acid, copper sulphate, potassium, sodium tartarate and anhydrous sodium carbonate were of BDH Analar grade. Urea used was from Eastman Chemical Company and L~Tyrosine from Sigma Chemical Company.

b. Assays Using Amino Acid p-nitroanilides As Substrates

N-Succinyl-, L—phenylalanine-, L-Alanine-, N-Acetyl L Alanine-, N Benzoyl-L—Arginine—, I—leucine-, and Glycine- p-nitroanilides were obtained from Sigma Chemical Company. Acetyl Tri-L-Alanine p—nitroanilide was from Mileb-Yeda Ltd., Formdimethy1amide BDH, and p-nitroanilime of General Purpose Reagent grade were used.

c. Assays Using Amino Acid B-naphthylamides As Substrates

2-naphthylamides of L-Alanine, ot-L-Aspartyl L-Arginine and N-Benzoyl L-leucine were obtained ffrom Koch—Light laboratories. N(g<$_r$ L-Glutamyl) p-napihthylamide was from Aldrich Chemical Company, OC-L—Glutamyl- and N-Benzoyl-D-L phenylalanine ^-naphthylamides from SERVA, and 2-naphthylamine ef General Purpose Reagent Grade were used. Methanol (Kodak grade) was used (50°/o strength) as solvent. Tween

80 (polyoxyethylene sorbitan men®—®leate) and Fast Garnet GBC from Sigma Chemical Company were the main components ef the colour reagent,

d. Buffer Solutions

Buffer solutions were prepared according to the methods of Gomori, modifying concentrations of stock solutions as required, Tris (hydroxymethyl) amino methane, citric acid and Acetic acid were from Eastman Chemical Company, and Sodium acetate from Fluka-Garantie. Sodium citrate, dibasic sodium phosphate ($Na_2HP0^2H_2$©), monobasic sodium phosphate and Sodium hydroxide were from BDH.

APPENDIX *TWO*

LIST OF ABBREVIATIONS

pna	p - nitroanilide
pNA	p - naphthy1amide
ala - pna	L - Alenyl - p - nitroanilide
ala - pNA	L - Alenyl - p - naphthylamide

BIBIOGRAPKY

Chandler A.C., and Read C.P. (1961) Introduction te Parasitology 10th Edition. J. Wiley and Sons Inc. N.Y. and London.

Bleed, D.C.(Henderson, J.A., and Radostits, 0.M (1979) Veterinary Medicine. 5th Edition. English Language Book Society and Bailiere Tindal. P. 789 - 791.

Tetzlaff, R.D., and Todd, A.C., (1973) Protective effects ef premonition and age group interaction of Haemonchus centertus in sheep. Am. J. Vet. Res. 34. 1549

Fry, N.C. (1958) Diseases of sheep in Ghana. Brit. Vet. J. 114. 209 - 214

Jackson, C (1965)* An annotated list of the helminth parasites cemmonly found in domestic animals in West Africa with notes on the control ef Haemonchosis and Stepihaneuresis. Ghana *J. Sci*. 5(1), 65 - 70.

Olsen, O.W. (1970) Animal Parasites. Their Life Cycles and Ecology. 3rd Edition. University Park Press, Baltimore, p. *kjO* - 434.

Stoll, N.R. (1929) Studies with the Strongyloid nematode Haemonchus contortus. Acquired Resistance of hosts under natural reinfection conditions out of doors. Am. J. HTg. 10 384 - 418

Wetzel, R., (1930) Biology of fourth stage Larvae •f Oxyuris equi. J. Paragitoi 17. 95 - 98.

9. Mettrick D.F, and Podesta R.B. (197^) Ecological and physiological aspects of helminth host interactions in the mammalian gastrointestinal canal. Adv. Parasitol 12 183 - 278

10. Coop R.L., Sykes A.R., and Angus K.W, (1972) The effect ©f daily intake ®f Ostertagia circumcincta larvae on body weight, feed intake and concentration ©f serum constituents in sheep. Res. Vet. Sci. 23 (1) 76-83

11. Sykes &.R. and C©@p R.L. (1977). Intake and utilization ©f food by growing sheep with abomassal damage caused by daily dosing with Ostertagia circumcincta larvae. J.Agric. Sci. 88 671 - 677•

12. Sykes A.R. (1978). Effect of subclinical parasitism in sheep. Vet. Record 102 (2) 32 - 34

13. Ross J.G. and Todd J.R. (1965)* Biochemical, Serological and Haematological changes associated with the infection of calves with the nematode parasite ostertagia ostertagi. Brit. Vet J t?i 55, - 64

14. Bird A.R. and Rogers W.P. (1956). The chemical composition of the cuticle of third stage nematode larvae. Bxpt. Parasitol 449 - 457

15. Slocombe J.O. (1974). Some analyses of exsheathing fluM from infective Haemonchus Contortus larvae from OnAario.
Int. J. Parasitol 4 397 - 402

16. F@ttr.ie P. J. J. (1931) . The hematology and pathology of Haemonchosis in sheep.
17th Report. Div. Vet. Sci. and An. Ind. Union of Sonth Africa pp. 495 - 572.

17. Andrews J.S. (1942). Stomach worm* Haemonchus contortus infection in lambs and its relation to gastric hemorrhage and general pathology.
J. Agric. Res. 65 1 — 1 8

18. Martin C.J. and Clunies-Ross I (1934). A minimal computation ef the amount of blood removed daily by Haemonchus fcontortus
J. Helminthol. 12. 137 - 142

19. Baker N.F., Cook E.F. Douglas J.R. and Cornelius C.E. (1959). The pathogenesis of Trichostrongyloid parasites III. Some physiological observations in lambs suffering from acute parasitic gastroenteritis
J. Parasitol. 45 643 - 657

20. Whitlock J.H, (1950) The anemias of Trichostrongylidosii
Cornell Vet. 40 288 - 299

21. Schalm O.W. (1956). The Blood and Blood Forming Organs. Diseases of Cattle. American Veterinary Publications* Evanston, Illinois.

22. Baker N.F. and Douglas J.R. (1957)• The pathogenesis of Trichostrongylid parasites. Ferrokinetic studies in ruminants.
Am. J. Vet. Res. 18 295 - 302.

23. Downey N.E. (1965). Some relationships between Trichostrongylid infestation and cobalt status in lambs: Haemonchus contortus infestation. Brit. Vet. J. 121 362 - 370

24. Silverman P.H., Mansfield M.E. and Scott H.L. (1970) Haemonchus Contortus infection in sheep: Effects of various levels of primary infection on nontreated lambs. Am. J. Vet. Res. 31 841 - 857

25. Theodorides V.J., Scott G.C. and Laderma M (1970) Strains of Haemonchus Contortus resistant against Bei^ imidazole anthelmintics. Am. J. Vet. Res. 31 859 - 863

26. Armour J. and Urquahart G.M. (1965), The control of helminths in ruminants. Brit. Vet. J. 121 392 - 397

27. Gibson T.E., and Everett G. (1968). A comparison of set stocking and rotational grazing for the control of Trichostrongylosis in sheep. Brit. Vet. J. 124287

28. Spedding C.R.W. (1956). The control of worm infestation by grazing management J. Helminthol. 2£ 179 - 186

29. Lindahl I.L., Kates K.C., Turner J.H., Enzie F.B. and Whitmore G.E. (1961). Effect of three management systems on the growth of lambs and development of internal parasitism J. Anim. Sci. 20199

30. Lindahl I.L., Kates K.C., Turner J.H., Enzie F.D. and Whitmore G.E. (1963) Effect of management systems on the growth of lambs and development of Internal parasitism I. Dry lot versus two pasture rotation systems*
J. Parasitol. *k9* (2) 209 - 217

31. Miller H.M. (1931). The production of artificial immunity in the albino rat to a metazoan parasite.
J. Prevent. Med. ^29 - ^52

32. Scot H.L. t Silverman P.H., Mansfield M.E. and Levine H.S. (1971)• Haemonchus contortus infections in sheep. Active and passive immunity in sheep given oral iron supplement.
Am. J. Vet. Res. *32* *2k9*

33. Stewart D.F. (1950) Studies on the resistance of Sheep to infestation with Haemonchus contortus and Trichostrongylus of sheep exposed its infestation V. The nature of the self cure phenomenon
Austral. J. Agric. Res. X 427 - ^39
Christie M.G. (1970) The fate of very large > doses of Haemonchus contortus and their effect on conditions in the ovine abomassum
J. Comp. Path. 80 89 - 100.

35. Jarret W.F.H., Jennings F.W., McIntyre W.I.M., Mullinger W., Sharp N.C.C., and Urquahart G.M. (1953) Immunological studies on Dictvocaulus Viviparous infection in valves - double vaccination with irradiated larvae
Am. J. V»t. Res. 20 522 - 526

36. Jairet W.F.H., Jennings F.W., McIntyre W.I.M., Mullinger W. and Sharp N.C.C. (1959) Studies on immunity to Haemonchus contortus- infection: vaccination of sheep using a single dose of irradiated larvae.
Am. J. Vet. Res. 20 527 -531

37. Wilson G.I. and Samson K.S. (197*0. Immunity to Haemonchus Spp in lambs. The immunizing effects of various methods of larval administration.
Res. Vet. Sci. 22 390 -394

38. Fisher E. and Aderhalden E. (1905) 2. Physiol Chem 46. 52 in: "A classification of proteolytic enzymes" Bergman, M. (1942) Adv Bnzvmol 2 4;f - 6 8

39. Bergman in (1942) A classification of proteolytic enzymes.
Adv. Bnzvmol 2 49-68

40. Gutteridge W.E. and Coobs G.H.
Biochemistry of Parasitic Protozoa. MacMillan Press, Ltd., London p. 89 - 104

41. Kelly J.D. (1973) N. Zeal. Vet. J.21,183
In: Veterinary Medicine by Blood D.C., Henderson J.A and Radostits O.M. English Language Book Society and Bailiere Tindall p. 756.

42. Fairbairet D. (i960) In: Nematology
Sasser J.N. and Jenkins W.R., eds. University of North Carolina Press, Chapel Hill, p 267 - 296.

43. Harned B.K. and Nash T.P. (1932) Theprotection of insulin by antiproteases and its absorption from the intestine.
J. Biol. Cham. *21** 443 - ^56

Sang, J.H. (1938) The antiproteolytic enzyme of Ascaris lumbrlooides var suis. Parasitol. 22. 141 - 155

von Bondsdorf, B. (1949) Blood ^ 91 - 102 Ins Biochemistry of Parasites by T. von Brand Academic Press, N.Y. and London p. 273.

de Waele A. (1933) Bull. Chn. Sci.. Acad Rov Belq. (5) .12 649 - 660 In Bueding E. (1949) Phvsiol Rev. 29 195 - 218

Rhodes M.B., Marsh C.L. and Kelley G.W. (1963) Trypsin and Cliymo trypsin Inhibitors from Ascaris Suum Bxpt. Parasitol 13 266 - 272

Von Brand, T (1973) Biochemistry of Parasites Academic Press, N.Y. and London p 253 - 324.

Smith E.L. (1948) Studies on dipeptidase II some properties of the glycyl-L-leucine dipeptidases of animal tissue. J. Biol. Chem. 176 9 - 1 9

Smith E.L. (1948) The glycyl gylcine dipeptidases of skeletal muscle and human uterus J. Biol. Chem. 173 571 - 584

Smith E.L. (1948) Studies on dipeptidases III Hydrolysis of methylated peptides: The role of cobalt in the action of glycylglycine dipeptidase J. Biol. Cham. 176 21 - 32

52. Smith E.L. (1948) The peptidases of skeletal, heart and uterine muscle
J. Biol. Chem. 173 553 - 569

53. Fruton J.S., Smith V.A and Driscoll P.E.(1948) on tho proteolytic enzymes of* animaltissuesVII A peptidase of calf thymus
J. Biol. Chem. 173 457 - 469

54. Zamecnick P.C., Stephenson M.L. and Copo,0. (1945) Peptidase activity of lymph and serum after bums.
J. Biol. Chem. 158 135 - 144

55. Smith E.L. and Bergman M. (1944) The peptidases of Intestinal mucosa
J. Biol. Chem. 153 627 - 651

56. Hartley B.S., (i960) Proteolytic Enzymes
Ann. Rev. Bioehom. 29 45 - 72

57* Barrot A.J. (1980) Introduction: the classification of proteinases. In: Protein Degradation inHealth and Disease. Ciba Foundation Symposium 75 Excerpta Modica, Amsterdam p. 1 - 9.

58. Blacklock D.B., Gordon R.M. and Fine J. (1930) Ann. Trop. Med. Parasitol. 24 43 - 54. In Biochomistry ef Parasites by T. von Brand (1973) Academie Press, N.T. and London p.

59. Roy D.N. (1937) The physiology of digestion in larvae of Gastrophilus equi
Parasitol. 29 150 - 162

Tatchell R.J. (1958) The physiology of digestion in the larvae of the herse bet-fly, Gastrephllus intostinalis (De Geer) Parasitel. 48. 448 - 458

Regers, W.P. (1941) Digestion in Parasitic Nematodes 111 The digestion of proteins J. Helminthel. 19 47-58

Lee D.L. (1958) Digestion in Loidvnema appendienlata (Leidy I850), a nematode parasitic in cockroaches Paraeitel 48 437 - 447

Rhodes M.B., Marsh C.L. and Ferguson D.L. (1969) Expt Parasite! 26150-155* In Biechemistry of Parasites by T. von Brand Academic Pres, N.Y. and London, 1973 P. 270.

Lee D.L. (1962) Hie histochemical localization of leucine aminopaptida.se in Ascaris Lumbricoides Parasitol *52* 533 - 538

D^vey K.G. and Kan S.P. (1967) Nature (Lond) 188, 493 - 494 cited in Biochemistry of Parasites by T. von Brand. Academic Press N.Y. and London 1973 P. 270

Rogers W.P. (1965) The role ©f leucine aminopeptidase in the moulting of nematode parasites. Comp. Biochem. Physiol 14 311 - 321

Ozerol N.H., and Silverman P.EE. (1969) Partial characterisation of Haemonchus contortus exsheathing fluid.
J. Parasitol. 55 79 - 87

Rogers W.P. (1970) The function of leucine amino peptidase in exsheathing fluid.
J. Parasitol. 56 138 - 143

Ozerol N.H. and Silverman P.H. (1972). Enzymatic studies en the exsheattufoant of Haemonchus contortus infective larvae: The role of leucine aminopeptidase.
Comp Bioehem Physiol 42 109- 121

Slocembe J.O.D. (1974) Some analysis of exsheathing fluid from infective Haemonchus contortus larvae.
Int. J. Parasitol 4 397 - 402

Malmstrong B.G. and Rosonburg A (1959) Mechanism of metal ion activation of enzymes
Adv. Bnzvmol. 21 131

Smith E.L. (1946) Manganese and L-leucin© aminopeptidase.
J. Biol. Chom. 163 15 - 22

Klotz I.M. (1954) Ins The mechanismof Enzyme Action. McElroy, W.D. and Glass B,eds. Johns Hopkins Press, Baltimore, p. 257*

74. Wold F> and Ball®** C.E. {1957) Studies *&n* th® *enzyme* Snolas© XX. Kinetic Studies J. Biol Cham 227. 313 - 328

75. Lowry, Rcsebrough, Farr and Randall (195'|) Protoin measurement with th® Fokin phowal reagent J. Biol. Chem. 193 265 - 276

76. Riels, W. (1974) 'Trypsin' In methods of Enzymatic Analysis IX Borgtnan H.U., od Academic Press Baltimore p. 1013.

77. ABsen M.L. (1939) Th© estimation of pepsin, trypson papain and cathepsin with Haemoglobin J. Gen. Phvsiel. 22 79-89

78. Thaeh R.E., and Newburger M.R. (1972) Research Techniques in Biochemistry and molecular Biology. W.A. Bon. p. 36

79. Frankel, S. (1970) In: Gradwohl's Clinical Laboratory methods and Diagnosis IX 7th Ed. Frankel S., Reitman S. and Sonnenworth A.C., ods. Mosby Co, Saint Louis, p. 45.

80. Hess, R. (1965) Arylamidase activity related to angi'e tonsinase B.B.A. *22* 316

81. Green, M.N., Tsotf, K-C, Bressler, R. and Selignan A.M. (1955). The colorimetric determination of leucine amino peptidase activity with L-leucyl-^-Naphthylamide hydrochloride A.B.B. 458

82. Clark J.M. and Switzer R.L. (1977)
Experimental Biochemistry 2nd Edition
Freeman and Co. San Francisco.

83. Eckert J. (1967) Z. Parasiteak 21 2°9 - 241. In:
Biochemistry of Parasites by T. von Brand (1973)
Academic Press, N.Y. and London p'254

84. Rogers W.P. (1940) Haematelogical studies ®n
the gut content ef certain Nematode and Trematode
parasites.
J. Helminth.Pl 18 53 — 62

85. 0elam, J.B. (1971) Studios on gut ultrastructure
and digestive physiology in Rhabdias bufanis
and R. snhaerocephala (Nematoda: Rhabditida)
Parasitel. 62 247 - 258

86. Celam, J.B. (1971) Studies on gut ultrastructure
and digestive physiology in Cyathostoma Lari
(Nematoda: Strongylida)
Parasitel 62 273 - 283

87. Timms A.R. and Bueding E. (1959) Brit. J.
Pharmacol Chomother 4 68-73 In! Biochemistry
of Parasites by T. von Brand (1973) Academic
Press, N.Y. and London p. 255*

88. C»k L.P., Grant P.T., and Kermack W.O. (1961)
Proteolytic enzymes of the erythropoietic forms
of Rodent and Simian species of malarial plasmadia.
Expt. Parasitol JM 322 - 379

89. Behai F.J., Klein R.A., and Dawson F.B. (1966) Separation and characterisation of aminopeptidase components of human liver.
A.B.B. 115 5^5 -554

90. Glenner G.G., and McMillan P.J. (i96?) A mammalian peptidase specific for the hydrolysis of N Terminal •t-L-Glutamyl and Aspartyl residues
Nature 194 867

91. Nagatsu, I., Nagatsu T., Yamamoto, T., Glenner G. and Mehl J.W, (1970) Purification of aminopeptidase A in human serum degradation of angiotensin . XX by the purified enzyme
B.B.A. 198 255 - 270

92. Hopson V.K., Makinen K.K., and Glenner G.G. (1966) Purification of a mammalian peptidase selective for N-Termlnal Arginine and Lysine residues Amino peptidase B. ABB 114 557 - 566

93. Behai F.J., Little G.H. and Klein R.A. (1969) Aryiamidase of human liver.
B.B.A. 178 118 - 127

94. Brecher A.S. and Suszkin J.B. (1969) Brain aryiamidase. Purification and characterisation of the soluble bovine enzyme.
Biochem. J. 112 335 - 342

95. Sjoholm, Ingver (1969) Oxytecinase and its possible significance in the degradation of oxytocin during pregnancy.
F.E.B.S. Labters 4 135-139

Derer F.E., Haley E.E., and Bueharian D.L. (1968) Th* hydrolysis of B Aspartyl peptidases by rat tissue.
A.B.B. 127 490 - 495
Armentr«lst R.V. (1969) Pyrrolidonecarbexylyl peptidase from rat liver.
B.B.A. 191 ' 756 - 759
Levene P.A., Steiger R.E., and Rethen R. (1932) The relation of chemical structure te the rate of hydrolysis of peptidases VTX.Hydrolysis of peptides by alkali.
J. Biol. Chem. 97 717 - 726
Synge R.L.M. (1945) The kinetics of low temperature acid hydrolysis of gra^ueedin and of some related dipeptides.
Biochem. J. *22** 351 - 355
Smith E.L., Spackman D.H. and Pelglase W.J. (1952) The specificity ef leucine aminopeptidase XII Action on diastereoisemers.
J. Biol. Chem. 199 801 - 817
Behai F.J., Asserson B., Dawson F. and Hardman J* (1965) A study of human tissue aminopeptidase
A.B.B. 111 335 -
Behai F.J. and Folds J.D. (1967) Arylamidase of Neisseria catarrttalis.

103. **Gemeri, G.(1955) Preparatien ef buffers fer use in Enzyme Studies Metiieds in Enzymelegy I 138-146.**