Peptidases of *Trypanosoma cruzi*

by

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Abstract

A survey of proteolytic activities in *Trypanosoma cruzi* (Y strain) was carried out. Protein substrate-containing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) revealed some distinct cysteine peptidase activities with acidic pH optima and an alkaline membrane-associated peptidase, putatively a metallopeptidase. The activities in the insect (epimastigote) and mammalian (trypomastigote) forms of the parasite were found to be similar except that the membrane associated putative metallopeptidase was absent from trypomastigotes. No secreted or shed peptidases were detected. Peptidases expressed by different strains of *T.cruzi* isolated from various areas of South, Central and North America were very similar, although some differences were noted.

Chromogenic and fluorogenic assays with a diverse set of peptidase substrates were also used to characterize *T.cruzi* proteolytic activities. Information on pH optima, substrate-specificities and catalytic class were obtained for these peptidases. Both exopeptidase and endopeptidase activities belonging to different catalytic classes were found. In one case it was possible to relate the activities detected by protein substrate SDS-PAGE with an activity detected by a fluorogenic substrate. Again, no secreted peptidase activities were detected. A

series of synthetic substrate analogue inhibitors whose action is restricted to cysteine and serine peptidases were used to gain further information on substrate-specificity for the activities of the cysteine peptidases.

The major alkaline peptidase activity of a number of trypanosomatids, particularly *Leishmania donovani* and *Crithidia fasciculata*, was investigated. Substrate-specificity, pH optima, kinetic data, electrophoretic mobility and inhibitor sensitivities were found to be similar to the enzyme characterized previously from *T.cruzi*. A number of non-trypanosomatid protozoa did not have an analagous activity.

An investigation into the use of radiolabelled total parasite DNA for the sensitive detection of trypanosomatid parasites is also presented. The specificity of the technique was evaluated and found to be sufficient for the unambiguous detection of *T.cruzi* and *T.rangeli* but not for discrimination between different species of *Leishmania*. The method was used for the analysis of *T.cruzi* and *T.rangeli* infections in field-caught triatomine insects from a rural area near Bogota in Colombia.

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Abbreviations

Ac, acetyl

Boc, t-butyloxycarbonyl

BSA, bovine serum albumin

Bz, benzoyl blocking group

DFP, Diisopropylfluorophosphate

DMSO, dimethyl sulphoxide

DTT, dithiothreitol

E64,
trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane

EDTA, ethylenediaminetetra acetic acid

IAAH, iodoacetic acid

Nmec, 7-amino methylcoumarin

NP-40, Nonidet P-40

o-phen, o-phenanthroline

PBS, phosphate buffered saline

PMSF, phenylmethylsulphonyl fluoride

-pNan, p-nitroanilide

RPMI 1640, Rosewell Park Memorial Institute 1640

SDS, sodium dodecyl sulphate

TCA, trichloracetic acid

TEMED, 4-N,N,N,N-tetramethylethylenediamine

TLCK, N-alpha-p-tosyl-L-lysine-chloromethyl ketone

Tos, p-toluene sulphonyl

TPCK, N-alpha-p-tosyl-phenylalanine-chloromethyl ketone

Z, carbobenzoxy blocking group

Chapter One

Introduction

South American Trypanosomiasis (Chagas' disease).

This disease is widespread in parts of South America. It is also found in Central America (Cedillos, 1975; Zeledon et al., 1975), Mexico and the Caribbean (Petana, 1978) although it may be less widespread and the symptoms less severe in these areas. However, recent work indicates that the prevalence of Chagas' disease in Mexico is comparable to that seen in South America (Salazar Schettino et al., 1988). The number of people in the world infected with *T.cruzi* has been estimated as approximately 11 million (WHO, 1960). Two cases of endogenous infection have been reported from the U.S.A. (Schiffler et al., 1984). The disease may be fatal, debilitating or asymptomatic. The reasons for the variation in the symptoms are not known but could relate to parasite, host or environmental factors or a combination of these.

The causative organism of Chagas' disease is the flagellated protozoan, *Trypanosoma cruzi*, which is transmitted to humans by Triatomine insects. Certain populations of these vectors have adapted to live in

association with man and these are responsible for transmission of most cases of the disease. New endemic foci of the disease are initiated by colonization of dwellings with vectors that have become infected by feeding on wild animals (Miles, 1979). Populations of wild and domestic mammals are important reservoirs of *T.cruzi* which contribute to the problem of controlling the disease (Miles, 1979).

Chemotherapy for Chagas' disease is currently very unsatisfactory and there is no vaccine available. Vaccination carries the risk of precipitating an autoimmune response which has been implicated in the pathogenesis of the disease and so potential vaccines must be evaluated carefully with regard to this. The most successful preventitive measures used now are insecticides (Dias, 1987) and the use of new materials for rural housing which make the houses less suitable for triatomine bug infestation.

Trypanosoma cruzi: classification.

The order, Kinetoplastida, of which *T.cruzi* is a member, are characterized by posession of a modified single mitochondrion. This includes a DNA packed unique structure called the kinetoplast. While there are some free living Kinetoplastida, all members of the

Trypanosomatidae family are parasitic. The family is comprised of the genera: *Crithidia*, *Blastocrithidia*, *Leptomonas*, *Herpetomonas*, *Endotrypanum*, *Phytomonas*, *Leishmania*, *Trypanosoma* and *Rhynchoidomonas*. The genus *Trypanosoma* was divided by Hoare (1964) into the sections stercoraria in which infective forms develop in the hind gut of the insect and salivaria in which the infective forms appear in the mouth parts. *T.cruzi* is a stercorarian and *T.rangeli* and the African trypanosomes are salivarians.

The accepted taxonomy of the Trypanosomatidae probably does not reflect phylogenetic relationships accurately since there is little agreement in this area as seen from the very different phylogenies proposed by Wallace (1966) and Baker (1965). Biochemical studies (including DNA sequencing) of kinetoplastids coupled with a better understanding of their transmission genetics may enable their phylogenies to be determined with more certainty in the future (Newton, 1979).

Trypanosoma cruzi: life cycle and morphology.

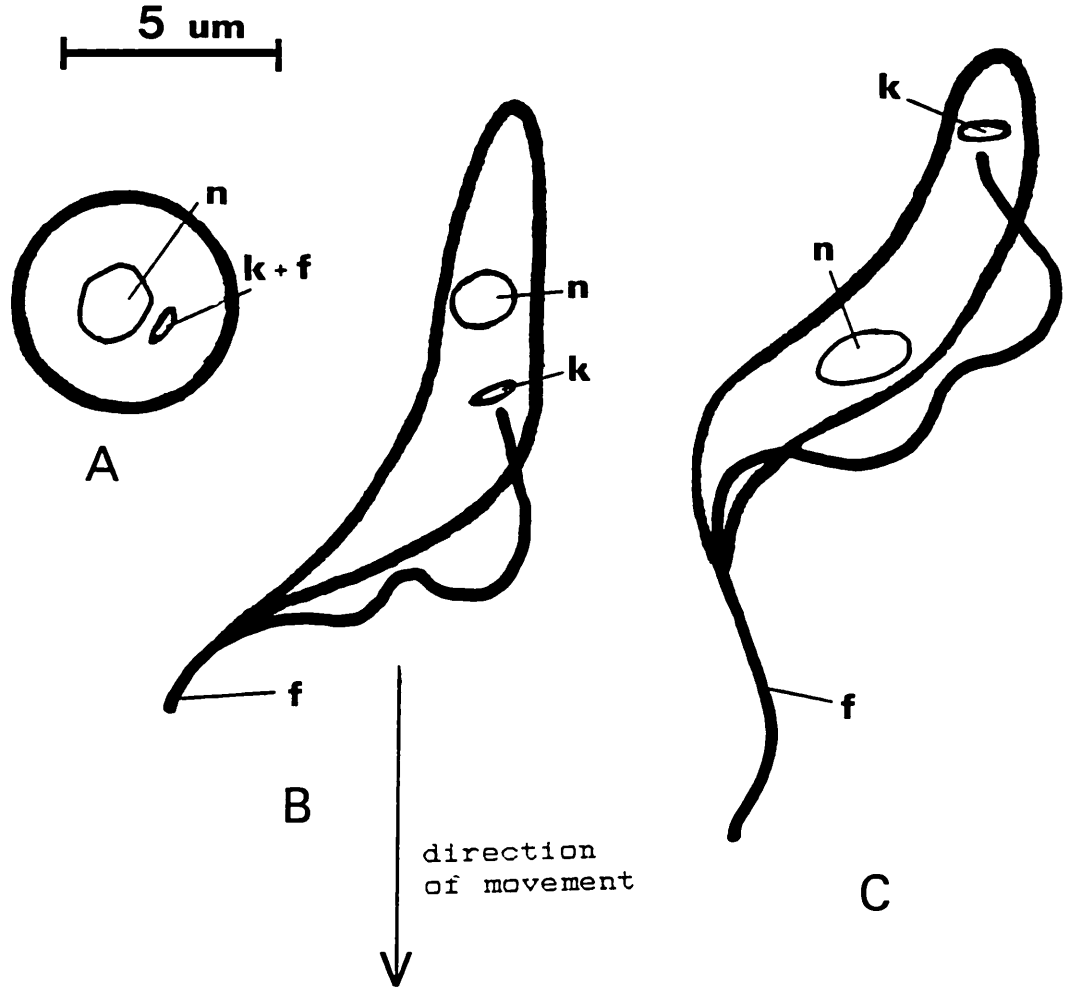
The discovery of *T.cruzi* and its role in the causation of the acute and chronic forms of what is now called Chagas' disease can be attributed almost entirely to Carlos Chagas (1909). Brumpt (1912) noted that *T.cruzi*

infection occurred by contamination with insect faeces rather than the inoculative route that Chagas had favoured. In addition to pioneering work on pathogenesis, Vianna (1911) showed that parasite cell division in the mammal only occured within host cell cytoplasm.

Infective, motile, metacyclic trypomastigotes (Figure 1 C) are shed in the faeces of the blood-sucking reduviid insect hosts of *T.cruzi*. These insects tend to defaecate during ingestion of the bloodmeal liberating infective parasites which may gain entry to the portal system of the host through the bite itself or through an intact mucosal surface such as the conjunctiva of the eye. Other routes of infection include ingestion of parasites, blood transfusions and transfer from the circulation of an infected mother to the foetus.

On entering the bloodstream the metacyclic trypomastigotes invade a number of cell types including macrophages, fibroblasts, muscle cells, neuronal cells and glial cells. Within the cell cytoplasm the parasites transform to non-motile, dividing, amastigote forms (Figure 1 A). After as many as 500 (Dvorak, 1976) amastigotes have been produced in the host cell they transform to motile, non-dividing, trypomastigotes which are similar in morphology to the metacyclic trypomastigotes (Figure 1 C). The mammalian host cell lyses liberating these into the local tissue spaces from where they may infect nearby cells or gain entry into the

Figure 1. Life cycle forms of *T. cruzi*. The nomenclature is based on that introduced by Hoare and Wallace (1966). A, amastigote; B, epimastigote; C; trypomastigote. Key: f = flagellum; n = nucleus; k = kinetoplast.


5 um
n
k + f
A
n
k
f
B
direction
of movement
k
n
f
C

portal system enabling them to infect cells at distant sites and also, if taken up in a bloodmeal, reduviid insect vectors.

Within the vector the non-dividing motile trypomastigotes transform into dividing, motile, epimastigotes (Figure 1 B). In the midgut and hindgut of the insect the epimastigotes divide and a proportion of them transform into non-dividing metacyclic trypomastigotes in the hindgut thereby completing the life cycle.

The pathology of Chagas' disease.

The acute phase may be characterized by a localized inflammation called a chagoma if the site of penetration was a break in the skin or Romana's sign if the site was the conjunctiva of the eye. As the parasitaemia develops, fever, subcutaneous oedema, lymph node enlargement and hepatosplenomegaly may occur. Myocarditis and meningoencephalitis may also occur in children and are often fatal. Andrade (1983) has estimated an approximate mortality of 10% during the acute phase of Chagas' disease. Parasite levels in the blood subside within four months through mechanisms of host immunity but very low parasite levels have been detected decades after the initial infection and these

are not thought to be due to subsequent reinfection.

Following the acute phase, which may pass unnoticed, an asymptomatic stage occurs which may last until death, by natural causes, many decades later. ECG (Andrade, 1983) and postmortem (Prata, 1976) information from subjects in this indeterminate phase of the disease shows that there is often a progressive cardiomyopathy occuring but that damage is below the threshold required for symptoms to appear.

The chronic cardiopathological form of the disease (reviewed by Andrade, 1983) commonly presents, many years after the initial infection, with palpitations, breathlessness, oedema of the lower limbs and precordial pain. Electrocardiogram abnormalities are common and to some extent diagnostic. Death from cardiac arrest, pulmonary embolism or intractable heart failure may occur.

The frequency of digestive syndromes is low in Venezuela (Koberle, 1974; Rezende 1976) while in parts of Brazil they can be more common than the cardiac syndromes (Koberle, 1974). Hypertrophy of the oesophagus and colon in response to destruction of neurons innervating these organs results in difficulty in eating and swallowing and other complications. Dietary, laxative and occasionally surgical intervention normally prevent death from the digestive syndromes.

The pathogenesis of Chagas' disease.

Vianna (1911) suggested that amastigotes that have failed to transform to trypomastigotes degenerate at the site of their release and cause a local response that is destructive to nearby ganglion cells. Koberle (1968) suggested that this was due to the release of a toxin from the degenerating parasites. However, it is now clear that the damage is largely caused by host inflammatory reaction. Cortisone, which blocks inflammatory responses, prevented neural degeneration in *T.cruzi*-infected mice implicating immune mechanisms in this aspect of pathology (Andrade and Andrade, 1966). Histological sections of infected tissue show considerable invasion by macrophages and lymphocytes which is consistent with immune mechanisms being involved in pathogenesis.

The delay between the parasitaemia and the onset of chronic symptoms was explained by Koberle (1968 and 1974) by postulating that the nerve and muscle damage caused in the acute phase accumulates and combines with normal, age-related, loss of neurons until a threshold of damage is reached at which time chronic symptoms appear. The progressive myocardial damage seen in the indeterminate phase of the disease (Andrade, 1983; Prata, 1976) is consistent with this model.

A mechanism involving autoimmunity can also

explain the delay between high parasitaemia and chronic symptom onset (reviewed by Hudson, 1985; Takle and Hudson, 1989). Chagasic serum has been shown to contain anti-self antibodies reacting with various cell types and extracellular components. Monoclonal antibodies that cross-react with epitopes on *T.cruzi* parasites and mammalian nerve and muscle cells have also been isolated (Woods et al., 1982). However, anti-self antibodies are found in a number of pathologies not thought to involve autoimmunity and some of the results demonstrating such antibodies have subsequently been retracted by the authors (reviewed by Kierszenbaum, 1985).

Diagnosis of Chagas' disease.

T.cruzi parasitaemias are rarely resolved completely and therefore a parasitological diagnosis can in principle be made even in the chronic phase of the disease. In the acute phase diagnosis can be made by microscopic examination of blood smears. Methods which concentrate the parasites in the blood increase the sensitivity of this technique (Budzko and Kierszenbaum, 1974; Hoff, 1974).

Parasite amplification methods (reviewed by Minter-Goedbloed et al., 1978) include: 1) xenodiagnosis in which uninfected insect vectors of *T.cruzi* are fed on

the patient and examined for *T.cruzi* parasites after a suitable incubation period; 2) haemoculture in which blood from the patient is inoculated into a suitable medium and 3) inoculation of mammalian hosts, for example mice, with blood from the patient.

Hybridization of labelled total parasite DNA (Ashall et al., 1988), purified kinetoplast DNA (Sanchez et al., 1984) and cloned parasite DNA sequences (Gonzalez et al., 1984) have potential for the sensitive and reliable diagnosis of Chagas' disease. Sample preparation and analysis may be expensive and time-consuming compared to microscopical examination of blood but could be practicable where large numbers of samples are to be analysed, as in an epidemiological survey, because the methodology involved lends itself to scaling up. For very sensitive detection the polymerase chain reaction (PCR) has considerable potential.

Diagnosis by immunological detection of *T.cruzi* antigens in human serum has been investigated by Araujo et al. (1981 and 1982).

Diagnosis by detection of host antibodies to *T.cruzi* antigens has been widely used. A complement-fixation test using a crude preparation of *T.cruzi* antigens first appeared in 1913. Serodiagnosis with purified *T.cruzi* antigens has done much to reduce false positives due to cross-reactions with other organisms such as *Leishmania* and *T.rangeli*. Gp90 (Schechter et al.,

1983) and gp25 (Scharfstein et al., 1985) both show promise although cross-reactions were reported in the case of the assay with gp90.

Chemotherapy of Chagas' disease.

Lampit, also known as nifurtimox, and benznidazole are used but have very unpleasant side effects. The drugs have very little effect on the chronic form of the disease presumably because the majority of parasites are sequestered inside host cells (Cancado et al., 1976).

Both drugs may work by reduction of their nitro groups and subsequent oxidation by oxygen to restore the nitro group with concomitant production of H_2O_2 within the parasite (Brown, 1987). Trypanosomes contain no catalase or glutathione peroxidase and hence are likely to be susceptible to oxidative and free radical attack.

Heterogeneity of *Trypanosoma cruzi*

The wide degree of variation in the severity and type of symptoms that occur in Chagas' disease is paralled by considerable heterogeneity amongst strains of the parasite with regard to many of the parameters that

have been investigated. This has led to the suggestion that different strains of the parasite might be responsible for the different manifestations of the disease (Miles et al., 1981; Dvorak, 1984). However despite attempts, there is still no good evidence in support of this view. Such evidence may be difficult to obtain since infections often involve more than one strain which may well confuse the issue since if the parasites are not cloned the strains that comprise the population may not be identified. If they are cloned, not all the strains present may be represented.

In general the parameters that have been used to group *T. cruzi* clones give concordant results (Tibayrenc and Ayala, 1988) allowing the species to be grouped into natural populations which may represent separately evolved clones derived by asexual reproduction. These parameters include growth kinetics (Dvorak et al. 1980), isozyme analysis (Ready and Miles, 1980) and DNA restriction site, or schizodeme, analysis of kinetoplast DNA (Morel et al., 1980).

An isozyme analysis involving over 500 independently isolated *T. cruzi* stocks placed the stocks into 43 different zymodemes. Within a zymodeme all members have the same isozyme patterns at the loci examined. Although some of the very similar zymodemes may have been related to each other, many of the zymodemes did not seem to be so in a hierarchical manner which

would allow phylogenetic relationships between the zymodemes to be inferred. Rather, the hypothesis that the zymodemes had been evolving independently for very long periods as asexual clones was supported (Tibayrenc and Ayala, 1988).

Peptidase nomenclature

Barrett and McDonald (1985) suggested that the term peptidase be used as an abbreviation of "peptide bond hydrolase" and so includes any enzyme that cleaves peptide bonds. Previously, "peptidase" was often used to describe an enzyme that cleaved near the N- or C-terminus of a polypeptide and that was therefore more active on short polypeptides than long polypeptides when activity was expressed in terms of weight, rather than moles, of substrate hydrolysed per unit of time.

The words "protease" and "proteinase" now seem to be used almost interchangeably in the literature. Grassmann and Dyckerhoff (1928) proposed the term "proteinase" for proteases that showed specificity for intact proteins as opposed to shorter peptides, for which these authors proposed the term "peptidase." Bergmann and Ross (1936) introduced the terms endopeptidase and exopeptidase which loosely speaking correspond to the terms "proteinase" and "peptidase" respectively proposed

by Grassmann and Dyckerhoff (1928) (Fig 2). The latter terminology is considered preferable because it is based on an understanding of the mechanism for the difference in substrate specificity (Barrett and McDonald, 1986).

Endopeptidases are inhibited by free alpha-amino and alpha-carboxyl groups whereas exopeptidases require these groups for activity. Some peptidases show both endopeptidase and exopeptidase activity and arbitrarily are classified as endopeptidases (Barrett and McDonald, 1986). The system of nomenclature proposed by Barrett and McDonald does not include the widely used term "protease". However, it seems unlikely that this word will be quickly lost from the literature. In this study the terminology suggested by Barrett and McDonald (1985) and Barrett and McDonald (1986) has been adopted, that is to say, any enzyme that cleaves a peptide bond is referred to as a peptidase, endopeptidase or exopeptidase.

Endopeptidases are further classified, according to the nature of their active site, into serine-, cysteine-, aspartic- and metallo- peptidases and these distinctions are used in the International Union of Biochemistry's classification of peptidases (Enzyme Nomenclature, 1984). Normally a peptidase can be assigned to one of these classes unambiguously through use of a panel of class-specific or associated inhibitors.

In contrast the exopeptidases are classified

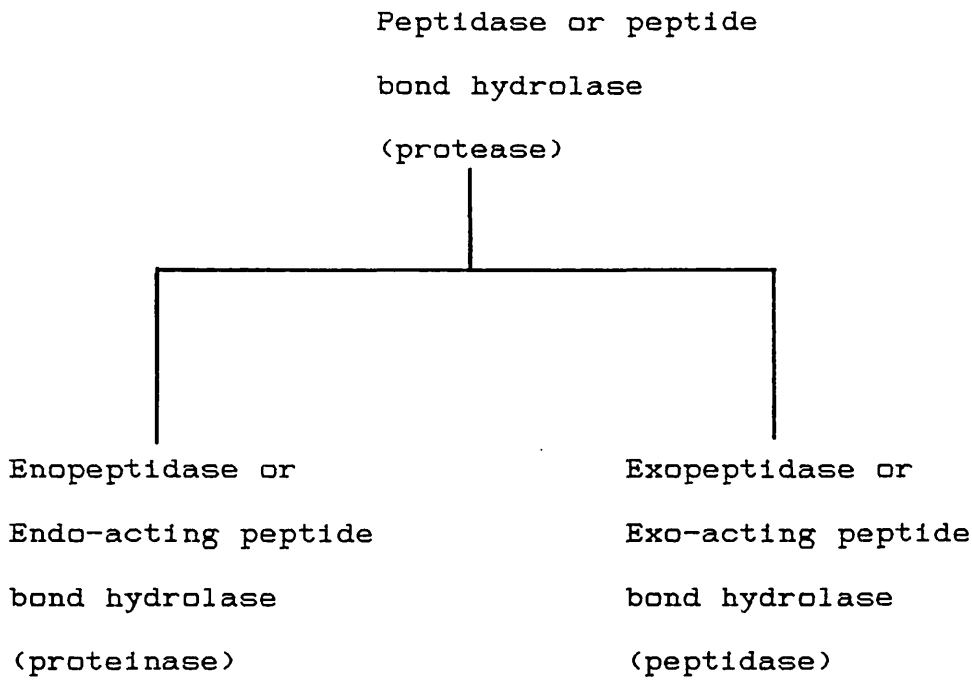


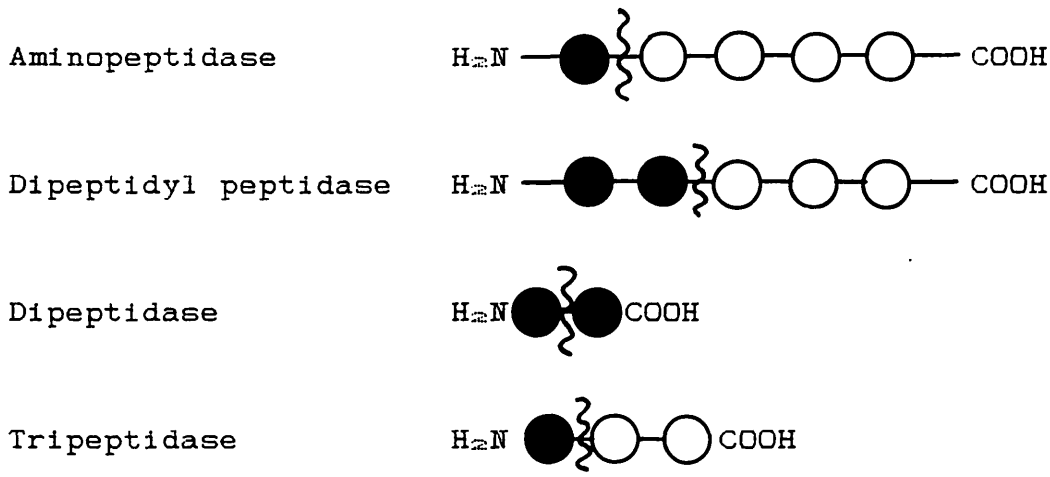
Figure 2. The system of nomenclature for enzymes that cleave peptide bonds proposed by Barrett and McDonald (1985 and 1986) and Bergmann and Ross (1936). The older system, proposed by Grassmann and Dyckerson (1928) which is still used in the literature is shown in parenthesis. The two systems are shown as corresponding but this is an approximation as exopeptidases do digest long polypeptides to some extent.

according to their substrate specificity even though they share at least some of the same catalytic mechanisms as the endopeptidases and have not been shown to use any different mechanisms. The features of the substrates used for classification of exopeptidases are whether it is the carboxy or amino terminal of the peptide that is attacked, whether the peptide to be cleaved is two, three or more residues in length and whether a single amino acid, a dipeptide or a tripeptide is cleaved from the substrate (Figure 3).

Peptidases

The substrate-binding site and catalytic site of a peptidase comprise the active site. They are thought to be separate in space and in many cases new enzymes appear to have evolved through changes to the binding site, which alter the specificity of the enzyme, while the composition of the catalytic site has remained constant. Trypsin, chymotrypsin and elastase are examples of enzymes related in this way. Site directed mutagenesis has also resulted in new enzymes with altered substrate specificity (Craik et al., 1987; Bone et al., 1989). For many serine peptidases substrate specificity has been modified during evolution by the addition of new protein domains that are quite separate from the active site

Aminopeptidases



Carboxypeptidases

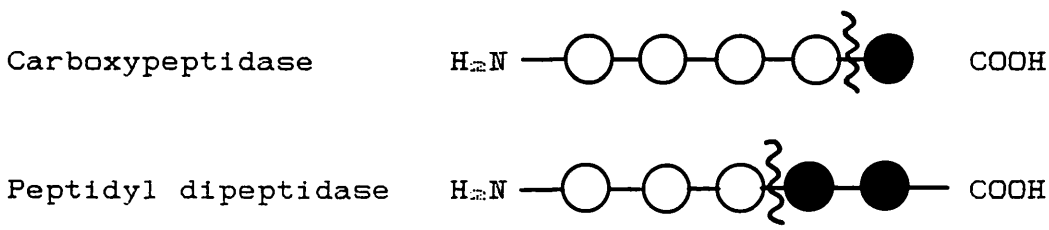


Figure 3. The system of nomenclature for exopeptidases taken from Barrett (1986). The circles represent the amino acid residues of the peptide; the broken line represents the peptide bond cleaved.

domain and which interact with the substrate (Neurath, 1984).

Figure 4 shows a diagramatic representation of the binding and catalytic sites of a peptidase based on the model proposed by Schechter and Berger (1967) to account for the results of substrate and inhibitor specificity studies on papain. The binding site is composed of a cleft with an extended polypeptide backbone binding site and a series of subsites, S_n and S'_n, which accommodate the amino acid residues of the substrate, P_n and P'_n. The peptide bond that is cleaved is indicated by the vertical, dashed line. The S and S' sites of peptidases can vary in their specificity for a particular amino acid. Some show very little discrimination between amino acid residues while, at the other extreme, some S and S' sites will only accommodate only one of the twenty amino acids. The S_1 site of the serine peptidase, trypsin, is only able to accept a lysine or an arginine. However, the nature of the amino acids occupying the other binding sites of trypsin exert much less influence on binding and catalysis by the enzyme. Cathepsin B, a cysteine peptidase, will accept only substrates with a large hydrophobic residue binding in its S_2 site (Shaw et al., 1983) while the closely related enzyme cathepsin L is tolerant of a wider range of amino acids at this site.

In many cases substrate-specificity is not determined solely by the linear order of the amino acids

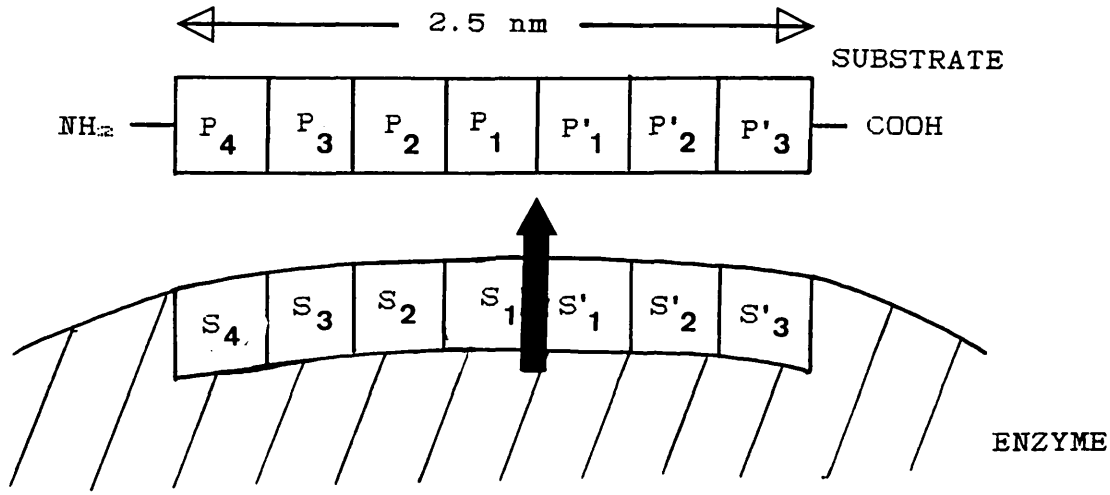


Figure 4. Scheme taken from Schechter and Berger (1967) for peptidase substrate binding subsites. The scheme, based on studies of papain, is generally applicable to peptidases although the contribution of different substites to binding varies greatly between enzymes. The arrow indicates the catalytic site and points to the bond on the substrate that is cleaved.

on either side of the bond to be cleaved. The HIV peptidase, for example, cleaves proteins at a number of distinct amino acid sequences.

Serine peptidases.

This class of enzyme seems to have evolved at least twice, that is by convergent evolution. The well represented trypsin superfamily is found in prokaryotes and eukaryotes while the subtilisin family has been found only in bacteria. The mechanism of catalysis is not fully understood but it is likely to be very similar in both the subtilisin and trypsin superfamilies because both groups of enzymes have their catalytically functional groups arranged in the same geometrical relationship (Robertus et al., 1972 a; Robertus et al., 1972 b and Segal et al., 1971). In other respects there is little homology between the two groups of enzymes.

The nucleophilic hydroxyl oxygen atom of the reactive serine residue attacks the carbonyl carbon of the peptide bond to be cleaved which results in release of the C-terminal-containing peptide product and covalent attachment of the N-terminal-containing peptide product to the enzyme via the serine oxygen atom. Subsequent attack of this acyl bond by water regenerates the serine hydroxyl group and liberates the N-terminal-containing peptide product (Figure 5). X-ray crystallography of different substrate analogues complexed with serine

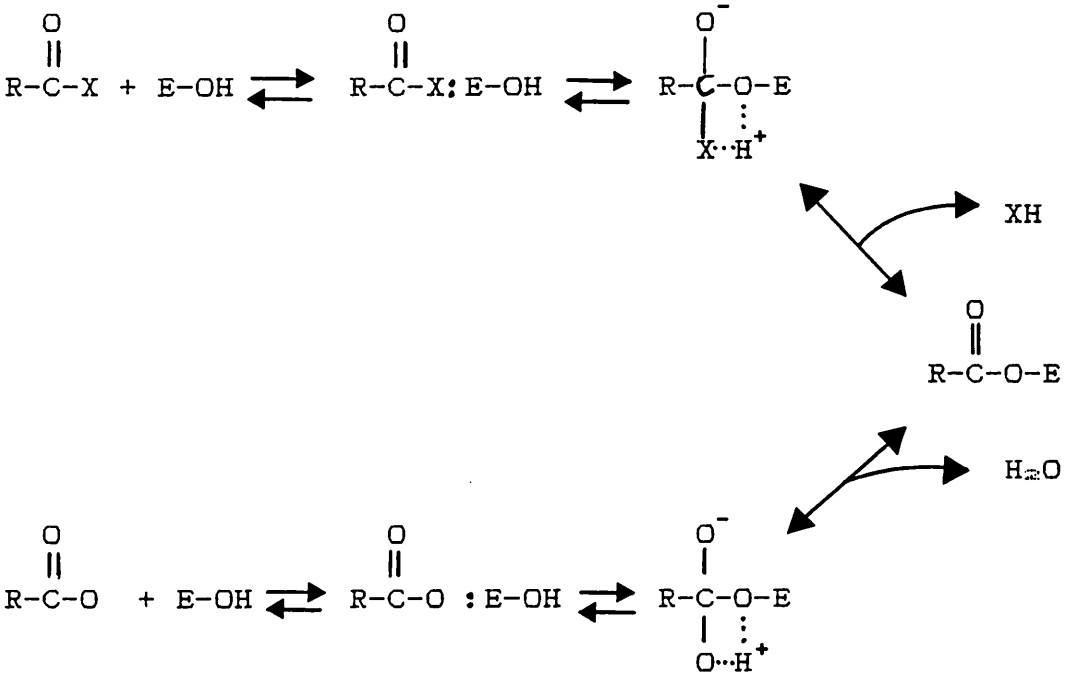


Figure 5. The reaction sequence for serine peptidases. Taken from Kraut (1977). If the substrate were a peptide then the leaving group, X, would be the alpha amino nitrogen of the N-terminal amino acid of the peptide product.

peptidases provide evidence for the Michaelis complex (Steitz et al., 1969 and Robertus et al., 1972b), the tetrahedral adduct (Matthews et al., 1975 and Poulos et al., 1976) and the acyl enzyme (Henderson, 1970). Kinetic data also support the existence of these intermediates (Hess et al., 1970; Fastrez and Fersht., 1973 and Bender and Philipp., 1973).

An imidazolium group of a histidine residue may assist the reaction by acting as a general base catalyst in accepting the proton from the serine hydroxyl group during its nucleophilic attack of the substrate. In the deacylation reaction the protonated form of this group would be able to act as a general acid catalyst favouring hydrolysis of the acyl bond of the enzyme-substrate intermediate. A pocket in the enzyme containing a positively charged side-chain is located so that it can help stabilize the negative oxyanion of the tetrahedral substrate-product transition state (Robertus et al., 1972 a) although its importance in catalysis has been questioned. There is now some evidence against the operation of a charge-relay system involving the so-called catalytic triad, ser-his-asp (Birktoft et al., 1978 and Matthews et al., 1977), in which the negative charge on a catalytic aspartate side-chain stabilizes the active site histidine during proton transfer from the reactive serine (Polgar and Halasz, 1982). The aspartate may act by increasing the ability of the histidine to

accept the proton from the serine oxygen during general base catalysis although for the second proton transfer, from histidine to the leaving group, the interaction of histidine with aspartate would be inhibitory. A crystallographic study of subtilisin of higher resolution than previous work did not give evidence for a hydrogen bond between the reactive serine and histidine (Matthews et al., 1977).

Cysteine peptidases.

The evolutionary superfamily that includes papain has well conserved members from all the major euykaryotes including the cathepsins B, H and L found in mammalian lysosomes (Takio et al., 1983). Two bacterial cysteine peptidases and the calpains from animals probably represent another three independently evolved families of this peptidase class.

The reactive nucleophile is thought to be a thiolate-imidazolium ion pair and in contrast to the serine peptidases attack on the carbonyl carbon atom of the scissile peptide bond is not thought to be assisted by general base catalysis. The thiol ester enzyme-substrate intermediate generated in the first stage of the reaction is then attacked by water with release of the second reaction product. There does not appear to be an equivalent to the serine peptidase catalytic triad and there is less evidence in favour of

an oxyanion hole than for the serine peptidases.

Aspartic peptidases.

This class of peptidase has been found only amongst eukaryotes and their viruses suggesting they have evolved more recently than the other peptidase classes. Sequence and X-ray crystallographic data for mammalian pepsin (Andreeva et al., 1984) and aspartic peptidases from *Rhizopus* (Bott et al., 1982), *Penicillium* (James and Sielecki, 1983) and *Endothra* (Pearl and Blundell, 1984) suggest that they have all diverged from the same ancestral protein and therefore comprise a single evolutionary superfamily.

The active site of aspartic peptidases is bilaterally symmetrical, or pseudosymmetrical, including the disposition of two aspartic acid residues which are within hydrogen bonding distances of each other (Hsu et al., 1977; Bott et al., 1982; Andreeva et al., 1984; and Blundell et al., 1985). In some virally coded aspartic peptidases symmetry at the active site is achieved by the association of two identical monomers, each contributing one of the active site aspartic acids (Navia et al., 1989). Carboxyl groups were originally implicated in the catalytic mechanism by the low pH optima of these peptidases and by inactivation with carboxyl blocking reagents, for example diazoacetylnorleucine methyl ester (Ragagopolan et al., 1966) and 1,2-epoxy-3-(p-nitrophenoxy)

propane (Chen and Tang, 1972).

The catalytic mechanism for cleavage of peptide bonds by aspartic peptidases is not known although it is clear that there is no covalent acyl or amide enzyme intermediate and that neither of the active site aspartic carboxyl groups nucleophilically attacks the peptide bond (Pearl, 1987). The active site aspartic acid groups could enhance the nucleophilicity of a water molecule which then attacks the carbonyl carbon or they could polarize the substrate carbonyl bond increasing the susceptibility of the carbonyl carbon atom to nucleophilic attack (Pearl, 1987)

Metallopeptidases

These are found in bacteria, fungi and higher organisms. Evolutionary relationships between metallopeptidases are unclear, particularly between some of the exopeptidases and endopeptidases. However, carboxypeptidase A, from mammals, and bacterial thermolysin appear to have evolved from different ancestral proteins. Zinc is thought to be the only metal naturally employed by the enzymes in nature although it is possible for zinc to be replaced artificially by other metals, for example cobalt, without losing enzymatic activity.

Two catalytic mechanisms have been proposed for carboxypeptidase A-catalysed cleavage of peptides

(Lipscomb et al., 1970). In the free enzyme the active site Zn^{++} is thought to be coordinated by three amino acid residue side chains of the enzyme and by a water molecule in the active site. The carbonyl oxygen of the scissile peptide bond of the substrate displaces the water and coordinates with the Zn^{++}. This increases the polarization of the carbon-oxygen bond of the carbonyl group, making the carbonyl carbon atom more susceptible to nucleophilic attack. The nucleophile is either: 1) a water molecule, one of the protons of which is lost to a carboxylate side chain group on the enzyme through general base catalysis or 2) an oxygen atom of a carboxylate side chain group on the enzyme which yields an acylated enzyme-substrate intermediate. Crystallographic and sequence data from thermolysin suggest that it has a similar catalytic mechanism to carboxypeptidase A although stereochemical restrictions appear to exclude the possibility of direct nucleophilic attack by an enzyme carboxylate residue. Thus, attack of the scissile bond by water is favoured (Kester and Matthews, 1977).

Peptidase inhibitors

Peptidase inhibitors are important for the study of the biochemistry and functions of peptidases. They

have potential for chemotherapeutic use and, indeed, captopril, an inhibitor of the metallopeptidase, angiotensin converting enzyme, is used in the treatment of high blood pressure. In some cases, chemical and X-ray crystallographic data allows an understanding of the inhibitory mechanism. In other cases similarities between the inhibitor and substrates of the enzyme in addition to knowledge of the reactive groups in the catalytic site of the enzyme suggest mechanisms of inhibitor action. Transition-state theory has been used to rationalize the inhibitory effects of various compounds on peptidases (Kraut, 1977).

Inhibitor specificity may primarily be determined by the nature of the binding sites of target peptidases or by the nature of the catalytic sites or by a combination of both. There are very few inhibitors which are absolutely specific in their action to one class of peptidase. Conversely, none of the inhibitors inhibit every member of a peptidase class. Thus a panel of inhibitors should be used in assessing the catalytic class of a peptidase.

Inhibitors of serine peptidases.

Inhibition by diisopropylfluorophosphate (DFP) is one of the best criteria for designating a peptidase into the serine class. DFP is highly toxic due to its inhibition of acetylcholinesterase which has an active

site serine residue.

Phenylmethylsulphonyl fluoride (PMSF) is safer and cheaper than DFP. It inhibits enzymes with essential, reactive serine groups but there are some serine peptidases that are resistant to it and some non-serine peptidases are inhibited by quite low concentrations of PMSF.

Blocked aminoacyl and peptidyl chloromethanes, for example tosyl-lysine chloromethyl ketone (TLCK) and tosyl-phenylalanine chloromethyl ketone (TPCK), are good inhibitors of serine peptidases but also inhibit many cysteine peptidases (Porter et al., 1971). This class of inhibitors is useful, however, because the aminoacyl or peptidyl portion of the molecule, which acts as a substrate analogue, can be changed thereby enabling inhibitors specific to enzymes with particular substrate specificities to be developed.

A number of natural and synthetic peptide aldehydes such as leupeptin and certain elastase substrate aldehyde analogs such as Ac-Ala-Pro-alaninal reversibly inhibit many serine peptidases but also some cysteine peptidases (Thompson, 1973).

Proteinaceous inhibitors tend to be class specific but do not inhibit all members within a class. (Alpha-2-macroglobulin, which inhibits peptidases from all classes by an unusual mechanism is an exception to this). They bind to the enzyme with a high affinity but

the peptide bond cleaves very slowly, ie the Kcat is low. Although these are substrate analogue inhibitors, interactions between the enzyme and inhibitor can involve amino acid residues of the enzyme that do not interact with the true substrate. It has been possible, by site-directed mutagenesis of such residues in tissue type plasminogen activator, to construct a variant enzyme which is resistant to plasminogen activator inhibitor-1. The altered enzyme is therefore expected to be an improvement on the recombinant t-PA presently used as an antithrombolytic reagent (Madison et al., 1989).

Inhibitors of cysteine peptidases.

Reagents that bind irreversibly to thiol groups tend to inhibit cysteine peptidases when used at low concentrations. This is because the active site cysteine is more reactive than most other cysteine residues in the protein. However, interaction with non-active site cysteines and amino groups results in inactivation of some enzymes of other classes. For this reason substrate analogue thiol blockers are superior reagents.

The use of fluorine instead of chlorine in peptidylhalomethanes reduces their action on most serine peptidases but does not affect their inhibition of cysteine peptidases since the sulphhydryl residue of cysteine peptidases is more reactive than the active site hydroxyl residue of serine peptidases. Peptidyl

sulphonium salts are also effective inhibitors of cysteine peptidases for similar reasons (Rauber et al., 1988). Peptidyl diazomethanes are very specific for cysteine peptidases (Shaw and Green, 1981).

Inhibition by the fungal compound, L-trans-epoxysuccinyl-leucylamido(4-guanidino)butane (E64), is diagnostic of cysteine peptidases (Barrett et al., 1982). The leucine residue of E64 interacts with the P'_1 site of the enzyme and the epoxy group reacts covalently with the active site. Like many of the diazomethanes, E64 is not toxic in mammals at concentrations far exceeding that required for peptidase inhibition and it therefore shows promise for *in vivo* experimental and chemotherapeutic use.

Cystatin, a proteinaceous inhibitor from egg white and other sources, inhibits most of the papain-related cysteine peptidases but not the calpains. The latter are inhibited by the proteins, calpstatin and α-cysteine proteinase inhibitor.

Inhibitors of aspartic peptidases.

Pepstatin, whose structure is: isovaleryl-val-val-sta-ala-sta (sta is the B-hydroxyl amino acid statine), is the most widely used inhibitor of aspartic peptidase. It binds reversibly to the enzyme. However, some fungal aspartic peptidases are resistant to peptstatin. Alterations in the structure of pepstatin

have been made in an attempt to increase the potency of the peptide for particular enzymes, particularly renin which is involved in the regulation of blood pressure.

The complex of diazoacetyl-norleucine methyl ester with copper ions has also been shown to inhibit aspartic peptidases (Ragagopalan et al., 1966). There are few other aspartic peptidase inhibitors reported in the literature.

Inhibitors of metallopeptidases.

These include metal chelators, especially o-phenanthroline which has a high affinity for Zn^{++}, which is thought to be the only ion naturally used by metallopeptidases. EDTA also chelates Zn^{++} and is therefore inhibitory to metallopeptidases.

Substrate analogues which include groups that coordinate the metal ion include the naturally occurring phosphoramidon (rhamnosyl-P-leu-trp) (Umezawa and Aoyagi, 1977), peptidyl hydroxamic acids and mercaptoacetyl-dipeptides.

α_2-Macroglobulin.

This very large plasma protein inhibits peptidases of all classes (Thomas et al., 1989) since it does not function through interacting with the peptidase active sites but sequesters the peptidase molecule. Peptidases cleave a "bait" region in α_2-macroglobulin

which causes a large conformational change in the inhibitor which effectively traps the peptidase (Kramer and Justus, 1988).

General functions of peptidases.

It seems likely that peptidases are essential components of all actively metabolizing cells. The range of functions in which they participate is very large. The variability shown by peptidases in their substate specificity, pH optima, cofactor requirements and control mechanisms facilitates their involvement in a wide variety of cellular processes. Historically, the function of peptidases in meeting nutritional requirements was the first to be discovered. Now a large and growing list of intra- and extra-cellular processes are known to involve the action of peptidases. Representative examples of these are briefly discussed here.

Nutritional functions.

Peptidases are required by most animals for nutritional purposes. Mammals, for example, secrete a variety of peptidases into the digestive tract. These include trypsin and pepsin. They also have peptidases anchored to the membranes of the brush border of the intestine. These include, for example, enteropeptidase,

which cleaves trypsinogen to yield trypsin, and aminopeptidases which liberate free amino acids from the oligopeptide products of protein digestion by endopeptidases.

Intracellular protein turnover.

The levels of most proteins in cells are maintained by a dynamic process of continuous degradation and synthesis although the half-lives of different proteins vary considerably. The variation in degradation rates between proteins and the pathways of degradation are not fully understood. Certain features of proteins have been identified which influence their rate of digestion. Proteins that lack a stable tertiary structure tend to be degraded rapidly be cells through a mechanism which may be initiated by binding of heat shock protein (hsp) 70-like molecules to the protein. Removal of the N-terminal methionine of newly synthesized proteins and acetylation of the N-terminal amino acid, whether it is methionine or the residue exposed after methionine removal, has been suggested to contribute to the way proteins are marked in order to direct them to the appropriate degradation pathway at the appropriate rate (Arfin and Bradshaw, 1988).

Binding of ubiquitin to proteins is thought to represent an important step in intracellular protein turnover and it is of interest that ubiquitin has

recently been shown to be a peptidase, although there is also evidence that it controls protein breakdown through delivery of proteins to other non-lysosomal peptidases.

Lysosomes are thought to play a role in intracellular protein degradation by a mechanism that is independent of the ubiquitin pathway.

Serum peptidases.

The majority of serum peptidases belong to the trypsin superfamily. However, unlike trypsin and other pancreatic peptidases such as chymotrypsin, elastase and they tend to have larger pre-sequences which are thought to have been acquired during their evolution from smaller enzymes (Neurath, 1984) and these sequences remain attached to the catalytic domain of the protein through disulphide or in some cases non-covalent bonds after proteolytic activation of the zymogen. The active sites, as in the case of the pancreatic serine peptidases, tend to be on the C-terminal side of the bond cleaved during zymogen activation. However, Factor B and C2 peptidases of the complement system have an unusual structure in which the reverse is true. The retention of the presequence by the active catalytic domain allows the peptidase to associate with various protein, ionic and phospholipid cofactors present in serum which modulate the specificity and activity of the enzyme. This enables the peptidases to participate in the intricate network of

specific cleavages and other interactions which underlie such processes as complement activation, blood clotting and fibrinolysis.

A number of conserved protein domains constitute the non-trypsin-like activation peptides of these serine peptidases. They include short consensus repeats, epidermal growth factor (EGF) repeats, thrombospondin repeats, immunoglobulin domains, fibronectin-like finger sequences and kringle sequences. Many of these domains are also found in non-peptidase proteins. Kringle sequences present on the activation peptide of prothrombin bind to cell membrane phospholipids and to cofactor proteins V and VIII. The binding of these components to prothrombin results in enhanced cleavage of prothrombin by factor Xa to yield thrombin (reviewed by Jackson and Nemerson, 1980). In many cases the mechanism by which cofactors activate plasma serine peptidases is not known. For example in the C3-C5 convertases of the classical complement system the catalytic site is in C2 but it is not clear whether association with C4 and C3 enhances its activity by facilitating binding of the C3 or C5 substrates or by inducing conformational changes in C2 which expose the catalytic site. Other mechanisms are also possible (reviewed by Reid and Porter, 1981).

Neuropeptide and prohormone processing.

Many bioactive peptides and proteins, including peptide hormones, growth factors and peptidases (zymogens), are synthesized in the cell as inactive precursors which must be proteolytically cleaved to release the bioactive component. The peptidases responsible for this have restricted substrate specificities and their activity and tissue and cellular location has to be controlled. In a number of cases the precursor protein and peptidase are produced together within the same secretory granule where hydrolysis of the proproteins occurs before the active products are released at their site of action (Gumbiner and Kelly, 1981).

These processing enzymes are often specific for paired basic residues, especially lys-arg, found in many preproteins, for example β-lipoprotein, proalbumin, prorenin, apolipoprotein A-II, protein C and many blood coagulation factors (references given in Cromlish et al., 1986). Single basic residues preceded by a proline residue are also often the targets of processing enzymes (Schwartz, 1986). The sequence surrounding the target may also be important in determining whether cleavage will occur at the target sequence, since pairs of basic amino acids are often present in other parts of the proprotein which are not cleaved by the processing enzyme. Alternatively, the selective cleavage may be due to the

accessibility of the cleaved sequence, for example at an exposed interdomain region of the proprotein.

In some cases the processing endopeptidase removes the basic amino acids that constitute the signal but where they do not a carboxypeptidase or aminopeptidase activity often removes them (Hook and Peng Loh, 1984; Fricker and Snyder, 1982).

Membrane transport of proteins.

It is now apparent that the majority of secreted and membrane bound proteins from prokaryotes and eukaryotes are directed through the endoplasmic reticulum by a hydrophobic signal leader sequence at the amino terminus of the protein. In the case of secreted proteins and those that are imported into various organelles, for example mitochondria, the leader sequence is then normally cleaved off by a signal peptidase. In most cases where the mechanistic class is known the signal peptidases are metallopeptidases (Mori et al., 1980; Mumford et al., 1980; Bohni et al., 1983).

Peptidases in growth and development.

In addition to important developmental processes which involve cellular invasiveness there is evidence that peptidases play a role in the embryonic development of *Drosophila melanogaster*. Two genes, which, based on their predicted amino acid sequence, code for secreted

serine peptidases have been cloned from *Drosophila melanogaster* (DeLotto and Spierer, 1986; Chasan and Anderson, 1989). Both these genes are required in order to set up a dorsal-ventral gradient of a nuclear protein, called dorsal, across the embryo. The level of this protein in cells of the embryo is thought to determine whether their subsequent development is appropriate to a dorsal, ventral or intermediate position. Interestingly, the serine peptidases seem to be required by the fly only at the time when the gradient of dorsal protein is forming, that is, just after fertilization. Thus, flies which lack the genes for either of the serine peptidases, but which receive an injection of cytoplasm from a wild type embryo of the same age, develop into normal adults.

It has not yet been shown whether or not it is the peptidase activity of these gene products that is responsible for their role in embryonic development. They could, for example, act by binding to a cell surface receptor without proteolysis occuring. However, their amino acid sequences are highly consistent with a function that involves extracellular proteolysis.

Pino-Heiss and Schubiger (1989) have demonstrated a correlation between the process of imaginal disc eversion (that is the extension of adult limbs from their concertina-like precursors in the pupa) with secretion of various serine peptidases. Activation of the zymogens for these prematurely with trypsin initiated precocious disc

eversion. Inhibition of the peptidases blocked the normal disc eversion.

A number of reports have shown a mitogenic effect of certain peptidases on tissue culture cells (Mazzei et al., 1966; Burger, 1970; Sefton and Rubin, 1971; Pohjanpelto, 1977; Chen and Buchanan, 1975). Some other mitogens have been shown to stimulate the release of peptidases from cells and peptidase inhibitors have been shown to inhibit the mitogenic action of tuberculin and other mitogens on lymphocytes (Weissman et al., 1972; Hatcher and Norin, 1982). Although the mechanisms involved and their relationship to physiological processes are unknown these results suggest a role for peptidases in the control of cell division.

Cyclins are highly conserved proteins which control the entry of cells into mitosis (Murray and Kirschner, 1989; Murray et al., 1989). Their levels increase before mitosis and they are rapidly destroyed at the end of mitosis. This destruction is thought to be due to a peptidase or peptidases. A cell-free system from clam embryos which retains the property of cyclin destruction at the end of mitosis is being used to investigate the properties of the putative peptidase and the features of the cyclins which make them good substrates for degradation (Ruderman, 1988).

Cell-cell signalling is important in developmental and other physiological events. Evidence

exists that a serine peptidase is involved in the initial step of transmembrane signalling during T-cell activation by other cells. Serine peptidase inhibitors prevented the phospholipase C-mediated cleavage of phosphatidylinositol in the T-cell plasma membrane when a cellular activation signal was present (Utsunomiya and Nakanishi, 1986).

Invasion of tissues by cells

Many normal and pathogenic processes involve the invasion of tissues by cells (Table 1). The types of evidence implicating peptidases in these fall into five classes.

1, Demonstration that a peptidase can digest the components of barriers to invasion. Basement membrane and extracellular matrix are secreted by some cell lines and these components can be purified and used as substrates to examine invasive cells for secreted or surface peptidases that can digest basement membrane or extracellular matrix material. Metabolic labelling of the cells that produce these components can make such assays very sensitive.

2, Correlation of the invasiveness of different cell and tissue types with peptidase levels. In the colonization of the bursa of Fabricius by haemopoietic cells, for example, Valinsky et al., (1981) found that plasminogen activator levels reach a peak level in the

Parasite invasion

Tumor invasion

Metastasis

Cell transformation

Angiogenesis

Rheumatoid arthritis

Infiltration by immune cells

Mammory gland involution

Uterine involution

Tooth eruption

Wound healing

Organogenesis in development

Ovulation

Trophoblast implantation

Cell migration in development

Fertilization

Table 1. Normal and pathogenic processes involving invasion of tissues (or cells) by cells.

bursa on the same day during development of the chick, or quail, that the migratory event occurs.

3, Correlation between the precise cellular or tissue location of a peptidase with the site of invasion. Within tumours peptidase levels have been found to be higher at the sites where invasion of normal tissue by the tumour is occurring.

4, Correlation of tissue resistance to invasion with the presence of peptidase inhibitors in those tissues. Cartilage is particularly resistant to tumour invasion which may in part be due to its mechanical properties. However, Kuettner et al., (1977) extracted samples of cartilage with 3M guanidine-HCl and found that this rendered them unable to resist invasion and degradation by tumour cells. Adding back fractions of the extracted material restored resistance to invasion to the cartilage and also prevented neovascularization, an invasive process, when added to rabbit cornea. The same fractions inhibited trypsin and collagenase activity.

5, Inhibition or stimulation of invasion through the application of exogenous antibodies, peptidase inhibitors or peptidase activators. The ability of mouse melanoma B16 cells to invade human amnion basement membrane was prevented by inhibitors of both plasmin and collagenase as well as antibodies against urokinase type plasminogen activator and collagenase. In the absence of plasminogen invasion was also blocked. These results

suggest that plasmin, generated by B16 cell urokinase type plasminogen activator, and active collagenase were both necessary for penetration of the basement membrane. The requirement for plasmin could be bypassed by the addition of mersalyl, an activator of latent collagenase. In the presence of mersalyl invasion could be inhibited only by inhibitors of collagenase. These experiments provide evidence for the involvement of a cascade in which urokinase type plasminogen activator produces plasmin from plasminogen which in turn activates latent collagenase. The collagenase then allows invasion by the tumour cells (Mignatti et al., 1986).

Barriers to invasion. The barriers that cells invade are skin, basement membranes, extracellular matrix and cell-cell junctions. Basement membranes are found in all organs where parenchymal cells interface with interstitial connective tissue, for example the basement membrane of blood vessels which lines the basal surface of the endothelial cells of the vessel. The basement membrane is composed of three layers: the lamina lucida directly below the cell membrane; the lamina densa which is composed of random collagen fibrils in the centre; and the reticular layer that anchors the fibrils to the underlying connective tissue. The major components of basement membranes are Type IV collagen, laminin and proteoglycans. Fibronectin and Type V collagen are found in some basement membranes but are also found in other

structures. Some cell lines retain the ability to secrete basement membrane in culture which has proved a useful source for *in vitro* studies of invasive processes.

Many epithelial cell sheets are composed of cells that touch at what have been called zona occluda which are structures located on the lateral membrane towards the apical pole of the cells. Cells may invade epithelia by digesting components of the zona occludens as well as the extracellular matrix found more basally.

In vitro assays for invasion. In order to analyse the biochemical events that are involved in invasion a number of *in vitro* systems have been developed. These include chick embryonic skin, chick embryonic heart, cultures of endothelial cells, isolated hepatocytes, bone or cartilage, rat urinary bladder, canine or bovine large vessels, chick chorioallantoic membrane, bovine lens capsule and human amnion (Mignatti et al., 1986). The extent of invasion is normally determined histologically. Quantification has proved difficult and in some cases there have been discrepancies between the invasiveness of cells in the *in vitro* systems and their invasiveness in the original host.

A quantitative assay for invasion using the human amnion has been developed by Liotta et al., (1980). This organ consists of a columnar epithelial cell sheet attached to a basement membrane which abuts a much thicker collagenous extracellular matrix containing

isolated fibroblasts. The amnion is about 0.5mm in thickness. The epithelial layer is removed by lysis of the cells and subsequent washing. Radiolabelled cells, whose invasive behaviour is to be investigated, are grown on, or added to the medium on, the exposed basement membrane. After various periods these cells are removed and the radioactivity remaining in the basement membrane and extracellular matrix is determined. The radioactivity is considered to be associated with cells that have penetrated the structure and are now buried within it and it therefore is a measure of the invasiveness of the applied cells. Such a system allows various exogenous agents to be tested for their effects on the invasiveness of cells.

Mignatti et al., (1986) provided evidence, using this assay system, that a peptidase cascade involving plaminogen activator, plasmin and collagenase, was involved in invasion of the amnion by a tumour cell line selected for high invasiveness. Inhibition of any of these three peptidases completely prevented invasion suggesting that they acted in a cascade rather than independently. Mersalyl, an activator of latent collagenase, could stimulate invasion even when plasmin formation had been prevented by depletion of plasminogen.

Functions of parasite peptidases.

Parasite peptidases have been considered here from a functional point of view. However it should be noted that knowledge of function is not necessary for a peptidase to be a good target for chemotherapy as in the case of lethal infections of group A *Streptococcus* in mice which can be cured by treatment with Z-leu-val-gly-CHN_2. This inhibitor, which is based on the active sequence of cystatin C, inhibits an intracellular cysteine peptidase produced by group A streptococcus but little is known of the function of the enzyme (Bjorck et al., 1989).

For some parasite peptidases a plethora of functions have been suggested and so the enzyme is included in more than one section. An elastase from *Pseudomonas aeuruginosa*, for example, has been implicated in tissue invasion, complement inactivation, immunoglobulin inactivation, lysozyme inactivation, proteinacious peptidase inhibitor inactivation and gamma interferon inactivation (Heck et al., 1986 b; Morihara et al., 1979).

Parasite peptidases and tissue invasion.

The life cycles of many parasites involve tissue invasion. In some cases the invasive process contributes to the pathology caused by the parasite. In *Pseudomonas*

aeruginosa infections , for example, invasion is associated with tissue damage which may lead to haemorrhage. In addition to facilitating invasion the tissue damage may result in an increase in nutrient availability for the bacteria. It is possible that bacterial invasion associated with tissue damage is not a good model for invasion by other parasites, for example *T.cruzi*, in which the process does not seem to directly cause tissue damage.

Bacterial invasion. A secreted elastase purified from *Pseudomonas aeruginosa* was shown to degrade types III and IV collagen and soluble laminin (Heck et al., 1986 a and b). The elastase also released fragments of laminin from rat kidney basement membranes (Heck et al., 1986 b). Inactivation of host proteinaceous inhibitors by the elastase (Morihara et al., 1979) may enable host peptidases to participate in the invasive process in addition to the bacterial enzyme. However, when mutant strains of *Pseudomonas aeruginosa*, lacking secreted peptidase activity, were compared with wild type strains in a rat model of infection, little difference in invasive capacity was seen (Doring et al., 1984). This casts doubt on the interpretations made from the *in vitro* studies using the purified bacterial enzyme at least for this model system.

It has been suggested that extracellular serine and cysteine peptidases of *Bacteroides gingivalis* are

important pathogenic factors in periodontal disease, or gum decay (Laughon et al., 1982; Uitto et al., 1989). In addition to causing direct tissue damage, which assists tissue invasion by the pathogen, they may activate host latent leukocyte collagenase leading to further tissue destruction (Fujimura and Nakamura, 1987; Uitto et al., 1989). Fluid from *B.gingivalis*-infected tissue was found to contain latent collagenase which could be activated by trypsin (Uito and Raeste, 1978).

In *Yersinia pestis*, the causative organism of plague, a plaminogen activator is encoded on a plasmid which has been shown to act as a virulence factor. The other genes on the plasmid are known and are thought unlikely to contribute to virulence (Sodeinde and Goguen, 1989). The plasminogen activator may activate host plasmin at foci of infection on the skin breaking down physical barriers to the bacteria and thereby facilitating their dissemination.

Streptococci produce streptokinase which, although not a peptidase itself, binds to plasminogen resulting in its autocatalytic activation to plasmin (Wulf and Mertz, 1968). This is thought to result in the lysis of clots and fibrin deposits thereby helping the infection to spread.

Peptidases from many other pathogenic bacteria have been isolated for which preliminary evidence, particularly substrate preferences, has been given for a

role in tissue invasion.

Schistosoma mansoni cercarial peptidase involved in skin penetration. The cercaria, the infective stage of schistosomes, gains entry to the portal system of man by invading intact skin. This involves penetration of the epidermal basement membrane, the dermal connective tissue extracellular matrix and an endothelial cell basement membrane. Secretions from the preacetabular glands of cercariae, including peptidases, have been thought to contribute to this process for many years (Lewart and Lee, 1954; reviewed in Stirewalt, 1974). A 28 - 30 kDa serine peptidase, purified from lipid-induced secretions of cercariae (McKerrow et al., 1985 b) and from cercarial extracts (Landsperger et al., 1982), has a number of characteristics which suggest it plays an important role in cercarial invasion of intact skin. DNA clones coding for the peptidase hybridized selectively to the cells of developing cercariae that are destined to become the acetabular glands (Newport et al., 1988; Dorsey, 1975). Northern blotting experiments showed that mRNA for the enzyme was only present during the cercarial stage. Further regulation of the peptidase activity is rendered likely by the presence of a zymogen sequence (Newport et al., 1988).

The enzyme digests keratin, laminin, type IV collagen, proteoglycans, fibronectin and elastin (Tzeng et al., 1983; McKerrow et al., 1985 a and b) which is

consistent with a role in skin penetration. The enzyme was able to digest these components not only when they were in solution but also when part of an extracellular matrix (McKerrow et al., 1985 b). The substrate analogue inhibitor, Suc-Phe-Gly-Ala-Leu-chloromethyl ketone, at 0.1mM apparently prevented cercariae from invading human skin without affecting cercarial motility (reviewed in McKerrow and Doenhoff, 1988). Sera from patients with *S.mansoni*, *S.japonicum* and *S.haematobium* all reacted with the cercarial peptidase and the enzyme therfore has potential for serodiagnosis and as the basis for a vaccine (Toy et al, 1987).

Invasion of cells.

Peptidases have been implicated in entry into host cells by *Plasmodium*, *Leishmania* and *T.cruzi* parasites. However, the best evidence of peptidase involvement in cell invasion is for sperm penetration of eggs at fertilization (Levine and Walsh, 1979).

Invasion of cells by *Plasmodium*. Peptidase inhibitors have been shown to block invasion of erythrocytes by merozoites of both *P.knowlesi* and *P.falciparum* (Banyal et al., 1981; Dluzewski et al., 1986; Hadley et al., 1986). In *P.berghei* a neutral endopeptidase was localized to the apical (invading) end of merozoites and both the peptidase and invasion of erythrocytes was blocked by leupeptin (Bernard and

Schrevel 1987).

Peptidases could act at any of a number of steps in the invasion process. These include specific host cell-parasite recognition; junction formation; induction of erythrocyte membrane invagination and removal of protein from the invaginating erythrocyte membrane.

Recently, a major blood stage antigen of *P. falciparum* which has a molecular weight of 111 kDa has been shown to have sequence homology to the active site region of the papain family of cysteine peptidases (Higgens et al., 1989). This antigen is processed to yield smaller fragments in the merozoite stage of the parasite. It is possible that the native peptidase or active fragments derived from it are involved in the invasion of erythrocytes by peptidases and it will be interesting to know if the blocking of invasion caused by peptidase inhibitors is mediated by inhibition of this enzyme. Alternatively, or in addition, the peptidase could function in haemoglobin degradation in the parasitophorous vacuole of the erythrocyte where the intact 111 kDa antigen is abundant.

Invasion of cells by *T. cruzi*. *T. cruzi* may invade different cell types using different mechanisms (Schenkman et al., 1988). There is evidence that macrophages are invaded by an endocytic mechanism (Nogueira, 1983) while for normally non-phagocytic cells internalization may be by penetrating the host cell

membrane and entering the cytoplasm directly or by endocytosis.

There is some circumstantial evidence that a peptidase may be involved in *T.cruzi* invasion of non-phagocytic mammalian tissue culture cells. Parasites freshly liberated from such cells were found to be less infective than those that were incubated for sixteen hours after liberation. During this time cell membrane components of the parasites showed changes in mobility on SDS-PAGE that suggested they had undergone proteolytic cleavage. Peptidase inhibitors blocked both the acquisition of enhanced cell invasiveness and the change in the surface components. Addition of exogenous peptidases accelerated both processes (Piras et al., 1983; Piras et al., 1985). However, there is little evidence implicating any of the known peptidases of *T.cruzi* in this processing step.

The major surface peptidase of *Leishmania* promastigotes: gp63. A 63 kDa surface component is found in Old and New World *Leishmania* species (Bouvier et al., 1987) and has been implicated in invasion of host macrophages for some time. Recently it has been shown that gp63 is a peptidase (Etges., 1986 a; Chaudhuri and Chang, 1988; Bouvier et al., 1987) held in the membrane by a glycophospholipid anchor (Etges et al., 1986 b). Gp63 purified from *L.mexicana* promastigotes degraded a broad range of mammalian serum proteins with a pH optimum

of 4 although in common with the *L.major* gp63 the purified *L.mexicana* gp63 degraded azocasein with a pH optimum of 7.5 (Chaudhuri and Chang, 1988). Inhibitor profiles, Zn^{++} labelling experiments, atomic emission and absorption experiments and sequence similarity to the active site of thermolysin together indicate that gp63 is a metallopeptidase (Bordier et al., 1989). The Old and New World *Leishmania* species have highly homologous gp63 sequences. However, Southern analysis using an *L.major* gp63 probe showed that *T.cruzi* and *T.brucei* did not contain homologous sequences (Miller et al., 1989). In addition to having a gp63 sequence that is highly similar to that of *L.major*, *L.donovani* also has a much less conserved gp63 homologue (Maingon et al., 1989).

At least three, non-exclusive, mechanisms had been proposed for the role of gp63 in macrophage invasion before it was known to be a peptidase. Firstly, binding of promastigote bound gp63 to the macrophage fucose / mannose receptor (Blackwell, 1985). Secondly, binding of gp63 to the macrophage fibronectin receptor or to fibronectin which is itself bound to macrophages (Ouaissi, 1988) and thirdly, interaction with components of the complement system so as to enhance phagocytosis of the parasites by macrophages (Russell and Wilhelm, 1986 and Mosser and Edelson, 1985 and Blackwell, 1985). The finding that gp63 from *L.major* and *L.donovani* has an Arg-Gly-Asp sequence (Webb et al., 1989 and McMaster et

al., 1989) would explain its affinity for the fibronectin receptor (Ouaissi, 1988) and for the complement receptor, CR3, of macrophages (Russell and Wright, 1989).

Chaudhuri and Chang (1988) provide evidence that gp63 cleaves C3 into smaller, discrete polypeptides at pH 7.5 and suggest that this process may be involved in the uptake of *Leishmania* parasites by macrophages. Thus, it may be that gp63 on the surface of *Leishmania* cleaves C3 to C3bi or some similar component and this allows *Leishmania* promastigotes to bind to macrophages via CR3. Certain proteolytic products of C3 reduce the production of microbicidal components of the complement system and this has been suggested as a further function of gp63 in infection.

Gp63 mRNA is produced by both promastigotes and amastigotes of *Leishmania* species (McMaster et al., 1989 and Medina-Acosta et al., 1989) and the strong immunological response to gp63 during *Leishmania* infections also suggests that gp63 is produced by amastigotes. However, gp63 is only detected on promastigotes and this has led to the hypothesis that amastigotes secrete gp63, perhaps by cleavage of the glycoinositolphospholipid tail by phospholipase C. The function of the secreted gp63 might be to degrade potentially microbicidal enzymes in the phagolysosome. Gp63 is associated with promastigotes in the gut of the insect host where it may serve a nutritive function

(Grimm et al., 1987) or inactivate host anti-parasite components in the insect. In conclusion gp63 has been found to have a number of well conserved biochemical features, including peptidase activity, which may participate in a range of functions none of which are clearly understood yet.

Peptidases involved in evasion of host immunity.

A number of parasite peptidases have been shown to degrade various components of the immune system including immunoglobulins, complement components and interferon and might therefore be involved in the evasion of host immunity. However, when the substrate specificity of the peptidase is broad, as in the case of the secreted elastase of *Pseudomonas aeruginosa*, such evidence is at best only circumstantial. In a few cases other evidence supporting a role in evasion of immunity for parasite peptidases is also available.

The streptococcal C5a peptidase. *Streptococcus pyogenes* is a common cause of bacterial throat and skin infections. Virulent *S. pyogenes* can inactivate the complement-derived chemotactic activity of human sera *in vitro* (Wexler et al., 1983). A surface bound peptidase of *S. pyogenes* specifically cleaves a lys-asp bond near the carboxy terminus of the human chemotaxin, C5a, markedly reducing the chemotactic activity of C5a (Wexler et al., 1985; O'Connor and Cleary, 1986)). The peptidase had

virtually no activity on a number of native proteins, for example bovine serum albumin, although after urea-induced denaturation some of these were digested slowly (Wexler et al., 1985). Little information on the biochemical properties of the C5a peptidase was reported by these authors except that it is a large surface exposed antigen.

Mutants of *S. pyogenes* which lacked the peptidase activity resulted in infections in mice which showed higher levels of polymorphonuclear lymphocyte invasion than infections with the wild type bacteria. However, in this particular murine model of infection the virulence (LD_{50}) of the mutant and wild type bacteria was the same (O'Connor and Cleary, 1987).

IgA1 peptidases of pathogenic bacteria. Various pathogenic species of bacteria including causative agents of gonorrhoea, meningitis and dental caries have been found to secrete peptidases that are highly selective for the hinge region of the heavy chain of IgA1 (Kilian et al., 1983; Plaut, 1983). Many non-pathogenic strains did not secrete an IgA1 peptidase suggesting that the peptidase is involved in pathogenesis.

A simplistic model for the function of the IgA1 peptidases is that they help the parasite evade host immunity by destroying IgA1. This could reduce the affinity of the IgA1 for antigen or may act solely by preventing the constant region-mediated effector function

of the antibody. An immune evasion function of the peptidases is supported by the role played by IgA in immunity at epithelial surfaces where it is the most prevalent immunoglobulin. However, the IgA1 isotype is less prevalent than IgA2 which lacks the hinge region and is not cleaved by IgA1 peptidases. This has raised doubt about the extent to which the peptidases could contribute to immune evasion.

IgA2 may have evolved from IgA1 as a means of avoiding destruction by these pathogens. More recently an IgA2 peptidase has been detected in species of pathogenic bacteria, although, little is known about the order in which either the IgA1 and IgA2 peptidases evolved or the order in which IgA1 and IgA2 evolved. Comparative sequence data for these proteins from many bacterial and host species might shed light on the evolutionary relationships between them and thereby lend support for or aginst a function of immune evasion for the peptidases. The IgA1 peptidases from two species of canine pathogenic *Ureaplasma* cleaved canine IgA1 but not murine or human IgA1. Conversely, the IgA1 peptidase from a human *Ureaplama* cleaved human IgA1 but not canine or murine IgA1 (Plaut, 1983).

Cleavage of bound immunoglobulins by *Schistosoma mansoni.* The schistosomula and young adult worm coat has receptors for the Fc part of immunoglobulins (Torpier et al., 1979). On binding, the immunoglobulin can still

interact with its specific antigen but this ability is progressively lost in the absence of serine peptidase inhibitors even though the Fc part of the bound immunoglobulin is not shed from the surface of the worm. This evidence and the release of immunoglobulin fragments from schistosomula led Auriault et al., (1981) to postulate that the surface bound immunoglobulins are cleaved by secreted *Schistosoma* peptidases.

These authors isolated a metalloaminopeptidase and a serine endopeptidase from secretions of schistosomula which they suggest may be responsible for the cleavage of the bound immunoglobulin. The endopeptidase was active against azocasein and had a pH optimum of 7 against this substrate. It was insufficiently characterized for any conclusion to be made regarding its relationship to the peptidase postulated to be involved in shedding of the shistosomula glycocalyx (Marikovsky et al., 1988 a and b).

Peptidases involved in removal of the *Schistosoma mansoni* glycocalyx. On penetrating the skin of its mammalian host *Schistosoma mansoni* transforms from a cercarial form, which is sensitive to the alternative complement pathway (ACP), to a young schistosomula which is resistant to the ACP. The loss of the cercarial outer coat, or glycocalyx, is thought to be responsible for this change since the glycocalyx is an efficient activator of complement. Marikovsky et al., (1988 a and

b) provide evidence that the glycocalyx is shed through the action of peptidases that are secreted by the parasites during transformation.

A 28 kDa serine peptidase and a 60 kDa peptidase have been purified from medium containing freshly mechanically transformed schistosomula. These secreted peptidases appear to be unrelated to each other or to the cercarial 30 kDa preacetabular gland peptidase thought to be involved in skin penetration (Mc Kerrow et al., 1985 b).

The shedding of the glycocalyx from freshly transformed schistosomula and the conversion of these schistosomula to ACP resistance occurs with the same kinetics as the release of the 28 kDa and 60 kDa peptidases. The purified 28 kDa peptidase cleaved radiolabelled surface molecules from schistosomula which had similar patterns of mobility on SDS-PAGE to the fragments shed from transforming schistosomula. Treatment of the schistosomula with the purified 28 kDa peptidase also rendered them resistant to, and unable to activate, the ACP (Marikovsky et al. 1988 b).

Peptidases invoved in parasite nutrition.

The best studied example of a parasite peptidase involved in nutrition is the *Schistosoma mansoni* haemoglobinase, first reported by Timms and Bueding (1959). A haemolytic agent is found in the oesophagus of *S. mansoni* adults (Kasschar and Dresden, 1986) which makes

haemoglobin available in the digestive tract of the parasite. Chappell and Dresden (1986) reported a number of proteolytic activities to be present in material regurgitated from the digestive tract of *S. mansoni* including a highly immunogenic 32 kDa peptidase which digested haemoglobin with a pH optimum of 5.

Lindquist et al., (1986) purified this component and found that it is an endopeptidase that requires a basic residue at the P_1 position of substrates and that it is inhibited by cysteine peptidase inhibitors. A 31 kDa peptidase which is probably the same component as that found in the regurgitant has been cloned and sequenced by Davis et al., (1987) and Klinkert et al., (1989). The sequence suggests that the enzyme is synthesized as a precursor of 41 kDa which is of interest since Chappell and Dresden (1986) found some activity with this molecular weight but otherwise with similar properties to the major species of 32 kDa.

For other parasites there is much less data on the role of peptidases in nutrition. However, it is likely that some of the bacterial peptidases involved in tissue degradation, which enhance invasion by the parasites, may provide amino acids for nutrition of the parasites. *T. cruzi* epimastigotes can grow with protein as the sole source of nitrogen (Avila et al., 1983) which suggests at least a potential involvement of peptidases in nutrition under natural conditions. Either the

exogenous proteins could be digested by extracellular peptidases or the proteins could be endocytosed or ingested by other means and degraded intracellularly.

Peptidases of African trypanosomes.

Extracts of various African trypanosome species hydrolysed protein substrates optimally at acidic pH. Haemoglobin and hide powder azure digestion was inhibited by E64, iodoacetamide, TLCK and leupeptin and was activated by DTT suggesting that a cysteine peptidase was responsible for digestion of both substrates (North et al., 1983). Hydrolysis of Bz-pro-phe-arg-pNan had similar pH and inhibitor profiles. However, azocasein digestion was not activated by DTT although its inhibitor profile suggested that a cysteine peptidase (or peptidases) was also responsible for its digestion (North et al., 1983).

Lonsdale-Eccles and Mpimbaza (1986) found similar peptidase activities to those described by North et al. (1983) and identified a 28 - 31 kDa cysteine peptidase activity using protein substrate-containing SDS-PAGE. The exact size of the peptidase varied with the species of African trypanosome. An acidic peptidase active on Bz-phe-arg-Nmec was found which was inhibited by Cbz-phe-ala-CHN_2 and Cbz-phe-phe-CHN_2 and which is similar to mammalian cathepsin L , and to the partially

sequenced peptidase from *T. cruzi* which has homology to cathepsin L (Cazzulo et al., 1989).

Pamer et al. (1989) also identified a 28 kDa acidic cysteine peptidase from *T. brucei* with similar properties to the cathepsin L-like enzyme described by Lonsdale-Eccles and Mpimbaza (1986). The activity was more abundant in the short stumpy forms than the long slender forms and procyclic trypomastigotes suggesting that it may therefore play a role in the transition between the mammalian and insect forms of the parasite.

A number of authors have reported hydrolytic activity in extracts of *Trypanosoma* species against Bz-arg-pNan at neutral to alkaline pH values. In *T. brucei* and in *T. cruzi* the enzyme is inhibited by TLCK, leupeptin and some heavy metal ions but not by iodoacetamide or PMSF. DFP and E64 at high concentrations inhibited the activity making it difficult to determine if a serine peptidase or cysteine peptidase was involved. However, diazomethylketones inhibit the activity in *T. cruzi* and *T. brucei* which supports the view that a cysteine peptidase is responsible for digestion of this substrate (Lonsdale and Grab, 1987; Ashall, 1989; Ashall et al., unpublished data).

In addition to gp63 (discussed on page 75) a number of peptidase activities have been detected and characterized in *Leishmania*. North and Coombs (1981) found that haemoglobin-containing polyacrylamide gels revealed eleven bands of activity after SDS-PAGE of *L.mexicana* amastigote extracts. Seven of these were also detected in extracts of promastigotes but the intensity of the bands was much weaker. These peptidase activities had acidic pH optima and from their sensitivity to inhibitors were probably cysteine peptidases. Consistent with this was the finding by Coombs (1982) that extracts of *L.mexicana* amastigotes digest azocasein much more rapidly than extracts of promastigotes.

Pupkis and Coombs (1984) separated two peptidase activities from *L.mexicana* using gel filtration and ion exchange chromatography. One of these almost certainly corresponds to gp63 while the other, a cysteine peptidase of 31 kDa with an acidic pH optimum, may be related to mammalian cathepsin L since it rapidly digested Cbz-phe-arg-Nmec. The enzyme was not found in promastigotes and therefore is probably responsible for some of the differences in peptidase activities between amastigotes and promastigotes detected by North and Coombs (1981) and Coombs (1982). A partially sequenced *T.cruzi* peptidase was shown to resemble mammalian

cathepsin L (Cazzulo et al., 1989) and it is therefore likely that a similar enzyme does exist in *Leishmania*.

The reason for the greater acid peptidase activity in amastigotes than promastigotes may reflect a need for the amastigotes to destroy host lysosomal enzymes present in the macrophage.

Peptidases of *Trypanosoma cruzi*.

In comparing the results of authors it is important to consider the different strains that may have been used, the conditions of the assays used and the methods for extract preparation. Unless a range of inhibitors is tested or a purification undertaken it is difficult to differentiate the activities of different enzymes. Some peptidases have been shown to have very different pH optima for different substrates for example gp63, a peptidase from *Leishmania* species, digested azocasein with a pH optimum of 7.5 but for other protein substrates it had a pH optimum of 4 (Chaudhuri and Chang, 1988). Thus pH optima are poor criteria on which to differentiate activities against different peptidase substrates. It is likely that a number of agents, for example denaturing compounds and temperature may in some cases exert their effects through the substrate rather than by a direct effect on the enzyme and therefore these

agents can be unreliable guides to enzyme characterization. It can not be assumed that enzymes in *T. cruzi* will have the same substrate specificities as those characterized from other organisms although a comparative approach can act as a useful guide to further experiments.

Steiger et al. (1979) performed assays for 18 acid hydrolases with extracts from four species of trypanosomatid: *Crithidia*, *L. donovani*, *T. cruzi* and *T. brucei*. For the latter two species both blood and culture (trypomastigotes and epimastigotes respectively in the case of *T. cruzi*) forms were used. [^{3}H]-acetyl haemoglobin was used as substrate at pH 3.6. The specific activity of acid peptidases of *T. cruzi* blood forms was found to be 7 fold higher than in epimastigotes. However, different strains of *T. cruzi* were used for the two forms and it is known that different strains and even different cultures from the same strain can show large differences in proteolytic activity (Torruela et al., 1981). *T. brucei* had high acid peptidase levels in both forms. *Crithidia* and *L. donovani* had considerably lower acid peptidase levels than the blood forms of the two trypanosomes. The authors argue that the high specific activities of the enzyme, which for *T. brucei* they present evidence of lysosomal location, in the blood forms of these parasites might reflect an adaption to the mammalian host related to the utilization of blood proteins.

Itow and Camargow (1977) obtained evidence for four different peptidases in *T.cruzi*: 1. An azocaseinase activity, which had a low pH optimum and was inhibited by TLCK but not PMSF. The activity had a temperature optimum of 60°C. Cazzulo et al. (1989) have purified and characterized this enzyme (see below) but no small synthetic peptide substrates digested by the enzyme have been reported. 2. An aminopeptidase activity was detected, in the soluble fractions of the cell extract, that cleaved leu-naphthylamide and lys-naphthylamide with a pH optimum of between 7 and 8. No evidence is presented that both substrates are being digested by the same enzyme. 3. Bz-arg-pNan detected an activity with an optimum pH of 8. The enzyme was inhibited strongly by PMSF, Zn^{++}, Pb^{++} and TLCK. It is probable that this enzyme is the same as the enzyme isolated by Ashall (1989). However, Ashall (1989) found that PMSF did not inhibit the enzyme. Both groups used the Y strain of *T.cruzi*. 4. Activity against Cbz-tyr-p-nitrophenil ester had a pH optimum of 7 and was enriched in the sediment of the crude homogenate which suggested it may be membrane associated. The activity was sensitive to PMSF, TLCK and TPCK suggesting it is due to a serine esterase with a similar substrate specificity to chymotrypsin. This enzyme was unstable when stored at -20°C.

Avila et al. (1979) report on five peptidase activities in a survey of acid hydrolases in *T.cruzi* Y

strain. The substrates used were gly-phe-naphthylamide, Bz-arg-naphthylamide, ala-naphthylamide, Bz-glu-tyr and acid denatured haemoglobin. Cysteine, Mg^{++} and Mn^{++} had differential effects on the rate of digestion of the first three substrates suggesting that different enzymes might be responsible for their digestion. The digestion of Bz-glu-tyr and haemoglobin was attributed to cathepsin A and D respectively. However no inhibitors were tested with these substrates and it is therefore difficult to determine the type of enzymes(s) which digest them or indeed if they are different from any of the other activities that were detected. The pH profile for digestion of Bz-arg-napthylamide was somewhat dissimilar to that obtained for Bz-arg-pNan by Ashall (1989) and Itow and Camargo (1977) although the similarity between the substrates would suggest that they were both digested by the same enzyme.

Rangel et al. (1981) have purified, to electrophoretic homogeneity, a peptidase from epimastigotes of *T.cruzi* Y strain that digests casein and haemoglobin with a pH optimum of 7 and, for haemoglobin, a second peak of activity at pH 3. The effects of various peptidase inhibitors and sulfhydryl agents indicated that the enzyme was a cysteine peptidase. A number of synthetic substrates were tested but none were hydrolysed by the purified enzyme. Polyclonal antisera raised against the purified enzyme

stained all life cycle stages of *T.cruzi* and electron microscopy of stained parasites showed the antibody binding to the surface of the parasites. However, the specificity of the sera for the peptidase was not demonstrated. The peptidase has been shown to hydrolyse human IgG, IgA and IgM at both pH 3.1 and pH 7.0. This, and the surface location, was used to argue that the enzyme may be involved in escape from the immune response.

Bongertz and Hungerer (1978) purified a peptidase from *T.cruzi* which digested Bz-arg-pNan and had an apparent molecular weight of 200 kDa. A peptidase with similar properties was purified and further characterized by Ashall (1989) and it is likely that this represents the same enzyme. The enzyme activity is present in epimastigote, trypomastigote and amastigote forms of the parasite and in sixteen different species of trypanosomatids from four genera: *Trypanosoma*, *Leishmania*, *Crithidia* and *Endotrypanum*. The enzyme was not found in a number of non-trypanosomatid protozoans or mammalian tissues. A series of substrates tested for digestion by the enzyme shows that only peptide bonds on the carboxyl side of arginine and (to a lesser extent) lysine residues are cleaved. All the substrates longer than Bz-arg-pNan that were tested were hydrolyzed more slowly than Bz-arg-pNan by the enzyme. This indicates that either the enzyme has a very specific sequence or

conformational requirement for its substrates or that it cleaves short peptides preferentially to longer ones. The enzyme has very little activity on the unblocked substrate, arg-pNan, indicating that it is not an aminopeptidase.

The peptidase was inhibited weakly by E64 but not by iodoacetic acid. Inhibition by E64 suggests that the enzyme is a cysteine peptidase. A series of peptidyldiazomethanes, peptidylfluoromethanes and peptidylsulphonium salts were also tested and those with arginine or lysine next to the reactive group gave significant inhibition confirming that the enzyme is a cysteine peptidase.

The azocaseinase activity first reported by Itow and Camargo (1977) has been purified by Cazzulo et al. (1989). The enzyme was shown to be a cysteine peptidase using a number of inhibitors. This result was confirmed by sequencing of the N-terminal 32 amino acids which showed homology to peptidases of the papain superfamily. The sequence was closest to that of cathepsin L, a lysosomal cysteine peptidase from mammals, and the enzyme was localized to the lysosomes of the parasite (Bontempi et al., 1989).

Aims of the project.

The aim of this project was to identify peptidase activities in *T.cruzi* using a wide range of detection methods, and to assign these activities to particular enzymes through the use of class- and substrate-specific peptidase inhibitors. In addition, an analysis of the major alkaline peptidase of *Crithidia* and *Leishmania* was made which showed that this enzyme was homologous, with respect to many criteria, with that from *T.cruzi* (Ashall, 1989).

A survey of the peptidolytic activities of a parasite should aid in understanding the general biology of the organism. In particular, peptidases may be involved in host-parasite interactions. Detailed investigations of individual peptidases would be facilitated as, for example, in determining what proteolytic events are or are not mediated by that peptidase. Knowledge of these peptidase activities may be of value in developing extraction procedures, from the parasite, which do not result in degradation of the components that are to be analysed. Finally, the development of selective chemotherapy for the disease may be benefited by knowledge of the peptidases present in the parasite since these are potential targets for the design of drugs.

Chapter Two

Materials and Methods

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

Two types of gel system were used: The LKB midget system (approximate dimensions of separating gels: 5 cm long and 0.75 mm thick) and the LKB standard system (approximate dimensions of separating gels: 12.5 cm long and 1.5 mm thick).

Electrophoresis was performed according to the method of Laemmli (1970). A 10% acrylamide gel [10% (w/v) acrylamide / 0.27% (w/v) bisacrylamide / 0.1% (w/v) SDS / 0.375M Tris-HCl, pH 8.8] was polymerized with TEMED, 0.01% (v/v) final concentration and ammonium persulfate, 0.05% (w/v). A stacking gel, consisting of 5.25% (w/v) acrylamide / 0.14% (w/v) bisacrylamide / 0.1% (w/v) SDS / 0.125M Tris-HCl pH 6.8, polymerized with 0.01% (v/v) TEMED and 0.075% (w/v) ammonium persulfate was generally used. Electrophoresis running buffer [0.1% (w/v)SDS / 0.4M Tris-HCl / 1.4% (w/v) glycine] was added to the lower and upper reservoirs.

If enzymic activity was to be assayed after separation, gels were electrophoresed at a constant low

voltage (50V) so as to avoid heat inactivation of enzymes. Otherwise a constant voltage of 150V was used.

Detection of peptidases in protein-containing polyacrylamide gels

Protein samples were separated by SDS-PAGE as described except that proteinaceous substrates [0.2% (w/v) gelatin unless stated otherwise] were incorporated into the gel matrix by dissolving the protein in the separating gel mixture before polymerization. Electrophoresis was then carried out at 50V and the gels removed and swirled in 2% (v/v) Triton X-100 for 20 minutes (LKB midget gels) or one hour (LKB large gels). The gels were incubated in an appropriate buffer overnight at 37°C. The gels were then swirled with Coomasie Brilliant Blue for 2 hours (LKB midget gels) or 12 hours (LKB large gels), destained and stored in 10% (v/v) acetic acid. Areas of peptidase digestion were revealed by a reduction in the degree of Commassie Blue staining (Lacks and Springhorn, 1980 and Lockwood et al., 1987).

Detection of peptidases in gels using chromogenic and fluorogenic assays.

1. Overlay method. Samples were separated by SDS-PAGE using the LKB midget system at 50V and gels were swirled in 2% (v/v) Triton X-100 for 20 minutes. The polyacrylamide gel was then placed on top of a 1% (w/v) agarose gel made in multi-range pH buffer for peptidase digestion. Before allowing the agarose gel to set, the p-nitroanilide substrate was added to a final concentration of 0.5 mM. Digestion of the substrate was revealed by the production of the yellow compound p-nitroaniline. This was photographed using a blue filter.

2. Gel strip method. SDS-PAGE of parasite extracts and subsequent swirling of the gel in 2% Triton X-100 was done as described above for the overlay method. Gel lanes were then cut transversely into strips with a razor blade and placed in cuvettes containing multi-range pH buffer at the indicated pH. These were then treated as for the appropriate chromogenic or fluorogenic assays.

Preparation of NP-40 extracts.

Cells were harvested by centrifugation at 2500 x g at room temperature for 10 minutes and the parasite

pellet resuspended at approximately 5 X 10^8 cells per ml in 1% NP-40 (v/v) / 75mM NaCl / 25mM Tris-HCl, pH 7.4. The suspension was kept on ice for 5 minutes and then microfuged at 12000 x g at room temperature for 2 minutes and the supernatant transferred to an Eppendorf tube. This extract was used in solution assays for peptidase activity immediately, or after storage at -20°C. For electrophoretic detection of peptidases an equal volume of 0.4M glycine / 0.1M Tris, pH 8.3 / 2% (w/v) SDS / 0.1% (w/v) bromophenol blue was added to the extract and the samples loaded onto gels immediately, or after storage at -20°C. Samples were not heated prior to electrophoresis.

Phase separation of membrane proteins using Triton X-114.

Cells were harvested as for NP-40 extracts. Parasite pellets were resuspended in 1% Triton X-114 / 25mM Tris-HCl, pH 7.4 / 150mM NaCl at a density of about 5 X 10^8 parasites per ml. The suspension was incubated on ice for 5 minutes and then microfuged at 12000 x g for 2 minutes at 4°C. The resultant supernatant was layered onto 0.4ml of a sucrose cushion [6% (w/v) sucrose / 150mM NaCl / 25mM Tris-HCl, pH 7.4 / 0.06% Triton X-114] and incubated at 34°C for 3 minutes to allow phase separation (Bordier, 1981). The sample was then centrifuged at 500 x g

for 5 minutes at 25°C. The detergent phase was transferred to a fresh tube. Triton X-114 was added to the aqueous phase to a final concentration of 1% (v/v) and the phase separation repeated. The two detergent phases were combined and the volume adjusted with 25mM Tris-HCl, pH 7.4 / 150 mM NaCl to equal that of the aqueous phase. The aqueous and detergent phases of the extract were either used immediately or after storage at -20°C for solution assays. For electrophoresis the phases were mixed with an equal volume of 0.4M glycine / 0.1M Tris, pH 8.3 / 2% (w/v) SDS / 0.1% bromophenol blue and electrophoresed without heat treatment.

Determination of protein concentration.

Protein concentration was determined using the BioRad protein microassay according to the manufacturer's instructions. This technique exploits the increase in absorbance of Coomassie Brilliant Blue at 595 nm when it binds to proteins (Bradford, 1976).

Bovine serum albumin (Sigma) was used to construct a standard curve. 200ul of BioRad dye solution were added to protein samples diluted to 800ul with 10mM Tris-HCl, pH7.6. The tubes were vortexed, incubated for 10 minutes to 1 hour at room temperature, vortexed vigorously, and the absorbance of the solutions read

immediately at 595nm. A standard curve was obtained using a series of known concentrations of bovine serum albumin, and concentrations of test proteins determined from the linear part of the standard curve.

Labelling of proteins with [^{3}H]-diisopropylfluorophosphate ([^{3}H]-DFP).

Samples to be labelled were treated with 50uCi of [^{3}H]-DFP (2Ci/m mole) (Amersham) in a total volume of 20-100µl of extract and then incubated for one hour at room temperature followed by four hours at 4°C. After incubation an equal volume of 0.4M glycine / 0.1M Tris-HCl, pH 8.3 / 2% (w/v) SDS / 0.1% (w/v) bromophenol blue was added and the samples heated to about 100°C for 3 minutes. Samples were then electrophoresed immediately or stored at -20°C.

Surface radioiodination of parasites

The glucose oxidase / lactoperoxidase method of iodination was used. These enzymes are immobilized on hydrophilic beads. The glucose oxidase generates hydrogen peroxide from glucose in the reaction mixture. The lactoperoxidase catalyses the oxidation of radiolabelled

iodide to iodine by the hydrogen peroxide. The radiolabelled iodine reacts with the tyrosine residues of proteins.

Lyophilized solid phase Enzymobead reagent (obtained from BioRad) was rehydrated according to the manufacturers instructions. Into 50 ul of the Enzymobead suspension was added: 10^7 cells in a volume of 70 ul of phosphate buffered saline (PBS) at pH 7, 1.0mCi $Na^{125}I$ in a volume of 10 ul and 25 ul of 2% (w/v) α/β-D-glucose (the glucose was mutarotated by making up the solution 16 hours before use).

The reagents were mixed and incubated at 25°C for 5 or 15 minutes. The cells and Enzymobeads were pelleted by centrifugation at 2500 x g for 3 minutes and the supernatant discarded. The pellet was washed twice in PBS and then extracted in 100ul of 1% (v/v)) NP-40 / 25mM Tris-HCl / 75mM NaCl by the method given in the section on preparation of extracts.

About half of the radioiodinated material was loaded onto one gel track for electrophoresis. After electrophoresis, the gel was dried down and exposed to XAR-5 film (Kodak) for 5 days at -70°C.

Staining gels for protein

1. Coomassie Blue. Gels were shaken in 0.1% (w/v) Coomassie Brilliant Blue / 45% (v/v) methanol / 10% (v/v) acetic acid, then destained with 45% (v/v) methanol / 10% (v/v) acetic acid for two hours, and transferred to 10% (v/v) acetic acid for storage. Gelatin-containing gels used for peptidase detection were destained by swirling with two changes of 10% (v/v) acetic acid.

2. Silver stain. Gels were swirled successively with the following solutions: 50% (v/v) methanol / 10% (v/v) acetic acid for two hours; 5% (v/v) methanol / 7% (v/v) acetic acid for two hours; 10% (v/v) glutaraldehyde for 30 minutes; four changes of 200ml double distilled H_2O over a period of two hours; 0.42% (v/v) ammonium hydroxide / 0.07% (w/v) NaOH / 0.8% (w/v) silver nitrate for 20 minutes; 5uM citric acid / 0.1% (v/v) formaldehyde until protein bands were clearly visible. The gel was immersed in 2.5% (v/v) acetic acid to stop the reaction. The gels could be stored in the last of these solutions for some days.

Parasites.

Table 2 shows the stocks of *T.cruzi* used with references where available. Epimastigotes were grown at 25°C in monophasic culture using RPMI 1640 medium

Strain	Origin
Y	Brazil / Man
Sylvio X-10	Brazil / Man
Esmeraldo	Brazil / Man
Vinc C68	Columbia / Man
Dog	USA / Dog
Armadillo	USA / Armadillo
California	USA / Man
Opposum	USA / Opposum
Can III	Brazil / man

Table 2. Strains of *T. cruzi* epimastigotes used and their origins. The Y strain isolated by Silva and Nussenzweig (1953) was used most extensively in this study.

supplemented with 10% (v/v) foetal calf serum (heat-treated at 50°C for 20 minutes) / 0.2mM glutamine / 100 units ml^{-1} penicillin / 100 ug ml^{-1} streptomycin / 20mM HEPES / 25 ug ml^{-1} hemin / 25 mg ml^{-1} trypticase. When the parasite exceeded 10^7 per ml they were diluted by adding two volumes of fresh medium.

T.rangeli epimastigotes were grown in Minimal Essential Medium supplemented with 20% (v/v) foetal calf serum without antibiotics.

Leishmania donovani promastigotes were grown in Schneider's medium supplemented with 10% (v/v) foetal calf serum (heat-treated at 50°C for 20 minutes) / 0.2 mM glutamine / 100 units ml^{-1} penicillin / 100 ug ml^{-1} streptomycin / 20 mM HEPES / 1 ug ml^{-1} hemin / 25 mg ml^{-1} trypticase. All other *Leishmania* parasites were provided by Dr David Evans, The London School of Hygiene and Tropical Medicine, London.

Crithidia fasciculata (strain HS-6) choanomastigotes were grown in the same medium as *T.cruzi* epimastigotes except that 1 ug ml^{-1} hemin was used.

African trypanosomes were provided by Dr Ken Hudson, Brunel University, London.

T.cruzi (Y strain) trypomastigotes were cultured using essentially the same method as Jaynes et al. (1988). Tissue culture flasks were sparsely seeded with Vero cells in RPMI supplemented with 10% (v/v) foetal calf serum and incubated for 24 hours at 37°C. *T.cruzi* trypomastigotes

were added at an approximate ratio of two parasites per Vero cell and incubated for 24 hour at 37°C. The medium was changed then and every 48 hours until the majority of infected cells released trypomastigotes which were collected and used immediately. The time taken from infection to parasite release was about one week with most Vero cells lysing on the same day. Except where stated otherwise parasites were obtained from the London School of Hygiene and Tropical Medicine.

Cryopreservation of Vero cells and parasites

Cells were resuspended at $2X10^6$ ml^{-1} for Vero cells and 10^8 ml^{-1} for parasites in the appropriate culture medium and an equal volume of freezing mixture [20% (v/v) DMSO / 40% (v/v) appropriate medium / 40% (v/v) foetal calf serum] added. One milliliter aliquots of cells were transferred to screw top ampoules which were placed in the freezing plugs of a liquid nitrogen tank. These were left overnight and then placed inside the tank.

Cells taken from liquid nitrogen were thawed in the ampoules at 37°C and added to 10ml of the appropriate medium. Medium was changed after 16 to 24 hours.

Chromogenic Assays

1. p-nitroanilide derivatives (Table 3). All p-nitroanilide derivatives were dissolved in DMSO to give a 20mM stock solution, which was stored at -20°C. Parasite detergent extracts (equivalent to about 10^7 parasites) or an equivalent volume of detergent extraction solution alone were added to 1ml of multi-range pH buffer (50mM NaH_2PO_4 / 50mM trisodium citrate / 50mM boric acid / 50mM Hepes) at the stated pH. Samples were preincubated for 15 minutes at 37°C. The reaction was started by adding 5ul of 20mM substrate to the 1ml reaction mixture and then mixing. After an incubation at 37°C the samples were microfuged for 2 minutes and the absorbance at 405nm (due to p-nitroanilide release) of the supernatants measured (Erlanger et al., 1961). For each substrate the length of the incubation was chosen to be within the time when the reaction rate was constant. For very slow reactions the shortest time which enabled an unambiguous change in absorption to be determined were used.

2. N-methylcoumarin derivatives (Table 4). These substrates were dissolved in DMSO to give a 10 mM stock solution, which was stored at -20°C. Fifty microlitres of parasite detergent extract (equivalent to about 5 X 10^5 parasites) or an equivalent volume of detergent extraction solution alone were added to 1.93 ml of

Bz-arg-
arg-
lys-
leu-
ala-ala-
Bz-tyr-
Cbz-phe-leu-glu-
Suc-ala-ala-pro-phe-
gly-
gly-arg
Ac-phe-
Boc-ala-ala-
ala-ala-ala-
ala-pro-
val-ala-
ala-ala-phe-ala-
ala-ala-val-ala-
Tos-gly-pro-arg-
Tos-gly-pro-lys-
Bz-pro-phe-arg-
Boc-leu-gly-arg-
Boc-bz-ser-gly-arg-
Boc-val-leu-gly-arg-
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Bz-val-gly-glu-

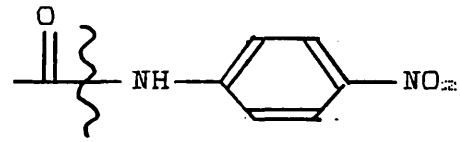


Table 3. p-Nitroanilide derivatives used and the structure of the chromogenic group. The bond that is cleaved to release the chromphore is marked with a wavey line.

Bz-arg-
Z-arg-arg-
Z-phe-arg-
leu-lys-arg-
leu-gly-arg-

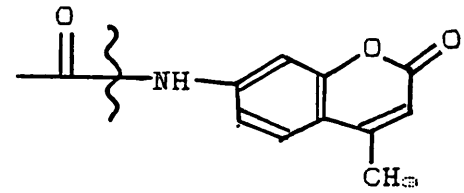
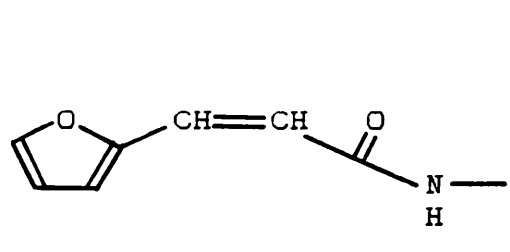


Table 4. 7-amido methylcoumarin derivatives and the structure of the fluorogenic group. The bond that is cleaved to release the fluoroescent group is indicated with a wavey line.



-leu-gly-pro-ala
-gly-leu
-phe-ala-gly-gly
-gly-leu-ala

Table 5. Furylacryloyl derivatives used as substrates and the structure of the chromogenic group. The bond that is cleaved to release the chromophore is indicated with a wavey line.

multi-range pH buffer (50mM NaH_2PO_4 / 50mM trisodium citrate / 50mM boric acid / 50mM Hepes) at the appropriate pH. Samples were princubated for 15 minutes at 37°C. To start the reaction 20 ul of substrate solution at the appropriate concentration was added to give a final reaction volume of 2 ml. The reactions were carried out at 37°C then stopped by addition of 1 ml of 100mM sodium monochloracetate / 30mM sodium acetate, pH 5.5 and the fluorescence determined with an excitation wavelength of 370nm and an emission wavelength of 460nm using a Perkin-Elmer LS-3 spectrofluoremeter. A 0.5uM solution of AMC was used to set the fluorescence signal to 1000 before the assay enabling readings to be converted from fluorescence units to concentrations (Barrett, 1981). The length of the incubations for the reactions were determined as for the p-nitroanilide derivatives.

3. Furylacryloyl derivatives (Table 5). The substrates were dissolved to give a 30mM solution in absolute ethanol which was stored at -20°C. To 2.85ml of multi-range pH buffer (50mM NaH_2PO_4 / 50mM trisodium citrate / 50mM boric acid / 50mM Hepes) was added 150ul of *T.cruzi* epimastigote NP-40 extract. Samples were preincubated for 15 minutes at 37°C before starting the reaction by addition of stock substrate solution to a final concentration of 0.3mM. The reaction was followed by monitoring the fall in absorption at 345nm.

4. Leu-ser-phe(NO_2)-leu-ala-leu-OMe. A 5 mg ml^{-1} stock solution of the substrate in water was made. 150ul of NP-40 extract was added to 0.85ml of multi range pH buffer (50mM NaH_2PO_4 / 50mM trisodium citrate / 50 mM boric acid / 50mM Hepes) and preincubated for 15 minutes at 37°C. Stock substrate was then added to give a range of final concentrations from 0.025 mg ml^{-1} to 1 mg ml^{-1}. The reaction was followed by monitoring the fall in absorption at 300nm.

5. Azocasein hydrolysis. Detergent extracts of parasites representing about 7.5ml of dense epimastigote culture (5 X 10^6 cells ml^{-1}) were added to 1.25ml multi-range pH buffer (50mM NaH_2PO_4 / 50mM trisodium citrate / 50mM boric acid / 50mM Hepes) at the stated pH. After a 15 minute preincubation at 37°C the reaction was started by adding 1.25ml of 20 mg ml^{-1} azocasein in multi-range pH buffer (50mM NaH_2PO_4 / 50mM trisodium citrate / 50mM boric acid / 50mM Hepes). One millilitre was taken immediately and added to 0.25ml of cold 25% (w/v) TCA, mixed and left on ice. The remainder of the reaction mixture was incubated at 37°C. At various time intervals 1ml of the reaction mixture was added to 0.25ml of cold 25% (w/v) TCA, mixed, and left on ice (reaction times were determined as for the p-nitroanilide substrates). The TCA precipitated samples were then centrifuged for ten minutes at 12,000 X g and the absorbance at 366nm of their supernatants determined.

Determination of pH optima.

Assays were performed as described above at a series of pH values. The pH of a multi-range pH buffer (50mM NaH_2PO_4 / 50mM trisodium citrate / 50mM boric acid / 50mM Hepes) was adjusted with 2M NaOH or concentrated HCl. The advantage of a single buffer system is that the concentration of impurities introduced with the buffer components will be the same for all the pH values. However, the concentration of Na^+ and Cl^- ions and any impurites in the NaOH and HCl solutions will be different for the different pH values.

Inhibitor profiles.

Assays were performed as described except that various inhibitors and other test compounds were included, at the concentrations stated in the text, in the preincubation. For the chromophoric protein assays test compounds were also included, at the same concentration as in the preincubation mixture, in the substrate solution.

For determination of IC_{50} values a series of inhibitor concentrations were used and the results

displayed graphically. The concentration that gave a 50% reduction in activity was determined from inspection of the graph.

Enzyme kinetics.

A preliminary experiment was done to show that, for the time course to be used and for the range of substrate concentrations to be tested, the increase in absorbance with time was linear. Assays were performed in duplicate with the range of substrate concentrations stated in the text. Plots of [S]/v versus [S] were used to determine Km and Vmax values (Cornish-Bowden and Wharton, 1988). The parameter -Km is given where the extrapolated line crosses the abscissa and Km/Vmax is the value on the ordinate where it is crossed by the extrapolated line.

Preparation of total parasite DNA.

About 10^{9} *T.cruzi* epimastigotes were harvested and washed once with phosphate-buffered saline. The parasite cell pellet was suspended in 1 ml of 1% (w/v) SDS / 25 mM EDTA / 50 mM Tris-HCl, pH 7.5 / 400 ug ml^{-1} proteinase K and incubated for 5 hours at 37°C. DNA was

extracted once with an equal volume of phenol saturated with water, twice with phenol-chloroform (1:1, v/v), and once with chloroform and then precipitated at -20°C with 2 volumes of absolute ethanol for 3 hours. The DNA was pelleted by centrifugation at 12000 X g for 10 minutes at 4°C, air dried for 60 minutes, and dissolved in 1 mM EDTA / 10mM Tris-HCl, pH 8 at a concentration of 0.5 mg ml^{-1} DNA.

Radiolabelling of DNA.

A DNA nick translation kit (GIBCO-BRL) was used. 1 ug of DNA was nick translated in 50 ul of 0.2 mM dATP / 0.2 mM dGTP / 0.2 mM dTTP / 50uCi dCTP (10 uCi ul^{-1}) / 100 U ml^{-1} DNA polymerase I / 0.001 ug ml^{-1} DNase I. The reaction was started by the addition of the enzymes. The tube contents were mixed and incubated at 16°C for 1 hour. The reaction was stopped by adding 10 ul of 0.25 M EDTA and the reaction mixture diluted to 0.5 ml with 1 mM EDTA / 10 mM Tris-HCl, pH 8 / 100 mM NaCl and applied to a column of 1 ml Sephadex G-25 equilibrated in 1 mM EDTA / 10 mM Tris-HCl, pH 8 / 100 mM NaCl. The column was centrifuged at 1000 X g at room temperature for 5 minutes to remove unincorporated nucleotides and the eluate was used for hybridizations.

Insect sample preparation.

R. prolixus was palpated to expel faeces onto a microscope slide. Salivary glands from these insects were dissected and dissociated mechanically into 25ul of phosphate-buffered saline (PBS) pH 7 on a microscope slide.

Detection of parasites and purified DNA by hybridization to labelled probes.

Seven microlitres of faeces and 7 ul of salivary gland material from each insect were applied to nylon membranes with a micropipette and the remainder used for microscopic examination. Tissue culture parasites were applied to the membrane in 5 ul of medium. DNA samples were applied to the membrane in 5 ul or less of 1mm EDTA / 10mM Tris-HCl, pH 8.

The nylon membranes were allowed to dry; floated on denaturant (0.4M NaOH / 1.5M NaCl) for 10 minutes, neutralized twice (3M NaCl / 0.5M Tris-HCl pH 7) for 3 minutes and then rinsed in 2 X SSC (SSC is 0.15M NaCl / 0.015M trisodium citrate pH 7). After air drying the membranes were baked (80°C) for two hours.

Membranes were prehybridized with 6 X SSC / 5 X

Denhardt's solution (0.02% (v/v) Ficoll / 0.02% (w/v) Bovine Serum Albumin / 0.02% (w/v) polyvinylpyrrolidine) / 0.5% (w/v) SDS / 0.5 mg ml^{-1} denatured herring sperm DNA at 68°C for at least one hour and then hybridized overnight at 68°C. Hybridization fluid was the same as the prehybridization fluid except that it included about 10^7 counts per minute of ^{32}P-labelled DNA for each 100 cm^2 of membrane.

The membrane was washed for 10 minutes at room temperature with 2 X SSC / 0.2% (w/v) SDS and then at 55°C with either 0.2 X SSC or 0.1 X SSC (low and high stringency respectively) for 25 minutes. The membranes were briefly blotted between filter paper sheets and wrapped in cling-film for autoradiography overnight using X-ray film with an intensifying screen at -70°C.

Chapter Three

Electrophoretic Detection of Peptidases of *T.cruzi*.

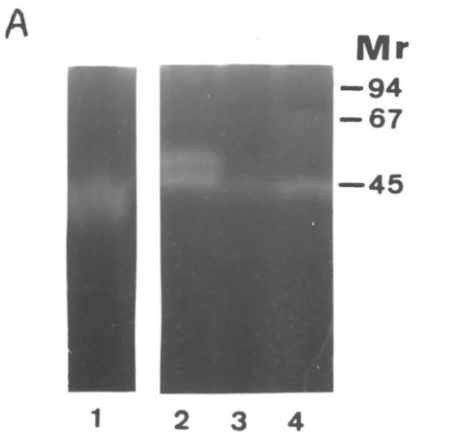
Peptidases of *T.cruzi* epimastigotes.

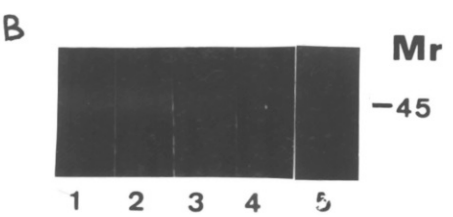
One percent NP-40 extracts of *T.cruzi* Y strain epimastigotes electrophoresed using a 10% polyacrylamide / 0.2% (w/v) gelatin separating gel with a large gel system (LKB) gave a single band of peptidase activity, at pH 5, with an approximate apparent molecular weight of 45 kDa (Figure 6 A lane 1). Extracts of *T.cruzi* Vinc C68 strain epimastigotes gave rise to a doublet while strains Can III and Esmeraldo 3 gave rise to single bands of a similar size to the Y strain band (Figure 6 A).

The band detected in extracts of *T.cruzi* (Y strain) epimastigotes was inhibited by 25uM leupeptin, 100uM TLCK and 0.5mM E64 (Figure 6 B). It was not inhibited by 1mM o-phenanthroline (Figure 6 B lane 2). These properties, particularly inhibition by E64 indicate a cysteine peptidase activity.

Five bands of peptidase activity were reproducibly detected in 1% NP-40 extracts of Y strain epimastigotes when a mini gel system (LKB) was used. These have been labelled a-e in Figure 7. One of these, band c, was not detected below pH 7 but the other activities (especially b) were increased in activity at

Figure 6. Peptidase gels using a large electrophoresis system (LKB). A, Electrophoresed extracts of different strains of *T.cruzi* epimastigotes incubated at pH 5. Lane 1, Y; lane 2, Vinc C68; lane 3, Esmeraldo 3; lane 4, Can III. B, Inhibitor profile of the Y strain epimastigote peptidase detected in LKB large gels. Gel strips were incubated at pH 5 in: lane 1, buffer alone; lane 2, 1mM o-phenanthroline; lane 3, 25 uM leupeptin; lane 4, 100 uM TLCK; lane 5, 0.5 mM E64.


A
Mr
94
67
45
1
2
3
4


B
Mr
45
1
2
3
4
5

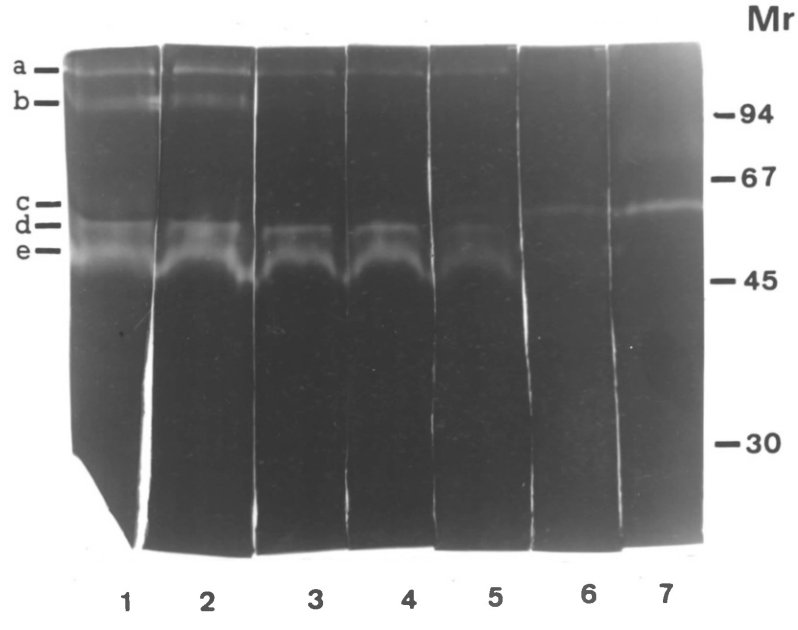


Figure 7. pH profile of peptidases of *T. cruzi* (Y strain) epimastigotes. Five peptidase activities, a-e, are apparent. The incubations were at pH 4 (lane 1); pH 5 (lane 2); pH 6 (lane 3); pH 7 (lane 4); pH 8 (lane 5); pH 9 (lane 6) and pH 10 (lane 7).

the lower pH values. At pH 10 the intensity of band c was increased and the other bands were not detected (Figure 7 lane 7).

No activity at acidic, neutral or alkaline pH was detected when 0.2% (w/v) haemoglobin was incorporated into the gel as substrate. 0.2% (w/v) casein gels gave a similar banding pattern and pH profile to that seen in the gelatin gels but with the exception that band c (Figure 7) was more intense and the remaining bands were less intense (data not shown). Gelatin was used as substrate for all subsequent electrophoretic experiments described here.

The reason for the difference between the banding patterns in large and mini gels is not clear. The longer electrophoresis running time required for the large size gel system may result in an appreciable loss of activity of some peptidases. It is also possible that the smaller amount of protein that has to be digested to give rise to a band, with the mini gel system compared to the large system, is responsible for the greater sensitivity of the mini gel system. The mini gel electrophoresis system was used for all the peptidase activity detection gels described in this chapter, other than that shown in Figure 6 A, because of its greater sensitivity and convenience.

Comparison of peptidases of different *T.cruzi* strains.

NP-40 extracts of seven strains of *T.cruzi*, isolated from different parts of South and North America, all showed similar peptidase banding patterns (Figure 8). Some differences were noticed which mainly relate to the relative intensities of bands. However, strain California contained at least one strong band of peptidase activity not seen in the other strains (Figure 8 lane 3). Further analysis of this band showed that, in common with band c of Y strain epimastigotes, it partitioned into the detergent phase of Triton X-114 extracts and was inhibited by o-phenanthroline (Figure 12 A and B, lanes 5 and 6).

Inhibitor studies.

Examples of gel strips incubated at pH 7.4 with different inhibitors are shown in Figure 9 A and B. 35uM TLCK and 0.2mM leupeptin inhibited all of the bands except a weak band of about 60 kDa (Figure 9 A lanes 1 and 2). 1mM o-phenanthroline, an inhibitor of metallopeptidases, did not inhibit these bands but did remove the 60 kDa activity (Figure 9 A lane 3). 0.2mM E64 inhibited all of the peptidases activities detected at pH 5 (Figure 9 B lane 2) while 1mM PMSF and 75uM pepstatin (Figure 9 B lanes 1 and 3) had no effect. These results, particularly inhibition by E64, indicate that cysteine peptidases are responsible for these bands. Consistent

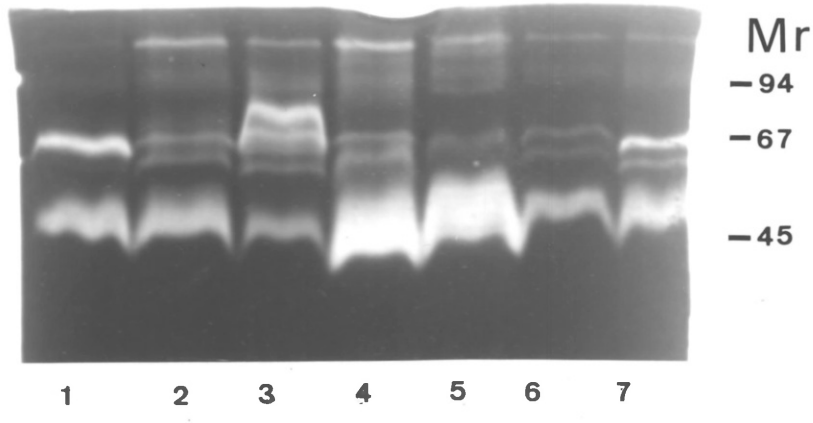
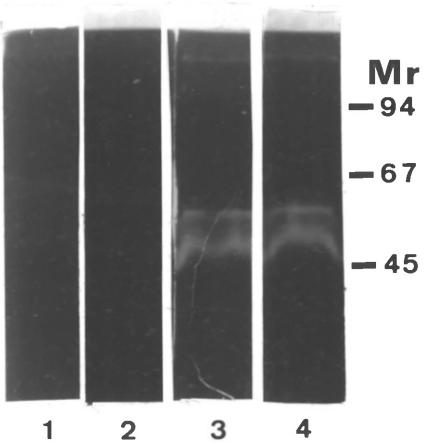


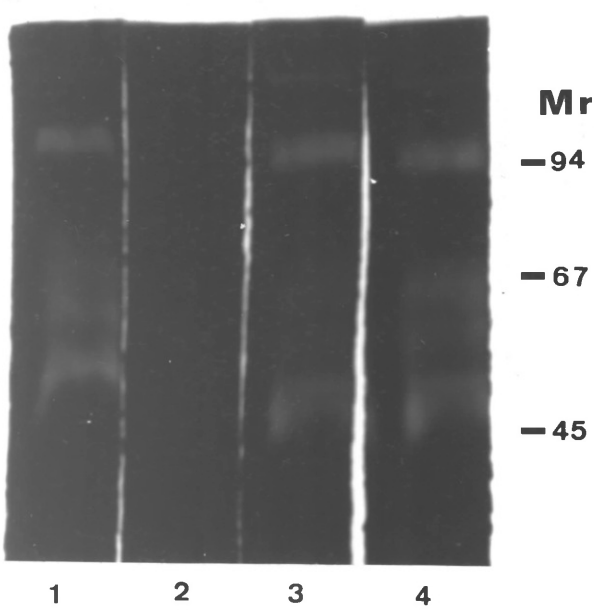
Figure 8. Peptidase activities of different *T.cruzi* strains at pH 7.4. One percent NP-40 extracts of epimastigotes were used. Lane 1 Dog Y; lane 2 Opossum; lane 3 California; lane 4 VinC 68; lane 5 Esmeraldo; lane 6 Armadillo; lane 7 Y.

Figure 9. Effects of peptidase inhibitors on *T. cruzi* peptidases. One percent NP-40 Y strain epimastigote extracts were used. (A) Gels were incubated at pH 7.4. Lane 1, 35uM TLCK; lane 2, 0.2mM leupeptin; lane 3, 1mM o-phenanthroline; lane 4, control. (B) Gels were incubated at pH 5 with 0.2% (w/v) SDS. Lane 1, 1mM PMSF; lane 2, 0.2mM E64; lane 3, 75uM pepstatin; lane 4, control.

A



B



with this is the lack of inhibition by pepstatin, an inhibitor of aspartic peptidases; PMSF, an inhibitor of serine peptidases and o-phenanthroline, an inhibitor of metallopeptidases. The band at about 67 kDa in Figure 9 B was not reproducibly detected. The sensitivity of the peptidase bands from Y strain epimastigotes to a panel of inhibitors is summarized in Table 6.

Both 1mM o-phenanthroline and 12.5mM EDTA, in addition to inhibiting the band of approximately 60 kDa, often activated the other bands (compare Figure 12 A with 12 B). Certain heavy metal ions are inhibitors of some cysteine peptidases and it may be that in some experiments these ions were present as impurities. Chelation of these ions by o-phenanthroline and EDTA might then have the effect of activating the cysteine peptidases. Indeed, the commercially available salts used for the buffers here did indicate low levels of heavy metal ion contamination. The combined concentration of heavy metal ions in the buffer used for incubation of the gels could be as high as 10uM which might partially inhibit some enzymes.

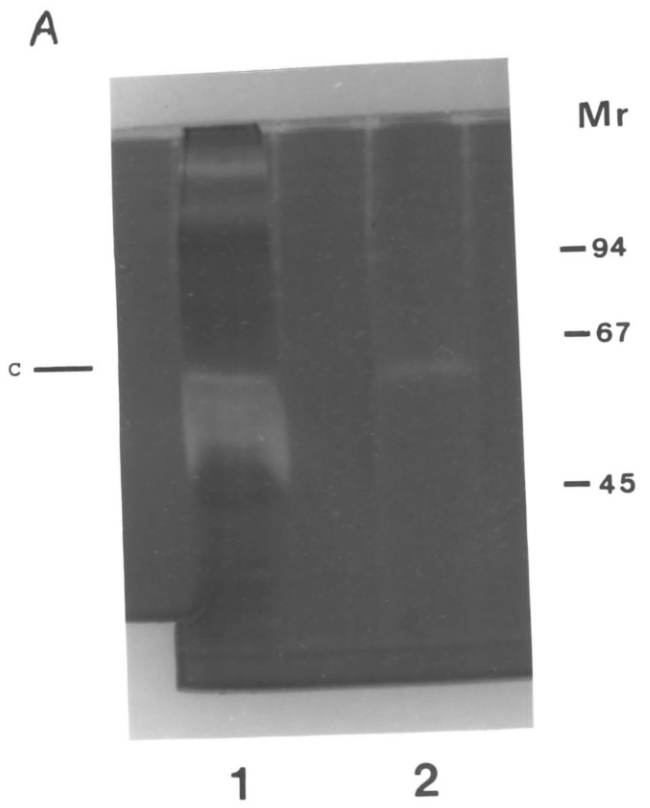
Triton X-114 separation of peptidases.

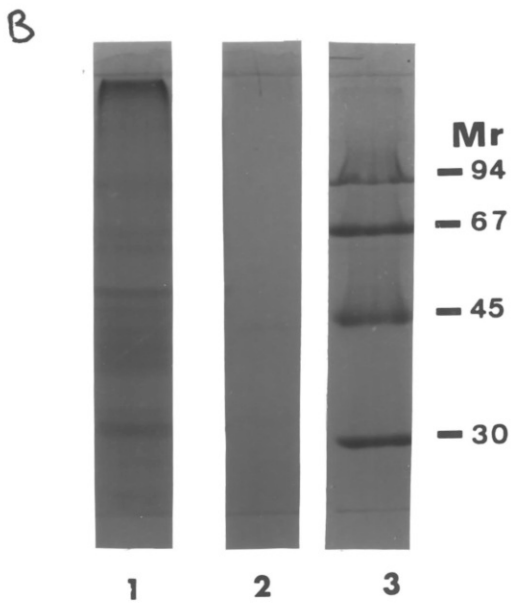
The aqueous phase of Triton X-114 extracts of epimastigotes retained all of the bands found in 1% NP-40 extracts (Figure 10 A lane 1). The detergent phase, however, was enriched for an activity of approximately

	Peptidase band				
	a	b	c	d	e
35uM TLCK	-	-	+	-	-
0.25mM leupeptin	-	-	+	-	-
12.5 mM EDTA	+	+	+/-	+	+
1mM o-phenanthroline	+	+	-	+	+
1mM PMSF	+	+	+	+	+
0.2mM E64	-	-	+	-	-
0.1 mM Zn^{++}	-	-	-	-	-
75uM pepstatin	+	+	+	+	+

Table 6. Inhibitor profile of *T.cruzi* peptidases detected by electrophoresis. + = activity; - = strong inhibition.

Figure 10. Triton X-114 phase separation of peptidases of *T. cruzi* Y strain epimastigotes. A, the aqueous phase (lane 1) of Triton X-114 extracts contained all the peptidase bands detected in 1% NP-40 extracts. The detergent phase (lane 2) was highly enriched for band c. The gel was incubated at pH 7.4. B, silver stained SDS-PAGE gels of the aqueous (lane 1) and detergent (lane 2) phases of Triton X-114 extracts. Molecular weight standards are shown in lane 3.


A
Mr
94
67
c
45
1
2


B
Mr
94
67
45
30
1
2
3

60 kDa (labelled "c" in Figure 10 A lane 2). The identity of this band was confirmed as the band labelled "c" in Figure 7 by its relative mobility, sensitivity to o-phenanthroline, insensitivity to other inhibitors (Figure 11 A), and by its alkaline pH optimum (Figure 11 B).

The detergent phase of Triton X-114 extracts showed very few bands on silver stained SDS-PAGE gels (Figure 10 B lane 2). In particular, a band of similar mobility to the peptidase activity present in the detergent phase could not be seen. The most intense band, of apparent molecular weight 42 kDa, was not a major component of the Triton X-114 aqueous phase material (Figure 10 B lane 1) suggesting that a separation of cell components had been achieved using the Triton X-114 phase separation technique.

All of the *T.cruzi* strains examined show a selective enrichment of band c in the detergent phase of Triton X-114 extracts. Figure 12 shows this for three *T.cruzi* strains. The major extra peptidase band of California strain NP-40 extracts (Figure 8 lane 3) was enriched in the detergent phase of Triton X-114 extracts and was also inhibited by o-phenanthroline (Figure 12).

Band c activity from Y strain epimastigotes was lost when the 1% NP-40 extract and the detergent phase of the 1% Triton X-114 extract was incubated overnight at 25°C or 4°C before adding loading buffer. Storage at

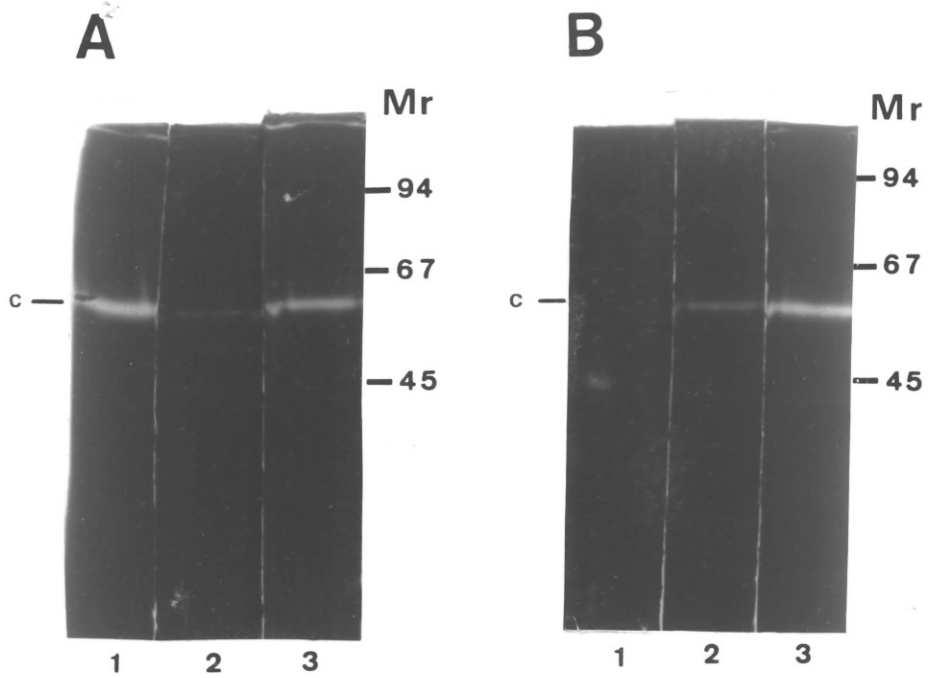
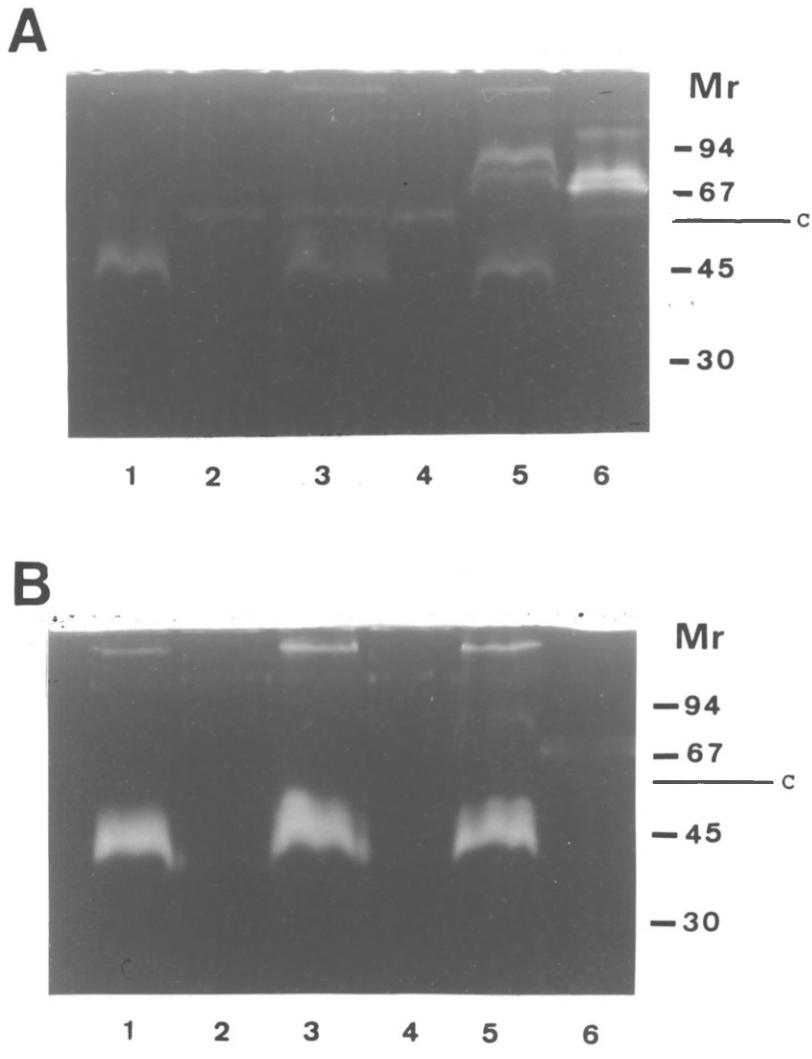


Figure 11. Inhibitor and pH profiles of the membrane-associated peptidase of *T. cruzi*. In A and B the Triton X-114 detergent phase of Y strain epimastigotes were electrophoresed. In A, incubations were at pH 8. Lane 1, control; lane 2, 1mM o-phenanthroline; lane 3, 35uM TLCK. B, lane 1, pH 5; lane 2, pH7; lane 3, pH 10.

Figure 13. Comparison of the membrane peptidase and surface iodinated proteins of *T. cruzi* (Y strain) epimastigotes. The 60 kDa activity labelled c, is present in lane 2 (control) but absent from lane 1 (1mM o-phenanthroline). At a similar mobility there is only a very weakly labelled radioiodinated component (lane 3).

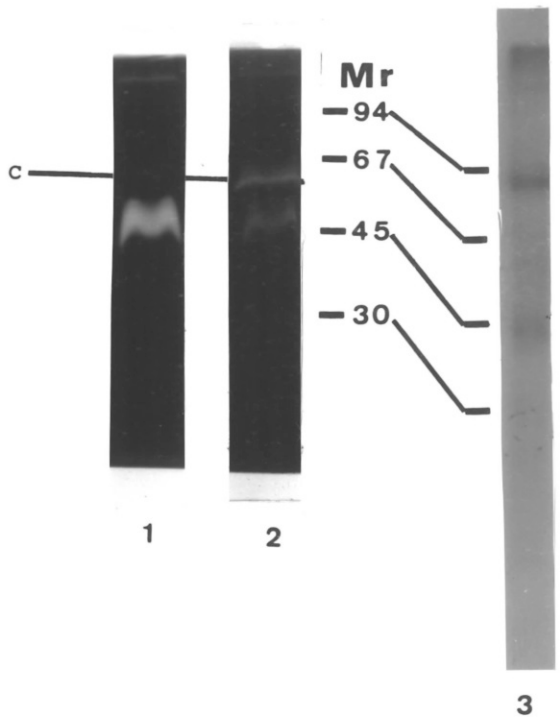

A
Mr
94
67
C
45
30
1 2 3 4 5 6
B
Mr
94
67
C
45
30
1 2 3 4 5 6

-20°C for up to a month before adding loading buffer did not lead to an appreciable loss of activity. A number of agents were found to be ineffective at stabilizing the metallopeptidase activity when included in the overnight incubation at 25°C. These included 25 mM B-mercaptoethanol; 12.5 mM DTT; 25% glycerol; and an inhibitor cocktail (12.5 ug/ml TLCK, 1 mM PMSF and 100 ug/ml leupeptin) with and without 1mM o-phenanthroline. These treatments did not alter the activity of band c when the 24 hour incubation at 25°C was ommitted. However, none of them significantly increased the stability of the enzyme.

The membrane-associated peptidase is not a major surface iodinated component of *T.cruzi* epimastigotes.

1% NP-40 extracts of radioiodinated *T.cruzi* Y strain epimastigotes showed qualitatively similar patterns in autoradiograms of SDS-PAGE gels to those described by other authors (Figure 13) (Snary and Hudson, 1979 and Nogueira et al., 1981). The most intensely labelled component had an apparent molecular weight of about 90 kD. This does not correspond to the mobility of any of the peptidase activities detected by substrate gel electrophoresis. A very weakly labelled band is present at about 60 kDa and could possibly represent the 60 kDa membrane-associated peptidase. However, other authors have not reported labelling such a

Figure 13. Comparison of the membrane peptidase and surface iodinated proteins of *T. cruzi* (Y strain) epimastigotes. The 60 kDa activity labelled c, is present in lane 2 (control) but absent from lane 1 (1mM o-phenanthroline). At a similar mobility there is only a very weakly labelled radioiodinated component (lane 3).


Mr
94
67
45
30
c
1
2
3

component by radioiodination of intact *T.cruzi* epimastigotes (Snary and Hudson, 1979 and Nogueira et al., 1981). This is in contrast to the situation in *Leishmania* in which a metallopeptidase of 63 kDa, gp63, is the most intensely labelled parasite component in radioiodination experiments (Chaudhuri and Chang, 1988).

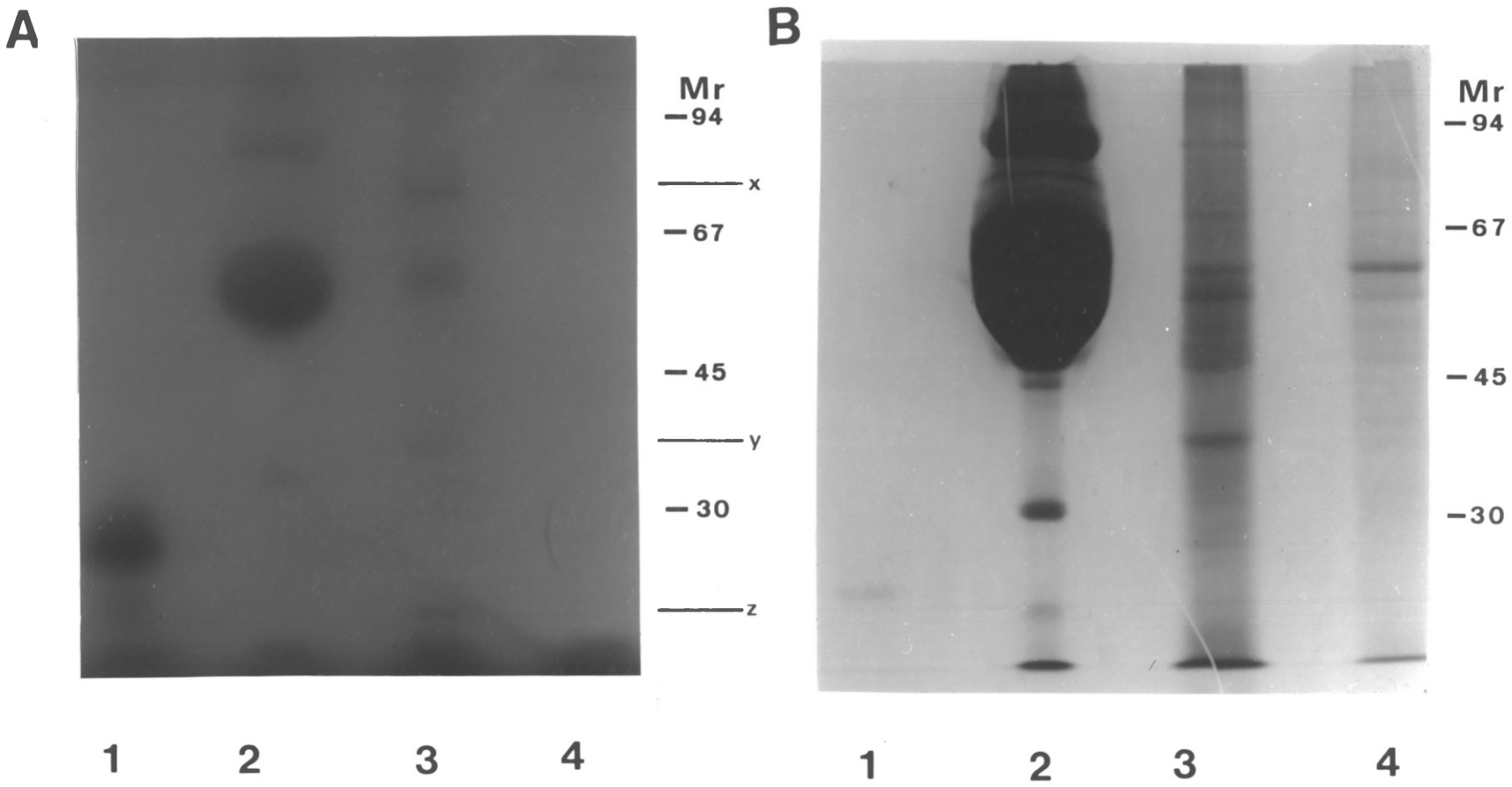
Activation of peptidase activities by SDS

It was noticed that 0.02% (w/v) SDS greatly increased the activity of the peptidases in some experiments if present during the digestion period. The effect was not seen if 0.02% (w/v) SDS was used only as a 30 minute incubation immediately prior to staining the gel. This suggests the effect may be directly on the peptidase activity. Alternatively, SDS may maintain the gelatin substrate in a conformation in which the accessibility of peptide bonds to the enzyme is increased.

[^{3}H]-DFP labelling of parasite extracts.

Components that labelled specifically with [^{3}H]-DFP were detected in epimastigote but not trypomastigote extracts of Y strain *T.cruzi* (Figure 14). The [^{3}H]-DFP labelled band in the epimastigote lane that approximately comigrates with the very heavily Coomassie blue stained band in the serum lane may represent BSA, which is known to react slowly with DFP. It is expected

Figure 14. [^{3}H]-DFP labelled *T.cruzi* (Y strain) epimastigote components. (A) Autoradiogram of SDS-PAGE gel of labelled extracts and (B) the same gel after staining with Coomasie brilliant blue. Lane 1, trypsin; lane 2, foetal calf serum; lane 3, 1% NP-40 extract of Y strain epimastigotes; lane 4, 1% NP-40 extract of Y strain trypomastigotes.


A
Mr
94
x
67
45
y
30
z
1
2
3
4
B
Mr
94
67
45
30
1
2
3
4

that some BSA may be present in the parasite extracts as the parasites were grown with foetal calf serum. The bands of apparent molecular weights 80 and 35 kDa and a very low molecular weight band (labelled x, y and z in Figure 14) in the epimastigote lane do not comigrate with any of the Coomassie Blue stained bands in the same lane (Figure 14; compare lane 3 of A with lane 3 of B) and are not represented in the serum lane and may therefore represent specific labelling of parasite proteins.

DFP irreversibly inhibits peptidases with hydroxyl groups in their active sites and it may be that the specific binding seen here is due to a serine peptidase. However DFP also inhibits various serine esterases, for example lipases, and so the labelled bands might also represent this class of enzymes.

Peptidases of *T.cruzi* trypomastigotes.

The patterns of Y strain trypomastigote and epimastigote acidic peptidases on gelatin SDS-PAGE gels were similar (Compare lanes 1 and 7 of Figure 15). The trypomsatigote peptidases had similar pH profiles to the epimastigote acidic peptidases (Figure 16). The trypomastigote peptidases were inhibited by 2mM IAAH and 0.2mM E64 (Figure 15 lanes 3 and 4) suggesting that, as for epimastigotes, they are cysteine peptidases. 35uM TLCK, an inhibitor of cysteine and serine peptidases, also strongly inhibited the trypomastigote and

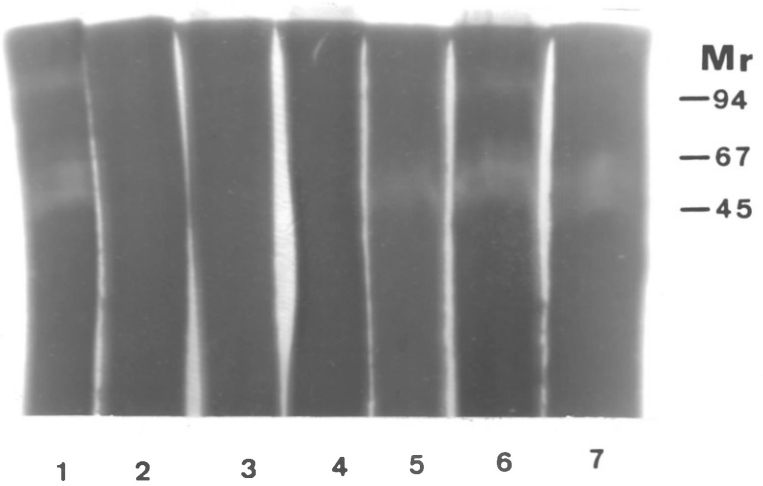


Figure 15. Inhibitor profile for peptidases of *T.cruzi* (Y strain) trypomastigotes at pH 5.5. Lane 1, no inhibitor; lane 2, 35uM TLCK; lane 3, 2mM IAAH; lane 4, 0.2mM E64; lane 5, 1mM DFP; lane 6, 75uM pepstatin; lane 7, *T.cruzi* (Y strain) epimastigotes without inhibitors.

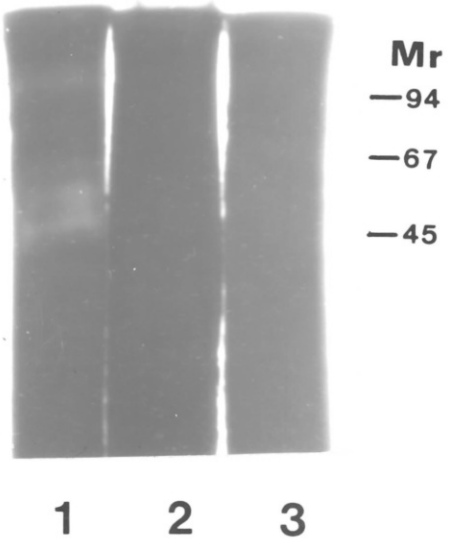


Figure 16. pH profile of peptidases of *T.cruzi* (Y strain) trypomsatigotes. Lane 1, pH 5.5; lane 2, pH 7.0; lane 3, pH 9.0.

epimastigote acidic peptidases (Figure 15 lane 2). 75uM Pepstatin, an aspartic peptidase inhibitor, had no effect on the activity of the peptidases (Figure 15 lane 6). 1mM DFP, a serine peptidase inhibitor, weakly inhibited the trypomastigote peptidases (bands at the higher molecular mass are not seen in the photograph but were visible, although faint, in the gel) (Figure 15 lane 5)

A band of about 30 kD was seen in some trypomastigote extracts but was never observed in extracts of epimastigotes (Figure 17). It is not clear why this activity varied between experiments more than the other activities.

Gelatin containing SDS-PAGE of 1% NP-40 extracts of trypomastigotes followed by digestion at alkaline pH values did not give rise to any peptidase activities (Figure 16 lane 3). This is in contrast to NP-40 extracts of epimastigotes in which a 60 kDa putative metallopeptidase activity with an alkaline pH optimum was detected (Figure 7 lane 7). This epimastigote peptidase was enriched in the detergent phase of Triton X-114 extracts (Figure 10 A). Detergent phases of Triton X-114 extracts of trypomastigotes, however, had no alkaline peptidase activity (Figure 18). The 60 kDa membrane associated putative metallopeptidase therefore appears to be a developmentally regulated activity absent from the trypomastigote stage.

A summary of the peptidase activities detected by

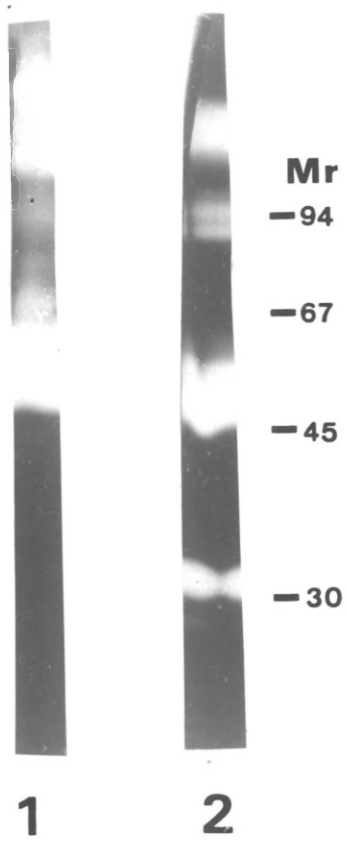
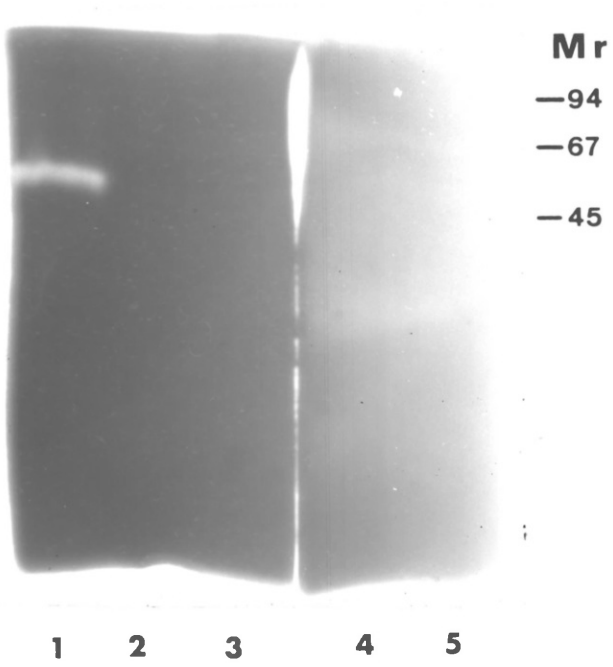


Figure 17. *T. cruzi* (Y strain) trypomastigote peptidases. Lane 1, epimastigotes; Lane 2, trypomastigotes.

Figure 18. Alkaline peptidases in the aqueous phase and detergent phase of Triton X-114 extracts of epimastigotes and trypomastigotes of *T. cruzi* (Y strain). Lane 1, detergent phase of epimastigotes; lane 2, aqueous phase of trypomastigotes; lane 3, detergent phase of trypomastigotes; lane 4, aqueous phase of trypomastigotes incubated with 1mM o-phenanthroline; lane 5, detergent phase of trypomastigotes incubated with 1mM o-phenanthroline.


Mr
—94
—67
—45
1
2
3
4
5

gelatin containing SDS-PAGE is shown in Table 7.

Secreted or shed peptidases from *T. cruzi* were not found.

No peptidases were detected in the medium of epimastigotes, trypomastigotes or amastigotes even when this was concentrated 10-fold by extraction of water through dialysis tubing covered with solid polyethylene glycol 20,000 (data not shown).

	Mr	pH	Class	
a	200	4	Cysteine	
b	120	4	Cysteine	
c	60	>10	metallo	(epimastigotes only)
d	55	5	cysteine	
e	45	5	cysteine	

Table 7 Summary of the properties of the major *T.cruzi* peptidase activities detected by SDS-PAGE.

Chapter Four

Detection of Peptidases of *T.cruzi* using Chromogenic and Fluorogenic Substrates.

Azocasein digestion by *T.cruzi* extracts.

Digestion of azocasein by 1% NP-40 extracts of Y strain *T.cruzi* epimastigotes was greatest at pH 5 but could not be determined at lower pH values due to precipitation of the azocasein. The activity at pH5 was increased approximately two-fold by including 3M urea during digestion. SDS at concentrations from 0.005% to 0.1% (w/v) further increased azocasein digestion by the extracts in the presence of 3M urea. Also, 0.3mM E64, 35uM TLCK and 60uM leupeptin strongly inhibited the digestion of azocasein (Figure 19). The action of E64 indicates that the activity is due to a cysteine peptidase (or peptidases). Neither 1mM PMSF (a serine peptidase inhibitor), 75uM pepstatin (an aspartic peptidase inhibitor) or 1mM o-phenanthroline (an inhibitor of metallopeptidases) had an effect on the rate of hydrolysis of azocasein (Figure 19). The activity was found only in the aqueous phase of Triton X-114 extracts suggesting it is not due to integral membrane components. The activity was not significantly reduced by incubation of the extracts at 25°C overnight.

Weak azocasein digestion was detected at pH 8 both

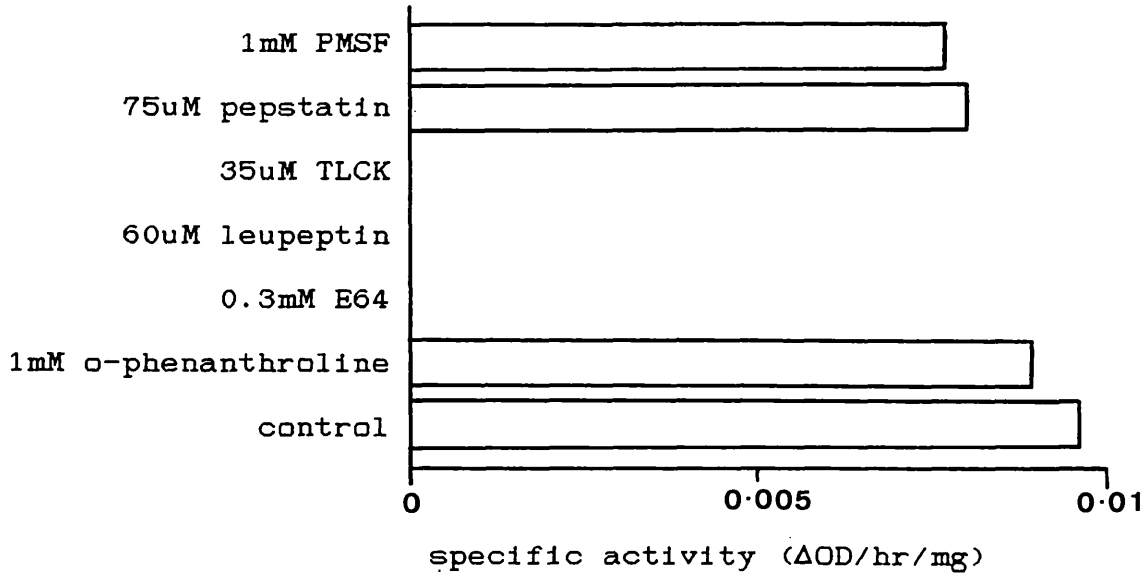


Figure 19. Inhibitor profile of azocasein digestion by a 1% NP-40 extract of *T.cruzi* (Y strain) epimastigotes.

in the presence and absence of 3M urea and this was found to be insensitive to 35uM TLCK but inhibited by 1mM o-phenanthroline. This activity was found largely in the aqueous phase but, unlike the TLCK-sensitive activity, was also detectable in the detergent phase. It is possible that this activity is due, all or in part, to the membrane-associated metallopeptidase of 60 kDa detected by SDS-PAGE (Chapter One, Figure 10).

Epimastigotes and trypomastigotes of Y strain *T.cruzi* hydrolysed azocasein when the viable parasites were added to the substrate (Figure 20). However, incubation of the parasites with azocasein resulted in some lysis which started early in the incubation and the activity detected may have been due to peptidases released from the cells rather than due to a surface or secreted peptidase.

Digestion of p-nitroanilide derivatives by *T.cruzi* extracts

Various amino acyl or peptidyl derivatives of p-nitroanilide were tested as substrates for a 1% NP-40 extract of Y strain *T.cruzi* epimastigotes in buffers ranging from pH 5 to pH 9 (Table 8 and Figure 21). The Bz-arg-pNan hydrolytic activity was consistent with that described by Ashall (1989). A characterization of this activity in some related trypanosomatids (Chapter 5) shows this enzyme to be a cysteine peptidase conserved amongst many trypanosmatids (all those tested) but absent from a number of non-trypanosomatid protozoa and other organisms (all those

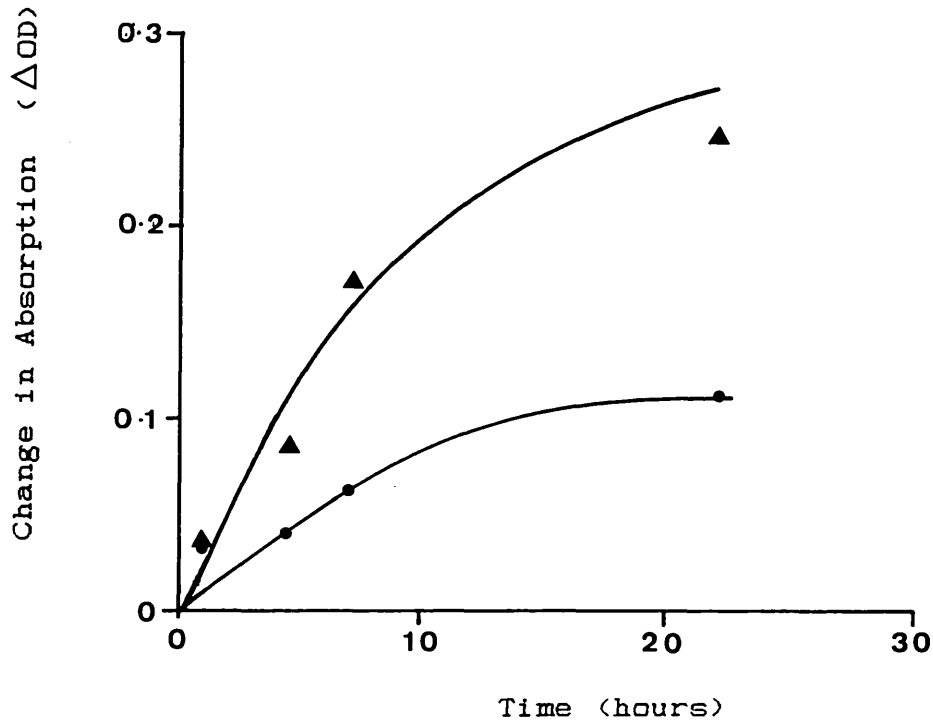
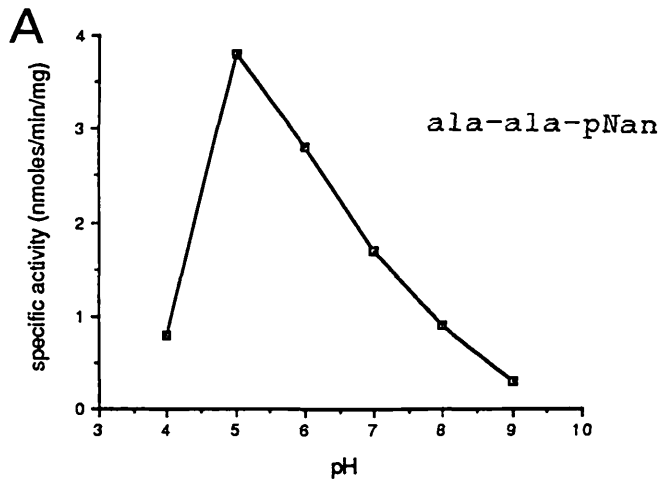


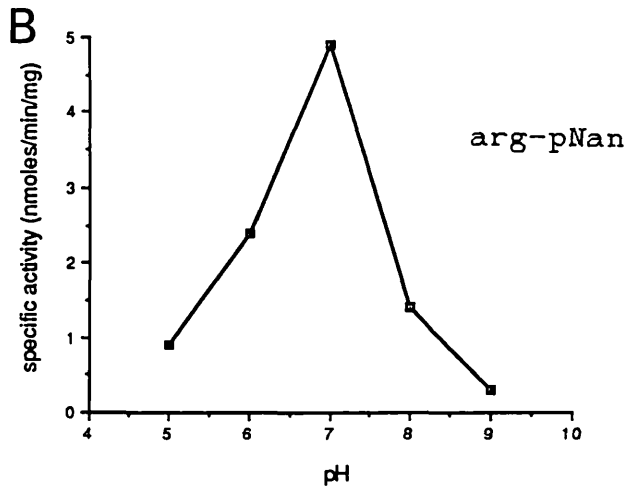
Figure 20. Digestion of azocasein by viable *T.cruzi* (Y strain) epimastigotes (▲) and trypomastigotes (●). Parasites were present at 10^7 per ml of azocasein solution.

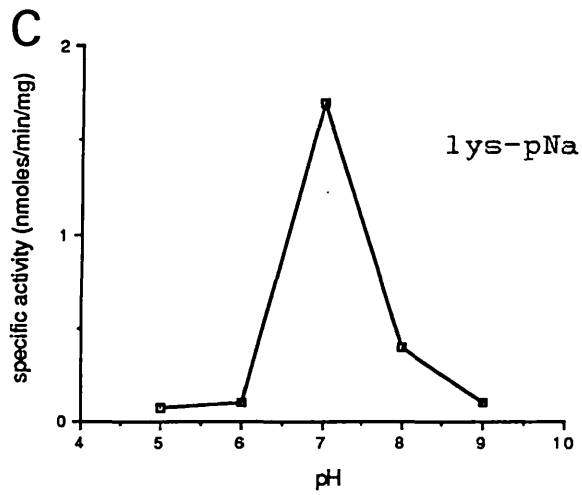
Substrate	specific activity (nmoles/min/mg)	optimal pH
Bz-arg-pNan	32.3	8
arg-pNan	4.0	7
lys-pNan	1.3	7
leu-pNan	4.1	7
ala-ala-pNan	3.0	5
Bz-tyr-pNan	0.5	5/6
Z-phe-leu-glu-pNan	0.4	7
Suc-ala-ala-pro-phe-pNan	0.4	7
Gly-pNan	0.0	-
Ac-phe-pNan	0.0	-
Boc-ala-ala-pNan	0.7	7
ala-ala-ala-pNan	0.2	7
ala-pro-pNan	0.0	-
ala-ala-phe-ala-pNan	0.1	5
ala-ala-val-ala-pNan	0.1	5
val-ala-pNan	0.0	-

Table 8. Specific activities of *T.cruzi* (Y strain) epimastigote extracts for digestion of some p-nitroanilide derivatives. Rates were determined at the optimal pH for each substrate and all substrates were present at 0.25mM.

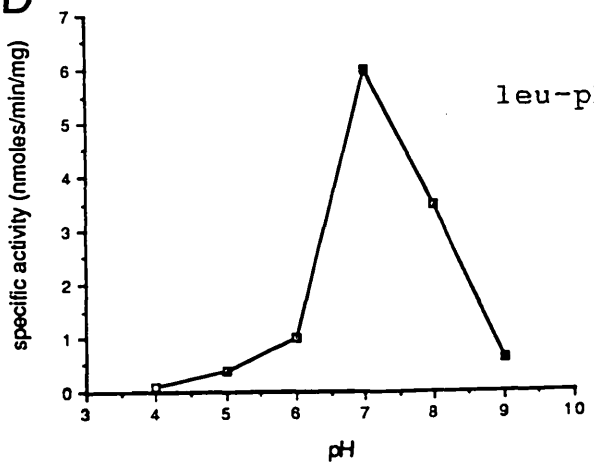
Figure 21. pH profile for hydrolysis p-nitroanilide derivative substrates by 1% NP-40 extracts of *T. cruzi* (Y strain) epimastigotes. A ala-ala-pNan; B arg-pNan; C lys-pNan; D leu-pNan; E Cbz-phe-leu-glu-pNan; F Bz-tyr-pNan; G Boc-ala-ala-pNan; H ala-ala-ala-pNan.


A
ala-ala-pNan
specific activity (nmoles/min/mg)
pH

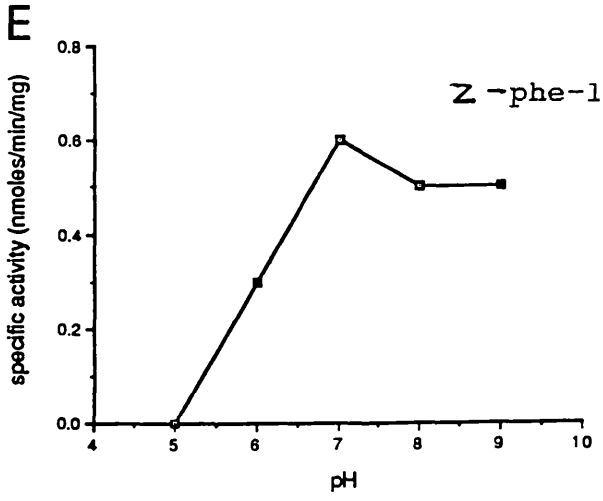

B
arg-pNan
specific activity (nmoles/min/mg)
pH


C
lys-pNan
specific activity (nmoles/min/mg)
pH

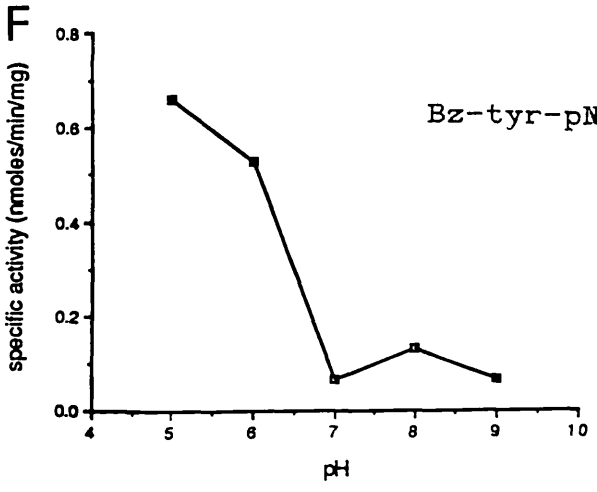
D

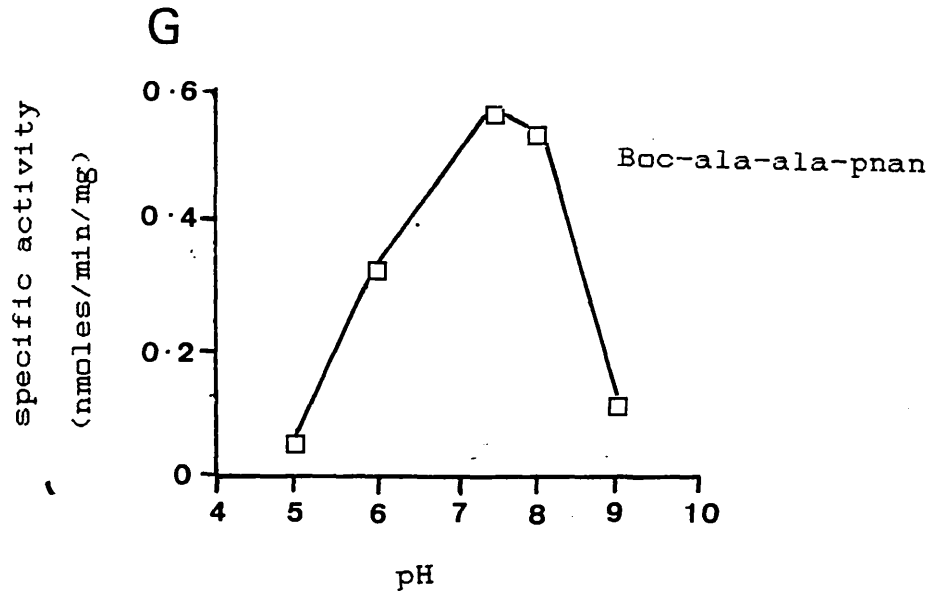

specific activity (nmoles/min/mg)
pH
leu-pNan

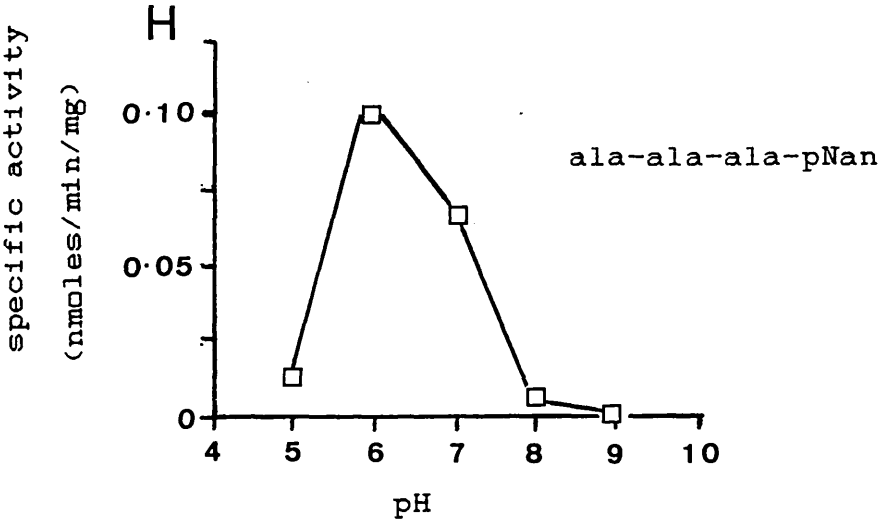
E


specific activity (nmoles/min/mg)
pH
Z-phe-leu-glu-pNan

F


specific activity (nmoles/min/mg)
pH
Bz-tyr-pNan


G
specific activity
(nmoles/min/mg)
0·6
0·4
0·2
0
4
5
6
7
8
9
10
pH
Boc-ala-ala-pnan


H
specific activity
(nmoles/min/mg)
0·10
0·05
0
4
5
6
7
8
9
10
pH
ala-ala-ala-pNan

tested).

Ala-ala-pNan. The rate of digestion of this substrate showed a broad pH optimum centered around pH 5 (Figure 21 A). 0.3mM E64 strongly inhibited this activity suggesting that it is due to a cysteine peptidase. 1mM IAAH completely inhibited the activity which is also consistent with this interpretation. The metallopeptidase inhibitors, o-phenanthroline, phosphoramidon, bestatin and EDTA had little effect on the activity (Figure 22). Also without effect were the serine peptidase inhibitors (PMSF, leupeptin, TLCK and Trypsin Inhibitor) and the aspartic acid inhibitor, pepstatin (Figure 22). The activity was enhanced about 1.7 fold by inclusion of 25mM β-mercaptoethanol during the digestion which is a characteristic shown by many thiol enzymes.

The ala-ala-pNan hydrolytic activity was found only in the aqueous phase of Triton X-114 extracts suggesting it is not due to integral membrane components. Incubation of extracts overnight at room temperature led to no loss of activity.

Boc-ala-ala-pNan This substrate was digested with a pH optimum of 7 (Figure 21). The activity was strongly inhibited by 1mM DFP but not by a range of other inhibitors (Figure 23). Inhibitors of metallopeptidases did have some effect: 1mM phosphoramidon inhibited the activity by about 50% and 100uM bestatin and 1mM o-phenanthroline gave a small amount of inhibition.

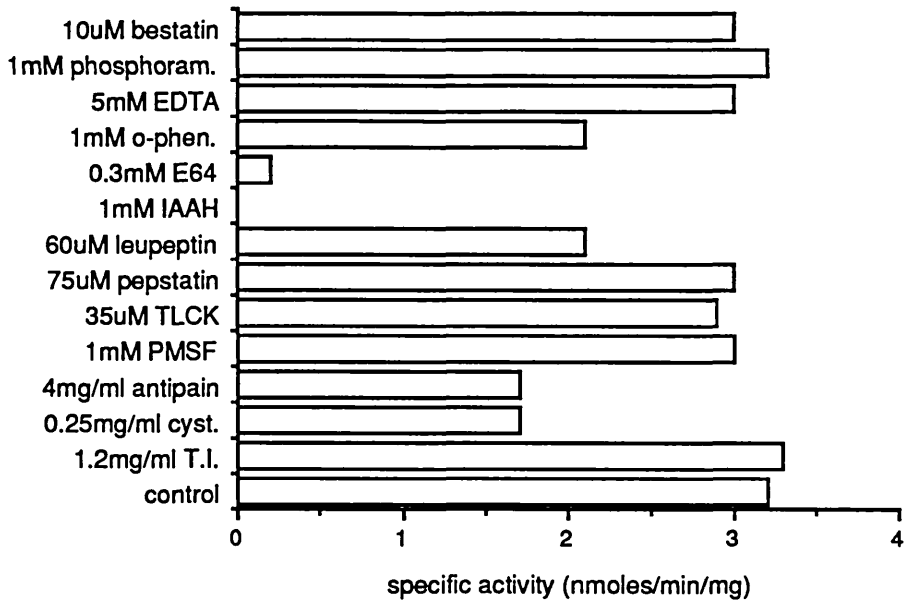
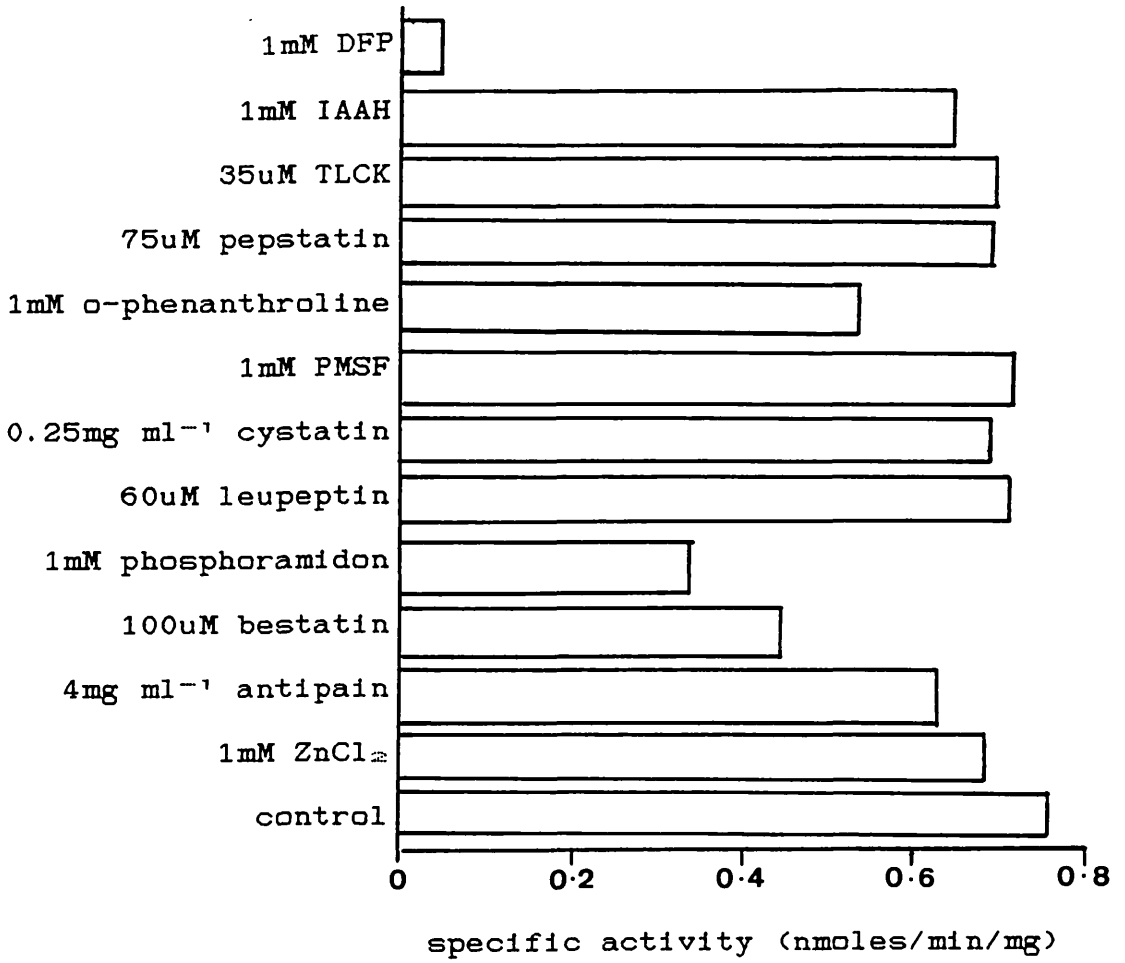


Figure 22. Inhibitor profile of ala-ala-pNan digestion by a 1% NP-40 extract of *T. cruzi* (Y strain) epimastigotes.

Figure 23. Inhibitor profile of Boc-ala-ala-pNan digestion by a 1% NP-40 extract of *T. cruzi* (Y strain) epimastigotes.


1mM DFP
1mM IAAH
35uM TLCK
75uM pepstatin
1mM o-phenanthroline
1mM PMSF
0.25mg ml⁻¹ cystatin
60uM leupeptin
1mM phosphoramidon
100uM bestatin
4mg ml⁻¹ antipain
1mM ZnCl₂
control
0
0·2
0·4
0·6
0·8
specific activity (nmoles/min/mg)

The strong inhibition by DFP implicates a serine peptidase although PMSF, another serine peptidase inhibitor, had very little effect.

Arg-pNan, lys-pNan and leu-pNan. These substrates all had similar pH profiles with an optimum of about pH 7 (Figure 21 B,C and D). Digestion of arg-pNan was not affected by inhibitors of cysteine, serine or aspartic acid peptidases but was strongly inhibited by 1mM o-phenanthroline and 100uM EDTA (Figure 24), suggesting that a metal ion is involved in the catalytic mechanism. (Figure 24 also shows the effects of a number of other agents on the arg-pNan hydrolytic activity of 1% NP-40 epimastigote extracts). Lys-pNan, leu-pNan, ala-pNan and val-pNan digestion was also inhibited by 1mM o-phenanthroline and 100uM EDTA but not by 75uM pepstatin, 1mM PMSF, 35uM TLCK or 1mM IAAH.

Unlike arg-pNan the N-terminally blocked substrate, Bz-arg-pNan, is not digested by the extracts in the presence of TLCK. Thus the enzyme (or enzymes) that hydrolyses arg-pNan may have a requirement for a free alpha amino group on the amino acid residue which is on the N-terminal side of the bond that is cleaved. A number of aminopeptidases, for example leucine aminopeptidases, have been shown to be metalloenzymes.

The arg-pNan hydrolysing activity was retained in the aqueous phase of Triton X-114 extracts suggesting that it is not an integral membrane component. Both activities

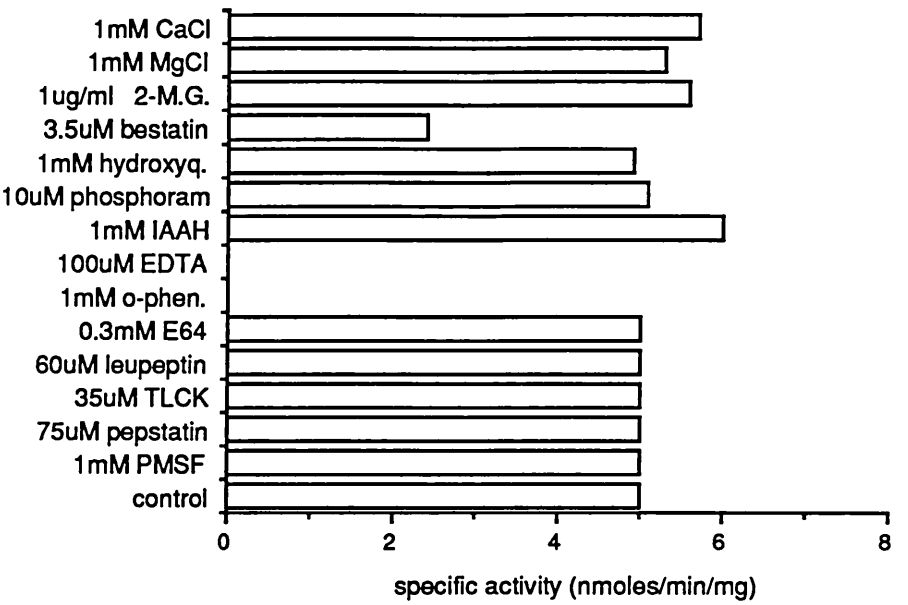


Figure 24. Inhibitor profile of arg-pNan digestion by a 1% NP-40 extract of *T.cruzi* (Y strain) epimastigotes.

were reduced after incubation of the extracts overnight at 25°C.

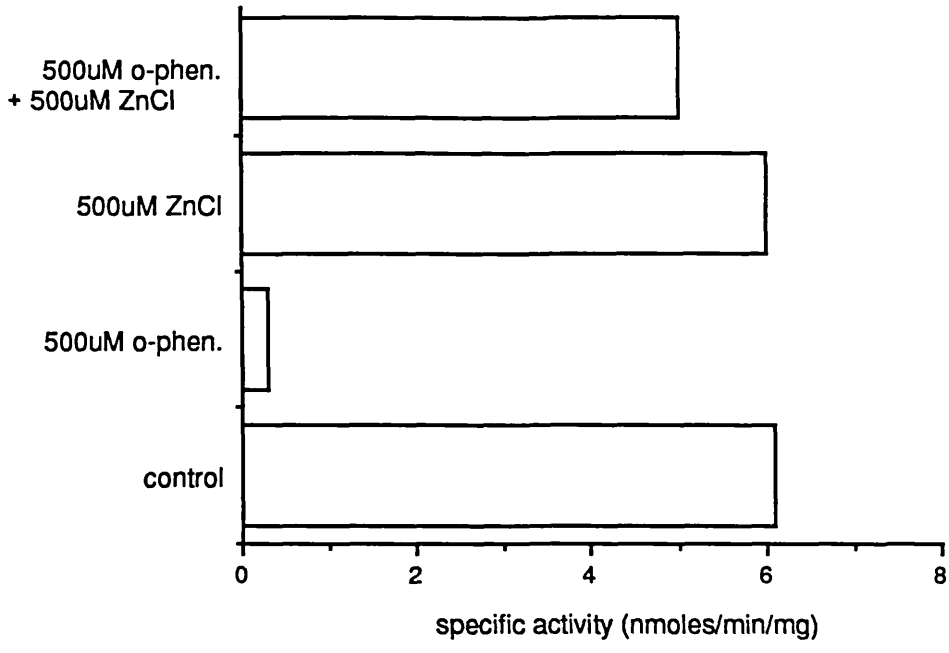
That the inhibition of leu-pNan and arg-pNan digestion by o-phenanthroline is due to chelation of a metal ion is supported by the observation that the inhibition was reversed by equimolar amounts of zinc ions (Figure 25). The zinc alone inhibited the activities to a small extent and the combination of o-phenanthroline and equimolar zinc resulted in a similar small degree of inhibition (Figure 25).

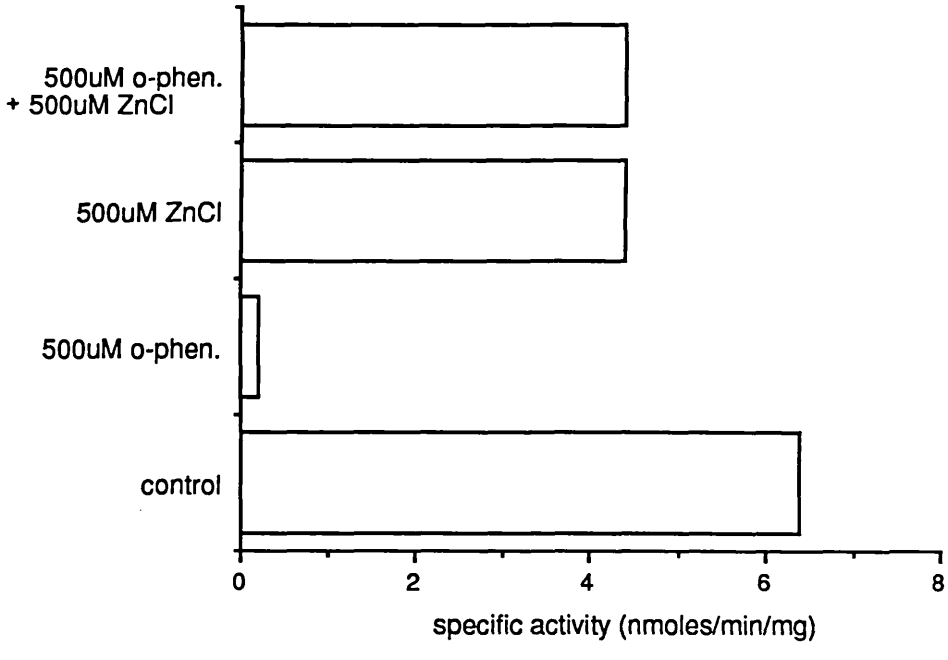
Ala-ala-ala-pNan. This substrate was digested with a pH optimum of 6 (Figure 21 H). The activity was inhibited by 1mM o-phenanthroline, 0.3mM E64 and 1mM IAAH (Figure 26). It is therefore likely that a combination of the metallo-aminopeptidase activity that hydrolysed ala-pNan and the cysteine peptidase activity that hydrolysed ala-ala-pNan is responsible for digestion of this substrate.

Digestion of N-methylcoumarin substrates by *T.cruzi* extracts

Z-arg-arg-Nmec. The hydrolysis of this substrate showed a similar pH profile (Figure 27 A) to that of Bz-arg-pNan (Ashall, 1989). The inhibitor profile (Figure 28 B) was also similar to that for Bz-arg-pNan (Figure 28 A). In particular TLCK gave complete inhibition and E64 partial inhibition of digestion of the two substrates. Both activities were stable when incubated overnight at

Figure 25. Reversal of o-phenanthroline inhibition of *T. cruzi* aminopeptidase activity by zinc ions. The substrates were arg-pNan (A) and lys-pNan (B).


500uM o-phen.
+ 500uM ZnCl
500uM ZnCl
500uM o-phen.
control
0
2
4
6
8
specific activity (nmoles/min/mg)


500uM o-phen.
+ 500uM ZnCl
500uM ZnCl
500uM o-phen.
control
0
2
4
6
8
specific activity (nmoles/min/mg)

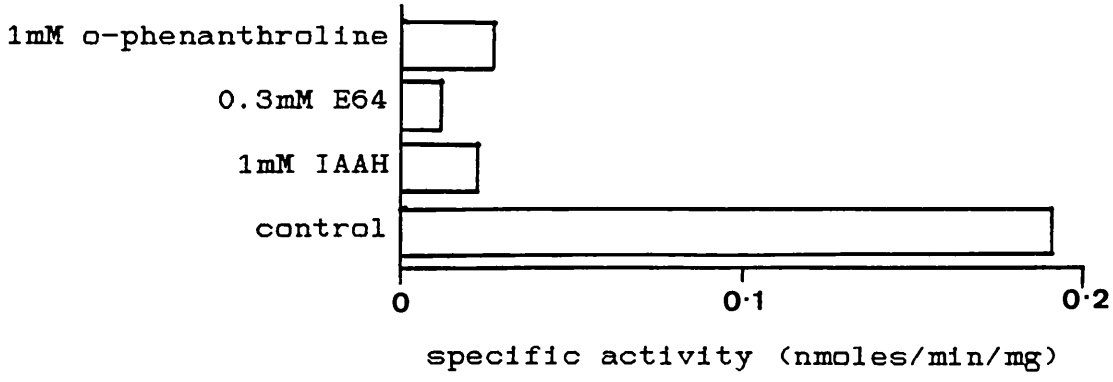
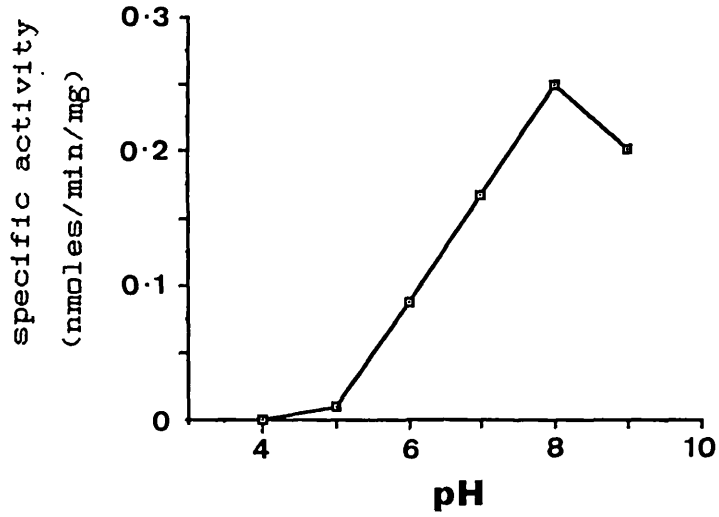


Figure 26. Inhibitor profile of ala-ala-ala-pNan digestion by a 1% NP-40 extract of *T.cruzi* (Y strain) epimastigotes.

Figure 27. pH profiles for digestion of Z-arg-arg-Nmec (A) and Z-phe-arg-Nmec (B) by 1% NP-40 extracts of *T. cruzi* (Y strain) epimastigotes.


specific activity
(nmoles/min/mg)
0·3
0·2
0·1
0
4
6
8
10
pH

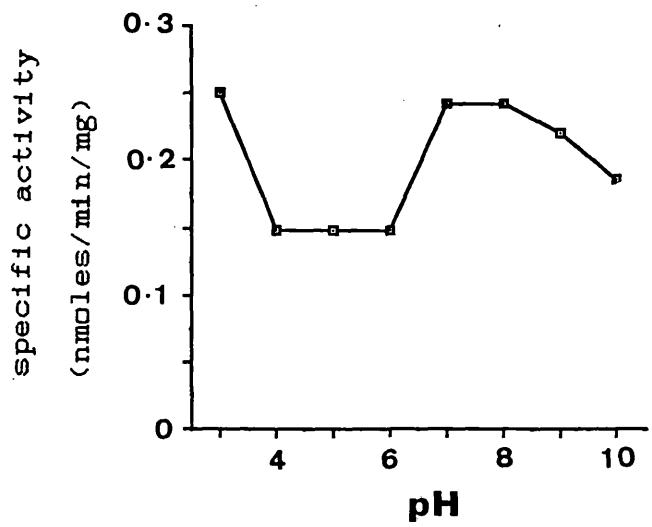
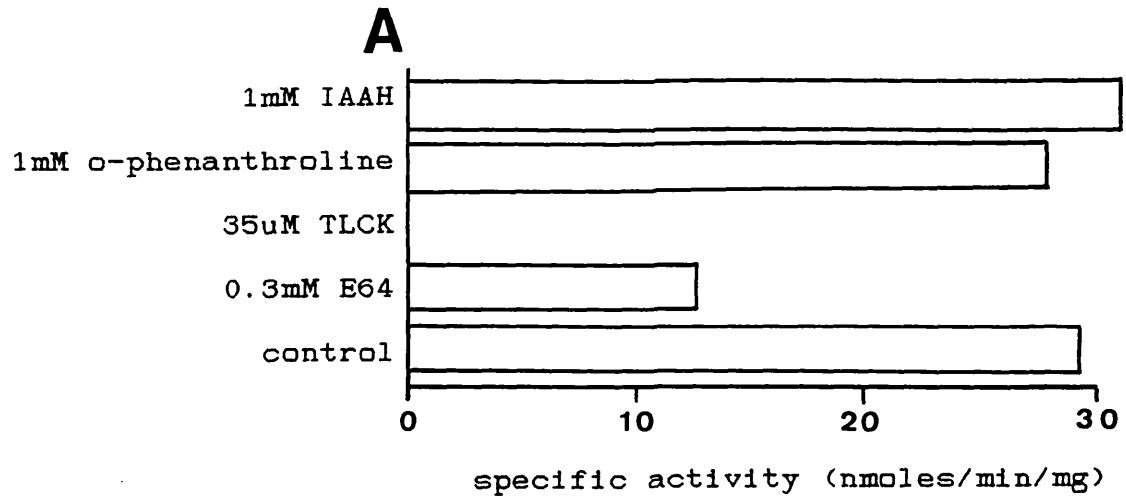
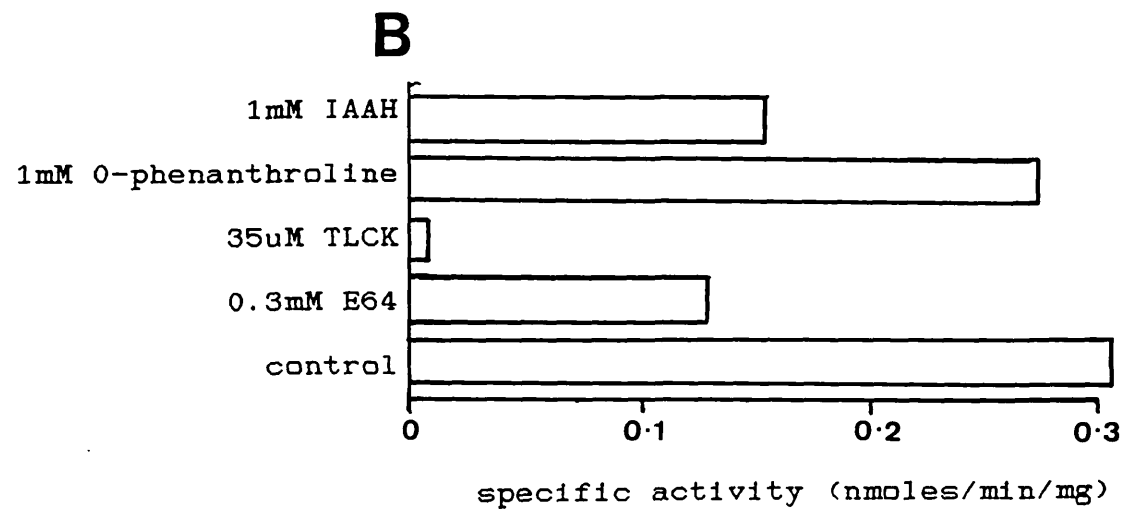
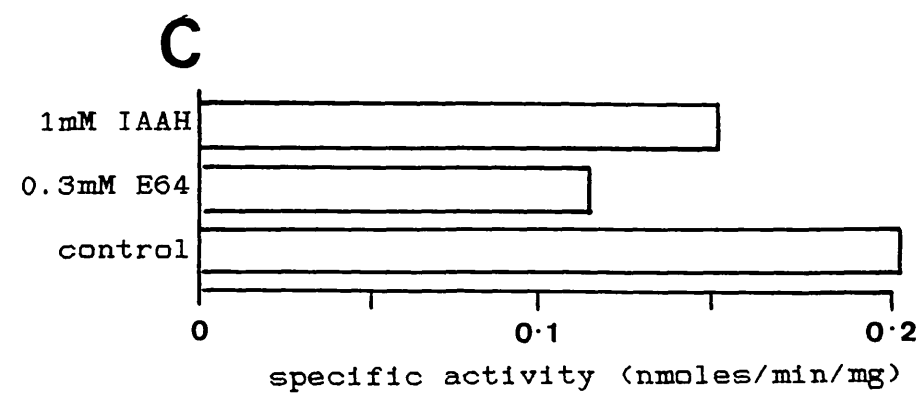

specific activity
(nmoles/min/mg)
0·3
0·2
0·1
0
4
6
8
10
pH

Figure 28. Inhibitor profiles of 0.25mM Bz-arg-pNan (A) 0.01mM Z-arg-arg-Nmec (B) and 0.01mM Z-phe-arg-Nmec (C) digestion at pH8 by 1% NP-40 extracts of *T.cruzi* (Y strain) epimastigotes.


A
1mM IAAH
1mM o-phenanthroline
35uM TLCK
0.3mM E64
control
0
10
20
30
specific activity (nmoles/min/mg)


B
1mM IAAH
1mM O-phenanthroline
35uM TLCK
0.3mM E64
control
0
0·1
0·2
0·3
specific activity (nmoles/min/mg)


C
1mM IAAH
0.3mM E64
control
0
0·1
0·2
specific activity (nmoles/min/mg)

25°C. It is therefore likely that the major cysteine peptidase of *T.cruzi* (Ashall 1989) is responsible for digestion of both substrates.

Z-phe-arg-Nmec. Two peaks of activity against this substrate were detected when pH was varied, one at pH 3 and one at pH 8 (Figure 27 B). When activity was measured at pH 8 the response to inhibitors was similar to that shown with Bz-arg-pNan and Z-arg-arg-Nmec (Figure 28 C) indicating that this activity is also due to the major cysteine peptidase of *T.cruzi* (Ashall, 1989). The inhibitor profile obtained when activity was determined at pH 3 (Figure 29) indicates that different cysteine peptidases hydrolyse Z-phe-arg-Nmec at pH 3 and pH 8. E64 strongly inhibits hydrolysis of Z-phe-arg-Nmec at pH 3 but not pH 8. IAAH, which does not inhibit the major *T.cruzi* cysteine peptidase or the Z-phe-arg-Nmec hydrolysing activity at pH 8, strongly inhibits the activity against Z-phe-arg-Nmec at pH 3 (Figure 29).

Digestion of furylacryloyl substrates and leu-ser-phe(NO_2)-leu-ala-leu-OMe by *T.cruzi* extracts.

These substrates are useful for the detection of peptidases which have strict specificity for the nature of the amino acids bound to their S' sites as well as their S sites. None of the substrates tested (shown in the Materials and Methods) were digested by extracts of *T.cruzi* (Y strain) epimastigotes.

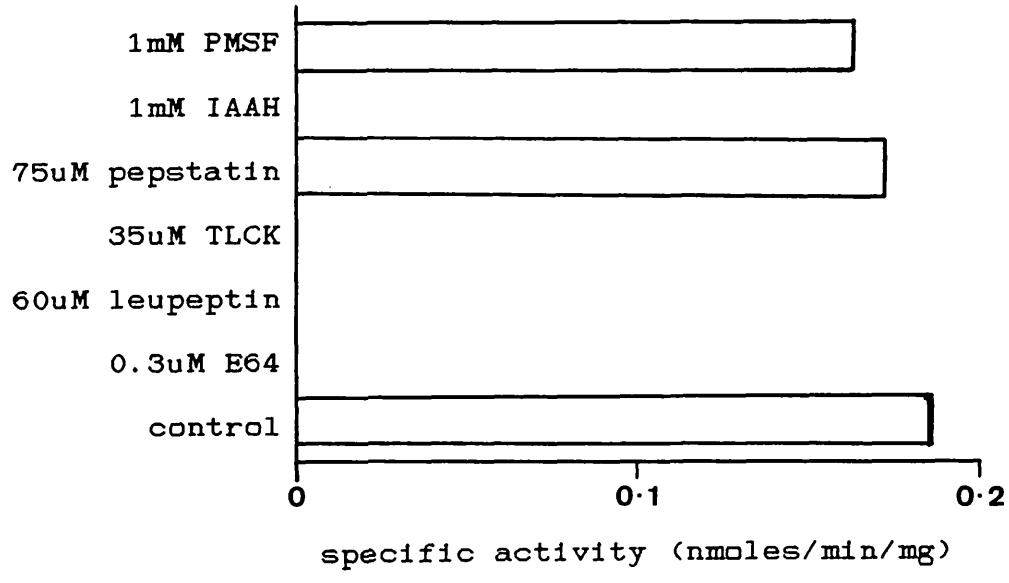


Figure 29. Inhibitor profile of Z-phe-arg-Nmec digestion at pH3 by a 1% NP-40 extract of *T.cruzi* (Y strain) epimastigotes.

Inhibition of *T.cruzi* peptidases by fluoromethylketones, diazomethylketones and sulfonium salts.

A series of peptidyl derivatives of these chemical groups were kndly supplied by Dr E. Shaw. These were tested as inhibitors of the peptidase activities detected using the chromogenic substrates. The chemical groups are known to inactivate cysteine peptidases by binding irreversibly to the sulfhydryl group at the active site. In the case of the fluoromethylketones reactivity tends to be greater against cysteine peptidases than serine peptidases. This is probably because the nucleophilicity of the sulfhydryl group of cysteine peptidases is sufficiently great to displace the fluorine from the reactive group of the inhibitor but the nucleophilicity of the serine oxygen is not. Chloromethyl-ketones, for example TLCK, will react with many members of both classes, because chlorine is a better leaving group than fluorine, and hence can not be used to distinguish these serine and cysteine peptidases.

The diazomethyl ketones are the most discriminatory of the reagents and have been shown to only inhibit cysteine peptidases despite the fact that they have been examined on a wide variety of serine and other peptidases (Shaw and Green, 1981). The level of inhibition achieved by these compounds depends on the affinity of the peptidyl part of the inhibitor for the

substrate-binding site of the enzyme and so inhibitor profiles can in principle be used to gain information on substrate preferences of cysteine peptidases and therefore to distinguish different cysteine peptidases.

The level of inhibition given by thirteen of these compounds, at a final concentration of 5uM, against digestion of four substrates by extracts of *T.cruzi* is shown in Table 9. The Bz-arg-pNan hydrolysing activity was strongly inhibited only by compounds with arginine or lysine in the P_1 position of the inhibitor, that is the amino acid position which is next to the reactive group of the inhibitor (Table 9). This is consistent with the known substrate specificity of the enzyme in *T.cruzi* that hydrolyses Bz-arg-pNan.

The activity that hydrolysed ala-ala-pNan was not inhibited strongly by any of the thirteen inhibitors possibly because the enzyme is a dipeptidyl aminopeptidase specific for alanine residues and none of the inhibitors tested were analogues of such a substrate.

Digestion of Z-phe-arg-Nmec was strongly inhibited by ten of the inhibitors (Table 9). Interestingly, Z-ala-phe-CH_2F did not inhibit while Z-phe-ala-CH_2F, in which only the order of the amino acids is reversed, strongly inhibited. This suggests that the S_1 and S_2 sites of the enzyme do not accommodate similar amino acids. That phenylalanine in the P_2 position gives stronger inhibition is consistent with the

	specific activity (nmoles/min/mg)			
Inhibitor	azo casein	Z-phe-arg-Nmec	ala-ala-pNan	Bz-arg-pNan
Control	100	100	3.3	25.0
Z-phe-ala-CHN_2	ND	5	2.3	23.0
Z-phe-phe-CHN_2	ND	10	3.3	24.0
Z-leu-lys-CHN_2	ND	5	3.0	1.0
Z-leu-met-CHN_2	ND	5	2.8	24.0
Z-phe-ala-CHN_2	20	10	3.1	24.8
Z-phe-ala-$CH_2S^+Me_2$	ND	5	3.3	24.5
ala-phe-lys-CH_2F	60	5	1.0	2.0
Z-phe-lys-$CH_2S^+Me_2$	ND	0	3.0	1.0
Z-ala-phe-CH_2F	100	100	3.3	24.0
Bz-phe-arg-$CH_2S^+Me_2$	ND	5	2.8	3.3
Z-tyr-ala-CH_2F	ND	20	3.1	24.0
Z-phe-^{1}Nva-CH_2F	ND	2	1.2	19.5
Z-leu-tyr-CH_2F	ND	35	3.3	24.0

Table 9. Specific activities of *T.cruzi* cysteine peptidases in the presence of various inhibitors. ^{1}Nva is a pseudoarginine.

enzyme being similar to cathepsin L. Inhibitors with leucine at the P_2 position also inhibited this activity (Table 9).

The large amount of parasites required for assay of the azocasein digesting activity precluded testing the effect of all the inhibitors on this activity. However the inhibitors tested had similar effects on both this activity and the activity against Z-phe-arg-Nmec. In particular Z-ala-phe-CH_2F gave no inhibition while Z-phe-ala-CH_2F inhibited the activity by about 80%. Thus, the azocasein and Z-phe-arg-Nmec digesting activities may be due to the same cathepsin L-like enzyme although the azocaseinase activity was enhanced by both 3M urea and 0.1% (w/v) SDS, whereas 3M urea and as little as 0.005% (w/v) SDS totally inactivated the Z-phe-arg-Nmec activity. These denaturing agents may be interacting with the substrates differentially in the two experiments and this could underlie their differential effect on digestion of the substrates. The inhibitor compounds are used at very low concentrations and exert their effects through specifically binding to the enzyme active site which makes them more reliable for differentiating the activities of cysteine peptidases when the substrates employed are very different as is the case here.

The relationship between peptidases detected by substrate gel electrophoresis and chromogenic assays

Ashall (1989) reported that the partially purified major alkaline peptidase of *T. cruzi* gave only very weak digestion of gelatin when analysed by substrate gel electrophoresis using gelatin as substrate. It is therefore unlikely that any of the proteolytic activities detected by gelatin-SDS-PAGE in this study are due to this enzyme even though both the major alkaline peptidase and the highest molecular weight activity detected by SDS-PAGE both had molecular weights of approximately 200 kDa.

When Y strain *T. cruzi* extracts were separated on gelatin-containing SDS-PAGE gels and the peptidase banding pattern compared with the activity of gel slices, taken from a neighbouring lane, for digestion of Z-phe-arg-Nmec the two peaks of fluorgenic substrate activity coincided with the peptidase bands (Figure 30). The resolution of the technique was not sufficient to determine if all or only some of the bands had Z-phe-arg-Nmec hydrolysing activity.

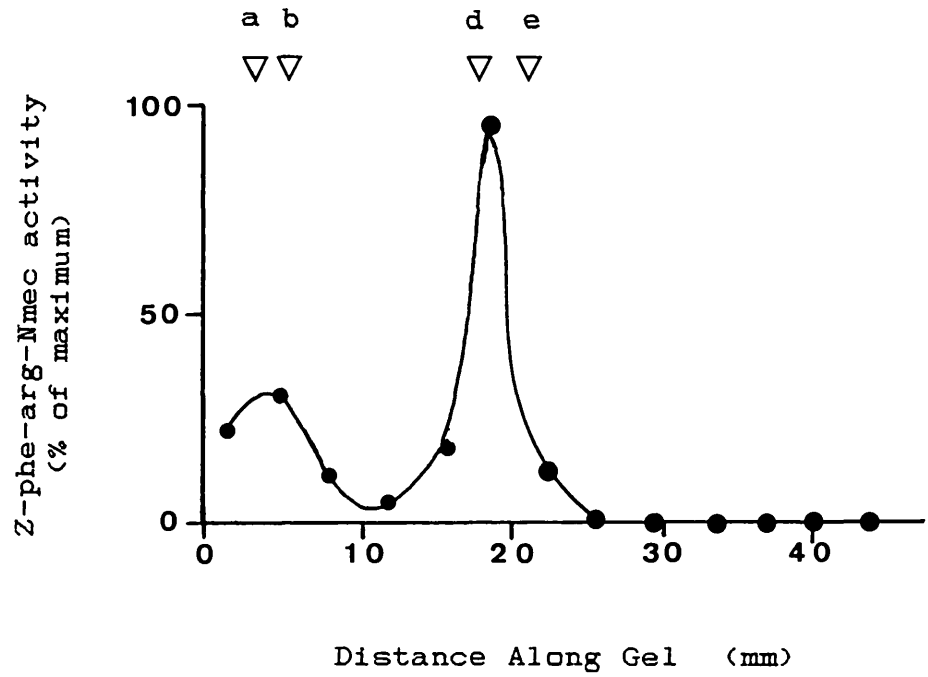


Figure 30. Relationship between peptidase bands detected by gelatin SDS-PAGE and the Z-phe-arg-Nmec acidic activity. The arrowheads labelled a, b, d and e represent bands of gelatin digestion seen in the gel lane adjacent to that used for Z-phe-arg-Nmec acidic activity determination. The bands correspond to those labelled a, b, d and e in Figure 7.

Chapter Five

A comparative study of the major alkaline peptidase activity of some trypanosomatids

An alkaline peptidase in *T. cruzi* has been identified and characterized by Ashall (1989). In this chapter the results of an investigation of some other trypanosomatids are presented which show that an identical or very similar enzyme exists in all the species examined. The data relating to *T. cruzi* and some of the entries in Table 10 were kindly supplied by Dr Ashall.

Digestion of three p-nitroanilide substrates by some trypanosomatids.

Extracts from 16 species representing 4 genera of trypanosomatid (shown in Table 10) all digested Bz-arg-pNan whereas the non-trypanosomatid protozoa examined did not digest this substrate. For all the trypanosomatids tested Bz-arg-pNan was digested better than the longer substrate, Tos-gly-pro-arg-pNan. Also, Tos-gly-pro-lys-pNan was digested 2- to 3-fold less readily than Tos-gly-pro-arg-pNan by the trypanosomatid extracts. The *T. cruzi* major alkaline peptidase prefers short substrates to long substrates and prefers arginine to lysine in the P_1 position of the substrate (Ashall, 1989). Therefore the substrate

Species	specific activity (nmoles/mg/min) Bz-arg-pNan	Tos-gly-pro-arg-pNan	Tos-gly-pro-lys-pNan
T. cruzi[a]	30.2	9.7	3.0
T. b. brucei (pros)	43.1	22.0	9.9
T. b. brucei (blood)[a]	21.2	13.4	7.6
T. b. gambiense[a]	17.6	8.6	3.0
T. b. rhodesiense[a]	11.3	8.5	3.8
T. evansi[a]	22.4	13.7	6.7
T. equiperdum[a]	10.9	5.6	2.2
L. donovani	8.4	5.0	2.3
L. arabica	26.2	16.4	6.0
L. hertigi	20.2	12.1	3.0
L. infantum	9.0	7.2	3.2
L. major	37.9	23.9	10.6
C. fasciculata	33.2	18.6	8.3
E. schaudinni	11.1	7.1	3.3
T. percae[a]	15.2	10.8	4.6
T. rangeli[a]	13.1	9.0	4.2
T. dionisii	16.9	8.3	3.4

Table 10. Specific activities for digestion of three peptidyl p-nitroanilide substrates at 0.25mM by 1% NP-40 extracts of various trypanosomatids. a, Result supplied by Dr Ashall.

preferences displayed by the trypanosomatids in Table 10 suggest that they may have a homologous enzyme.

L.donovani and *C.fasciculata* pH profiles for Bz-arg-pNan digestion.

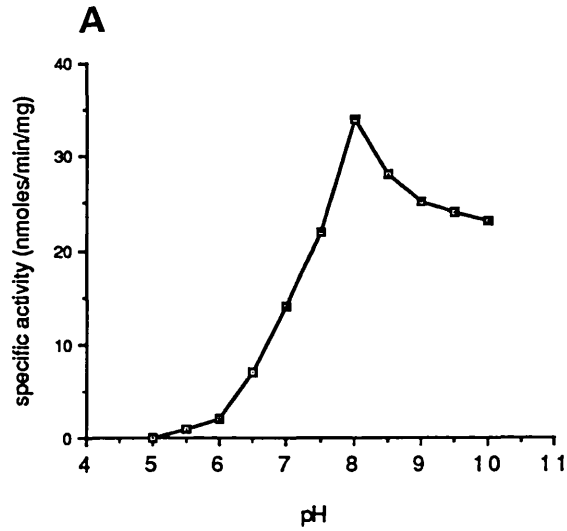
In common with the *T.cruzi* enzyme, the Bz-arg-pNan hydrolysing activity of extracts from *L.donovani* and *C.fasciculata* were optimal at about pH 8 (Figure 31).

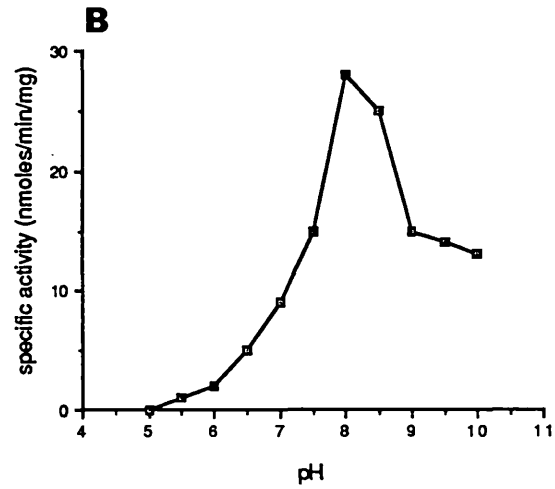
Inhibitor profiles for Bz-arg-pNan digestion by *L.donovani* and *C.fasciculata*

The Bz-arg-pNan hydrolysing activity of extracts from both these species was strongly inhibited by 55uM leupeptin and 3uM TLCK but was only partially inhibited by 0.2mM E64 (Figure 32). This is also apparent from the IC_{50} values which are low for leupeptin and TLCK but high for E64 (Table 11). For all three species, low concentrations of mercury (II) and zinc ions and the diazomethylketone, Z-leu-lys-CHN_2, inhibited digestion of Bz-arg-pNan (Table 11). Amastigotes, as well as promastigotes, of *L.donovani* showed partial inhibition of the Bz-arg-pNan hydrolysing activity by 0.2mM E64 and nearly complete inhibition by 55uM leupeptin and 3uM TLCK (Figure 32 D).

Although the different species show substantial variation in absolute sensitivity of the Bz-arg-pNan hydrolysing activities to some of the inhibitors, the relative values are similar and indicate a homologous

Figure 31. pH profiles of Bz-arg-pNan hydrolysis by extracts of *T.cruzi* (A), *L.donovani* (B) and *C.fasciculata* (C).


A
specific activity (nmoles/min/mg)
40
30
20
10
0
4
5
6
7
8
9
10
11
pH


B
specific activity (nmoles/min/mg)
30
20
10
0
4
5
6
7
8
9
10
11
pH

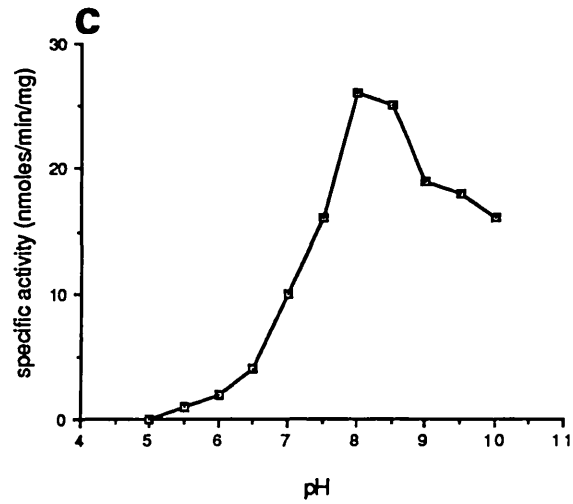
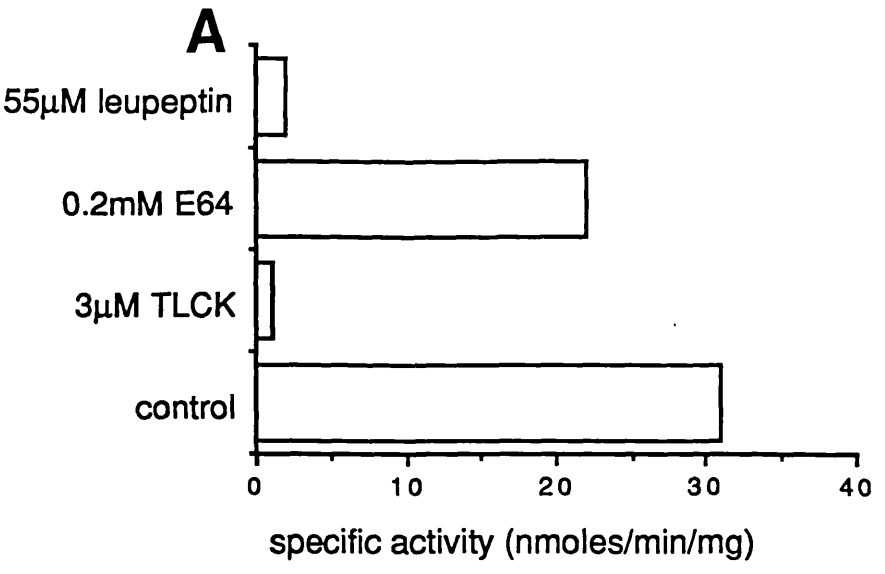
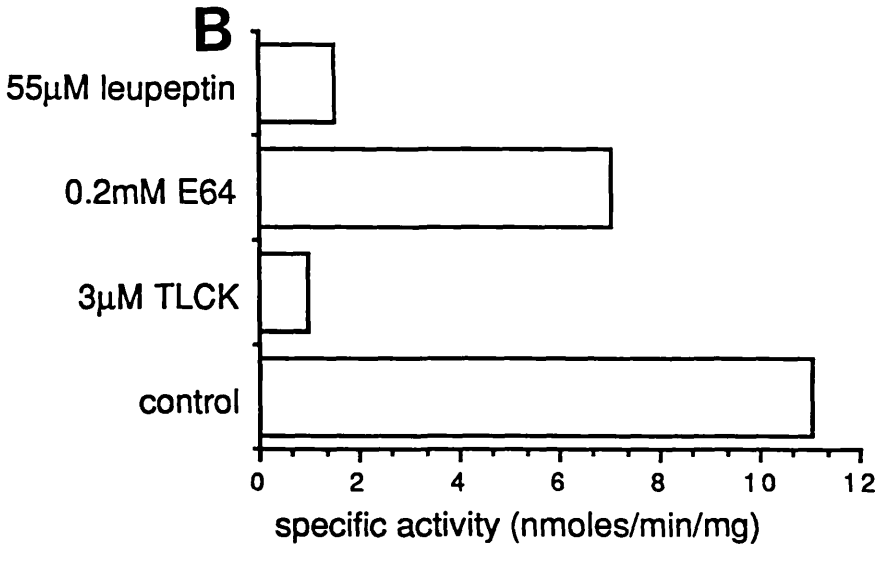
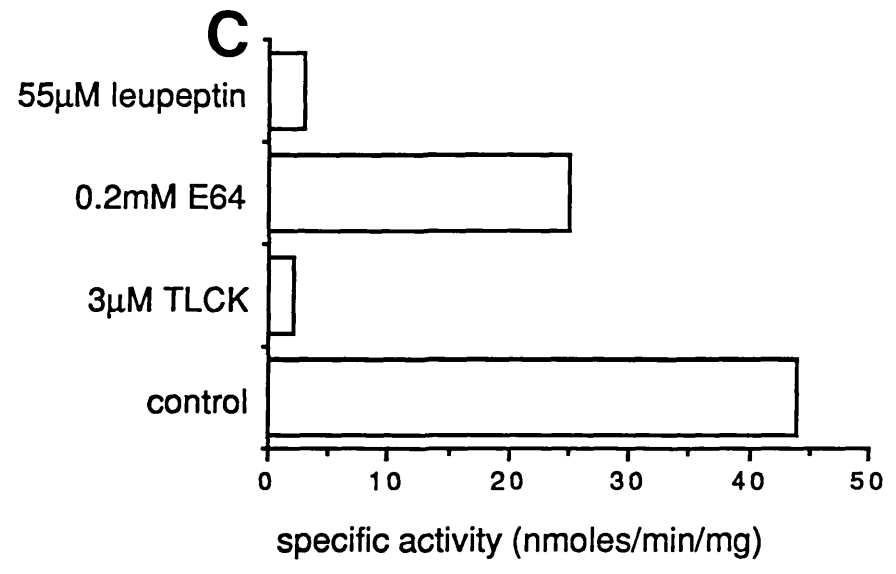
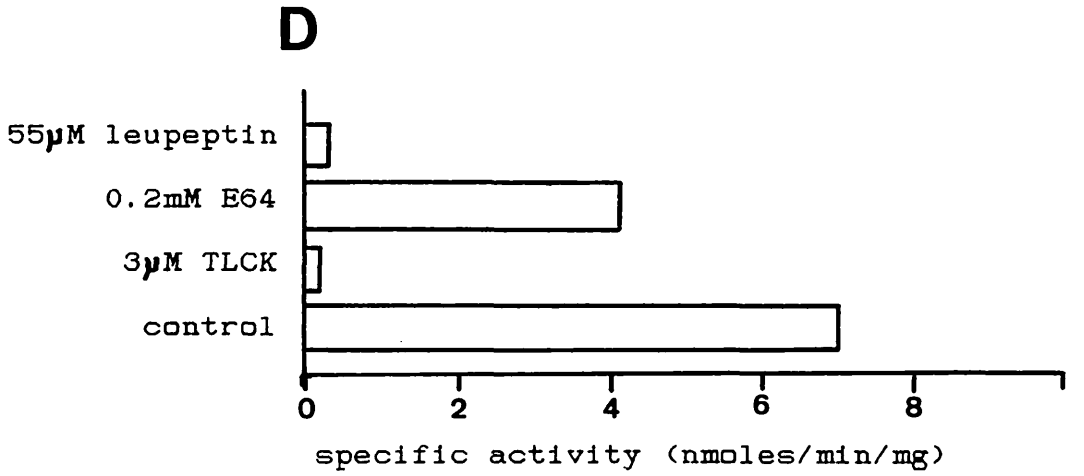

C
specific activity (nmoles/min/mg)
30
20
10
0
4
5
6
7
8
9
10
11
pH

Figure 32. Inhibitor profiles of the Bz-arg-pNan hydrolysing activities of extracts of *T.cruzi* (A), *L.donovani* promastigotes (B), *C.fasciculata* (C) and *L.donovani* amastigotes (D).


A
55μM leupeptin
0.2mM E64
3μM TLCK
control
0
10
20
30
40
specific activity (nmoles/min/mg)


B
55μM leupeptin
0.2mM E64
3μM TLCK
control
0
2
4
6
8
10
12
specific activity (nmoles/min/mg)


C
55μM leupeptin
0.2mM E64
3μM TLCK
control
0
10
20
30
40
50
specific activity (nmoles/min/mg)


D
55μM leupeptin
0.2mM E64
3μM TLCK
control
0
2
4
6
8
specific activity (nmoles/min/mg)

	IC_{50} values		
Inhibitor	*T.cruzi*	*L.donovani*	*C.fasciculata*
TLCK	0.6uM	0.03uM	0.5uM
leupeptin	34uM	3.3uM	32uM
E64	0.2mM	1.3mM	0.85mM
Z-leu-lys-CHN_2	5uM	0.6uM	0.25uM
Hg^{++}	5uM	ND	ND
Zn^{++}	0.7mM	0.5mM	ND

Table 11. IC_{50} values of peptidase inhibitors for Bz-arg-pNan hydrolysis by 1% NP-40 extracts of *T.cruzi*, *L.donovani* and *C.fasciculata*.

activity to the one described for *T.cruzi* (Ashall, 1989).

Digestion of various substrates by *L.donovani* and *C.fasciculata*.

In Table 12 the specific activities for digestion of various substrates, all at 0.25mM, are given for *C.fasciculata* and *L.donovani*. Bz-arg-pNan is digested most rapidly by *C.fasciculata* and Bz-arg-pNan and gly-arg-pNAN are digested most rapidly by *L.donovani*. Both extracts digest Tos-gly-pro-arg-pNan two to three times more rapidly than Tos-gly-pro-lys-pNan. The substrate, arg-pNan, which differs from Bz-arg-pNan only by the lack of the benzoyl amino blocking group, is resistant to digestion by extracts from both species. The small amount of activity seen against arg-pNan is probably due to the aminopeptidase activity described in Chapter 4. The profile of substrate preferences for the two extracts is similar to the profile seen for *T.cruzi* extracts (Ashall, 1989). In general, the longer the peptide part of the substrate the lower is the specific activity.

Peptidyl p-nitroanilide substrates were digested by promastigotes and amastigotes of *L.donovani* with similar specific activities (Table 13) suggesting that the same soluble alkaline peptidase is present in both stages of *L.donovani*. Amastigotes, in commmon with promastigotes digested gly-arg-pNan more rapidly than Bz-arg-pNan. This is in contrast to extracts of *T.cruzi* and *C.fasciculata*,

Substrate	*T.cruzi*	*L.donovani*	*C.fasciculata*
Bz-arg-pNan	32.3	11.1	37.4
Bz-pro-phe-arg-pNan	12.8	3.7	18.1
Boc-leu-gly-arg-pNan	12.7	8.5	28.1
gly-arg-pNan	26.1	11.4	25.2
Boc-Bz-ser-gly-arg-pNan	18.6	5.8	23.7
Tos-gly-pro-lys-pNan	3.2	2.4	9.3
Boc-val-leu-gly-arg-pNan	11.0	4.7	18.9
Tos-gly-pro-arg-pNan	11.1	5.0	18.9
arg-pNan	1.5	0.9	0.7
lys-pNan	0.3	0.4	0.6
Bz-gly-pro-citrulline-pNan	0.0	0.0	0.0
Bz-tyr-pNan	0.2	0.0	0.0
Ac-phe-pNan	0.0	0.0	0.0
Suc-ala-ala-pro-phe-pNan	0.0	0.0	0.0
Z-gly-gly-leu-pNan	1.5	0.0	0.0
Bz-val-gly-glu-pNan	0.0	0.0	0.0
Boc-ala-ala-pNan	0.0	0.0	0.0
ala-ala-pNan	0.5	0.1	0.4

Table 12. Specific activities of 1% NP-40 extracts of *T.cruzi*, *L.donovani* and *C.fasciculata* for various substrates at pH 8. The substrates were all present at 0.25mM.

Substrate	specific activity promastigotes	amastigotes
Bz-arg-pNan	11.1	8.3
arg-pNan	0.9	0.4
Boc-leu-gly-arg-pNan	8.5	5.3
Boc-bz-ser-gly-arg-pNan	5.8	4.3
Bz-pro-phe-arg-pNan	3.7	4.0
Tos-gly-pro-arg-pNan	5.0	5.4
Tos-gly-pro-lys-pNan	2.4	1.8
Bz-gly-pro-citrulline-pNan	0.0	0.0
gly-arg-pNan	11.4	9.9

Table 13. Specific activities for the hydrolysis of some substrates by extracts of promastigotes and amastigotes of *L.donovani* at pH 8. The substrates were present at 0.25mM.

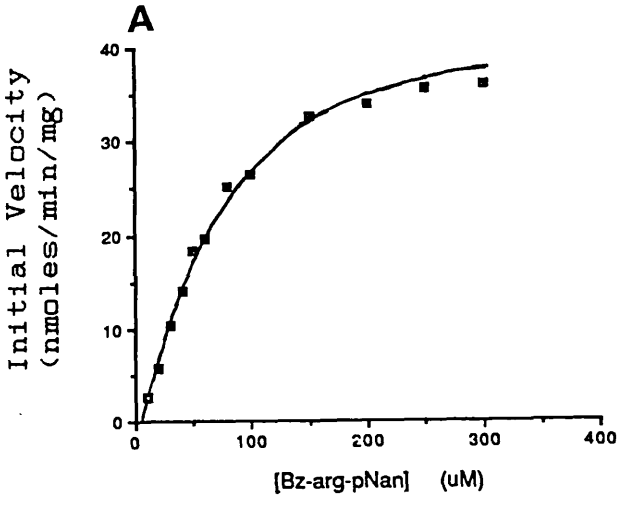
although in these organisms gly-arg-pNan was digested faster than any of the substrates tested except Bz-arg-pNan.

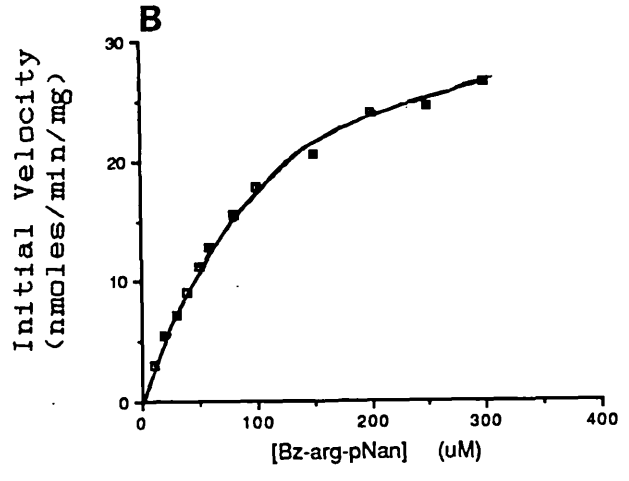
Enzyme kinetics of the major alkaline peptidase from *L.donovani* and *C.fasciculata*.

The rates of digestion of four peptidyl p-nitroanilide substrates at a range of concentrations were determined for extracts of *L.donovani* promastigotes and *C.fasciculata*. Initial velocities versus substrate concentration ([S]) for Bz-arg-pNan are plotted in Figure 33. Plots of [S]/initial velocity versus [S] were linear (Figure 34) suggesting that both of the enzymes showed Michaelis-Menten kinetics. The Km values of the enzyme from both species for different substrates do not differ substantially (Table 14) and it can be shown that the differences in Vmax account for differences in the rate of hydrolysis of these substrates shown in Table 12.

Plots of initial velocity versus substrate concentration for different peptidyl N-methylcoumarin substrates for extracts of *C.fasciculata* show that at substrate concentrations below about 5uM the rate of hydrolysis of Z-arg-arg-Nmec is greater than that of Bz-arg-Nmec (Figure 35). This is reflected in the lower Km for Z-arg-arg-Nmec (about 1uM) than Bz-arg-Nmec (about 15uM) (Figure 36 and Table 15). The low Km for Z-arg-arg-Nmec suggests that the physiological substrate of the enzyme may be cleaved on the C terminal side of two arginine residues.

Figure 33. Bz-arg-pNan concentration versus initial velocity for 1% NP-40 extracts of *T.cruzi* (A), *L.donovani* (B) and *C.fasciculata* (C).


A
Initial Velocity
(nmoles/min/mg)
40
30
20
10
0
0
100
200
300
400
[Bz-arg-pNan] (uM)


B
Initial Velocity
(nmoles/min/mg)
30
20
10
0
0
100
200
300
400
[Bz-arg-pNan] (uM)

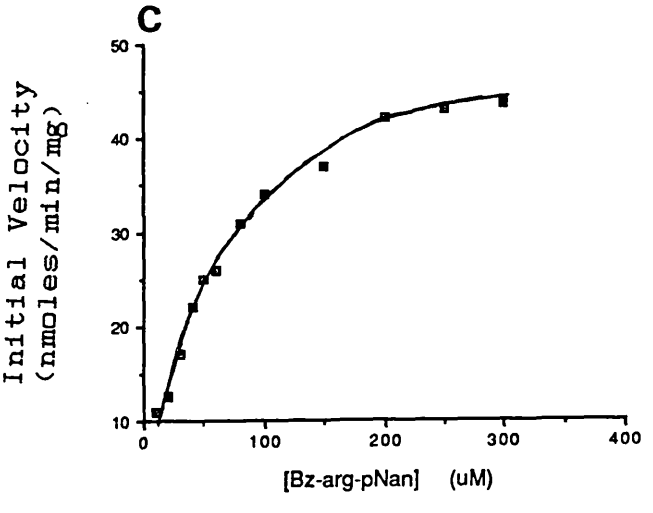
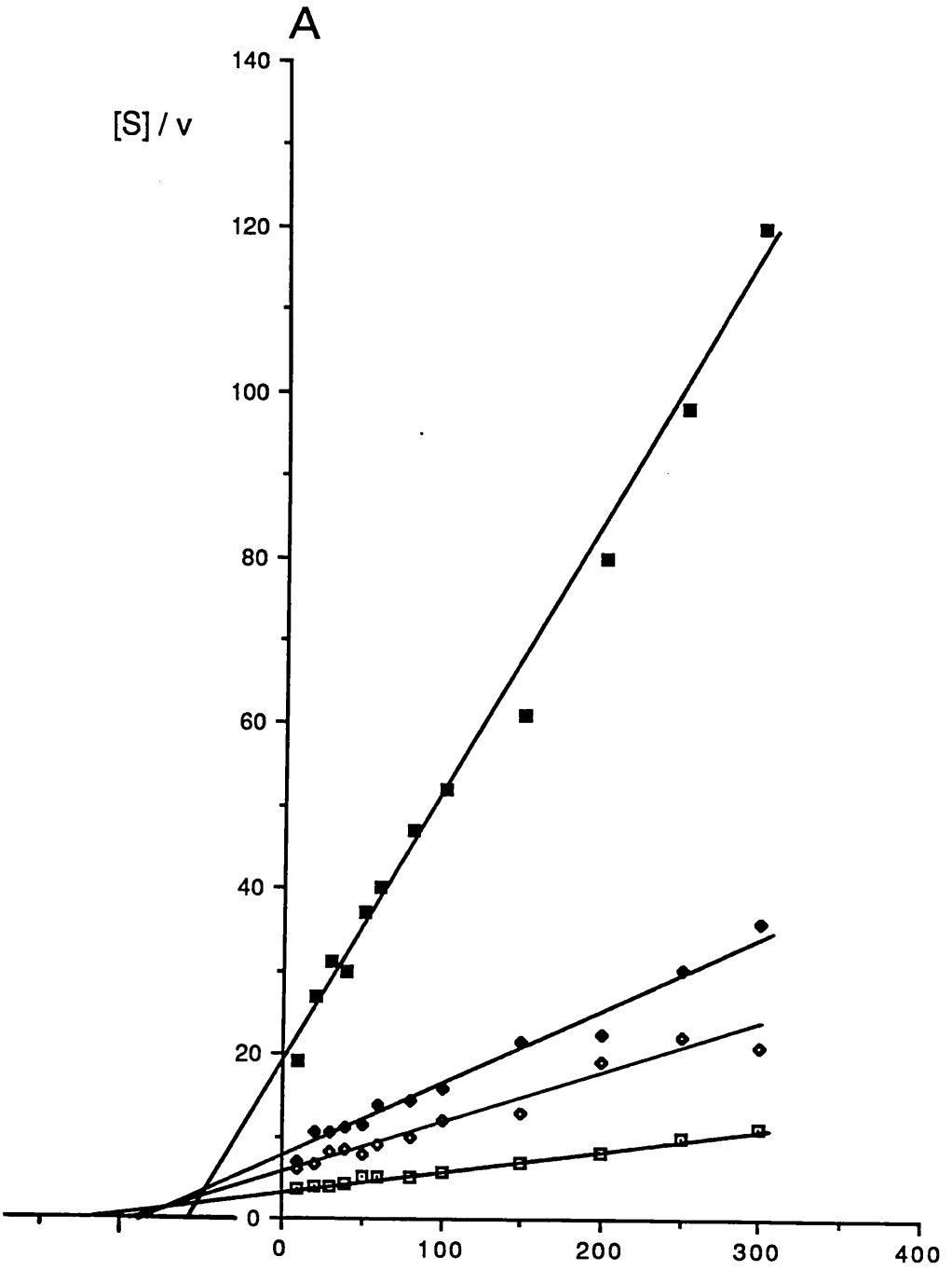
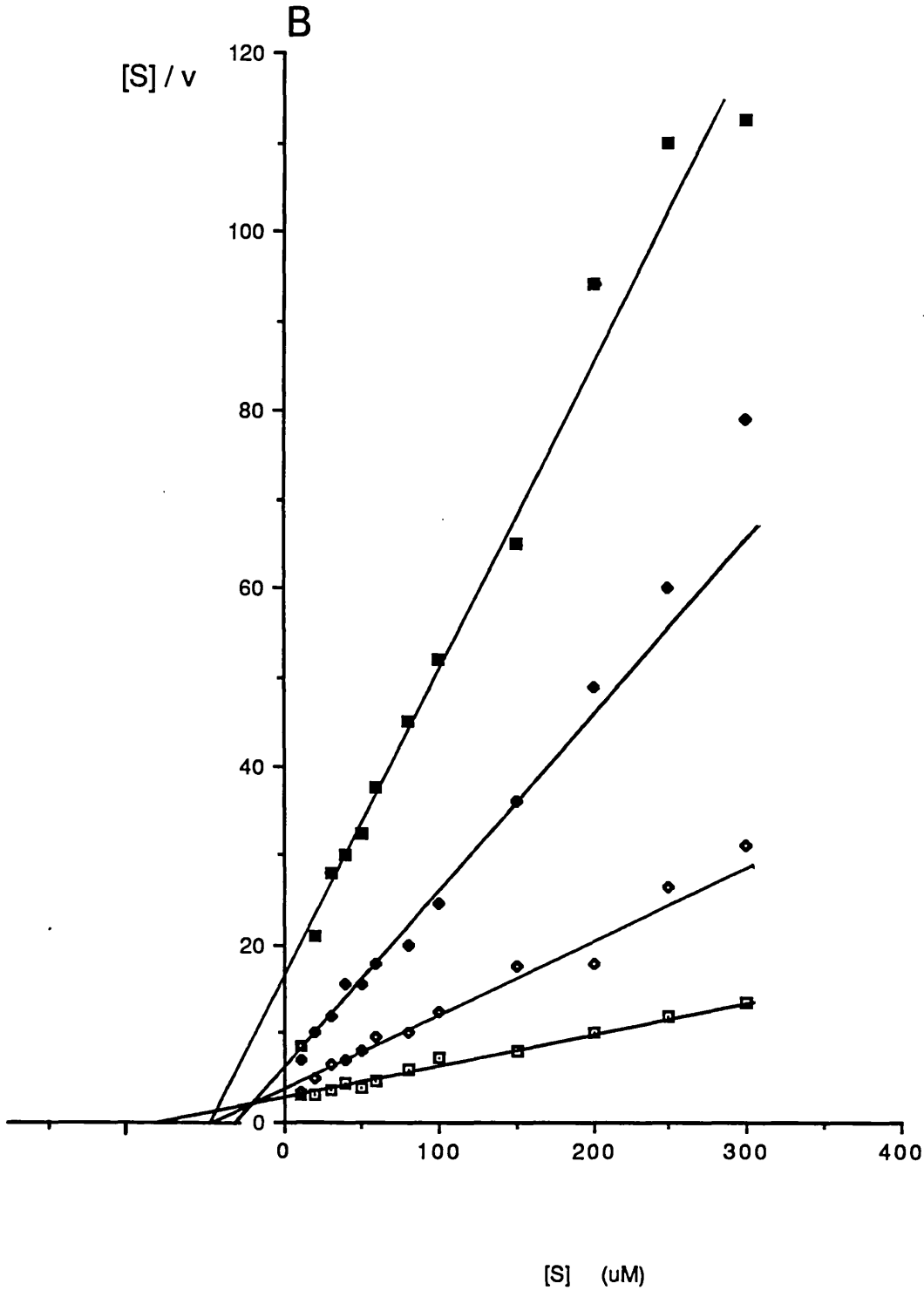

C
Initial Velocity
(nmoles/min/mg)
50
40
30
20
10
0
100
200
300
400
[Bz-arg-pNan] (uM)

Figure 34. Linear kinetic plots for digestion of four p-nitroanilide substrates by 1% NP-40 extracts of *L.donovani* (A) and *C.fasciculata* (B). Bz-arg-pNan (⧈); Tos-gly-pro-arg-pNan (◆); Tos-gly-pro-lys-pNan (■); Boc-val-gly-arg-pNan (◇).


A
[S] / v
140
120
100
80
60
40
20
0
0
100
200
300
400
[S] (uM)


B
[S] / v
120
100
80
60
40
20
0
0
100
200
300
400
[S] (uM)

	Km values (uM)		
Substrate	*T.cruzi*	*L.donovani*	*C.fasciculata*
Bz-arg-pNan	29	110	75
Tos-gly-pro-arg-pNan	30	95	26
Tos-gly-pro-lys-pNan	24	60	45
Boc-val-gly-arg-pNan	25	90	60

Table 14. Km values for four substrates from three trypanosomatids. The data for *T.cruzi* is from Ashall (1989). The values for *L.donovani* and *C.fasciculata* are from the kinetic plots in Figure 34.

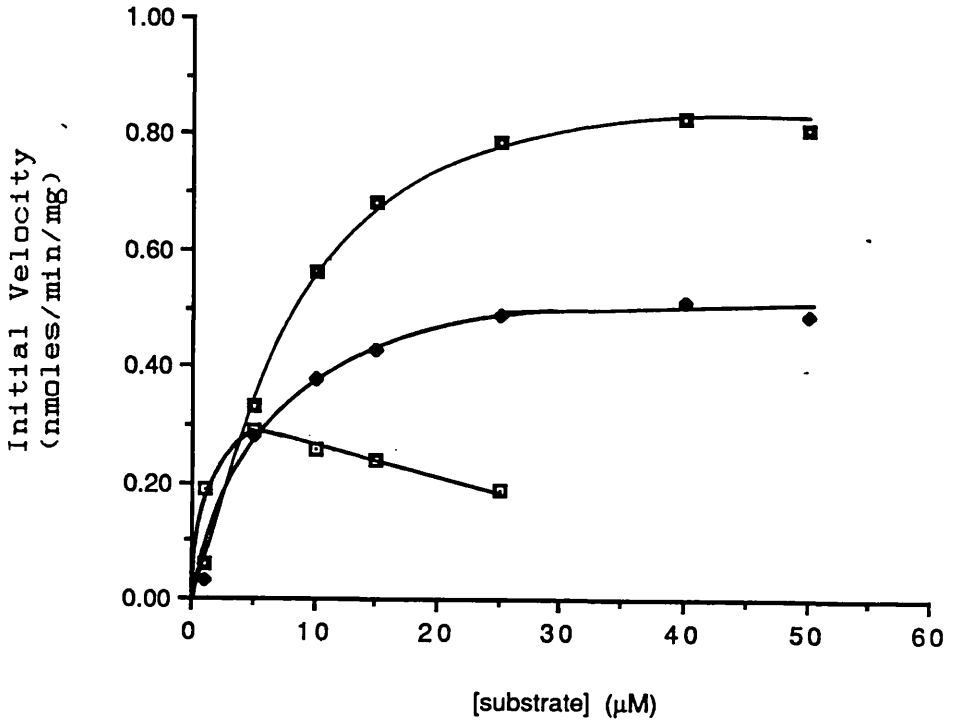
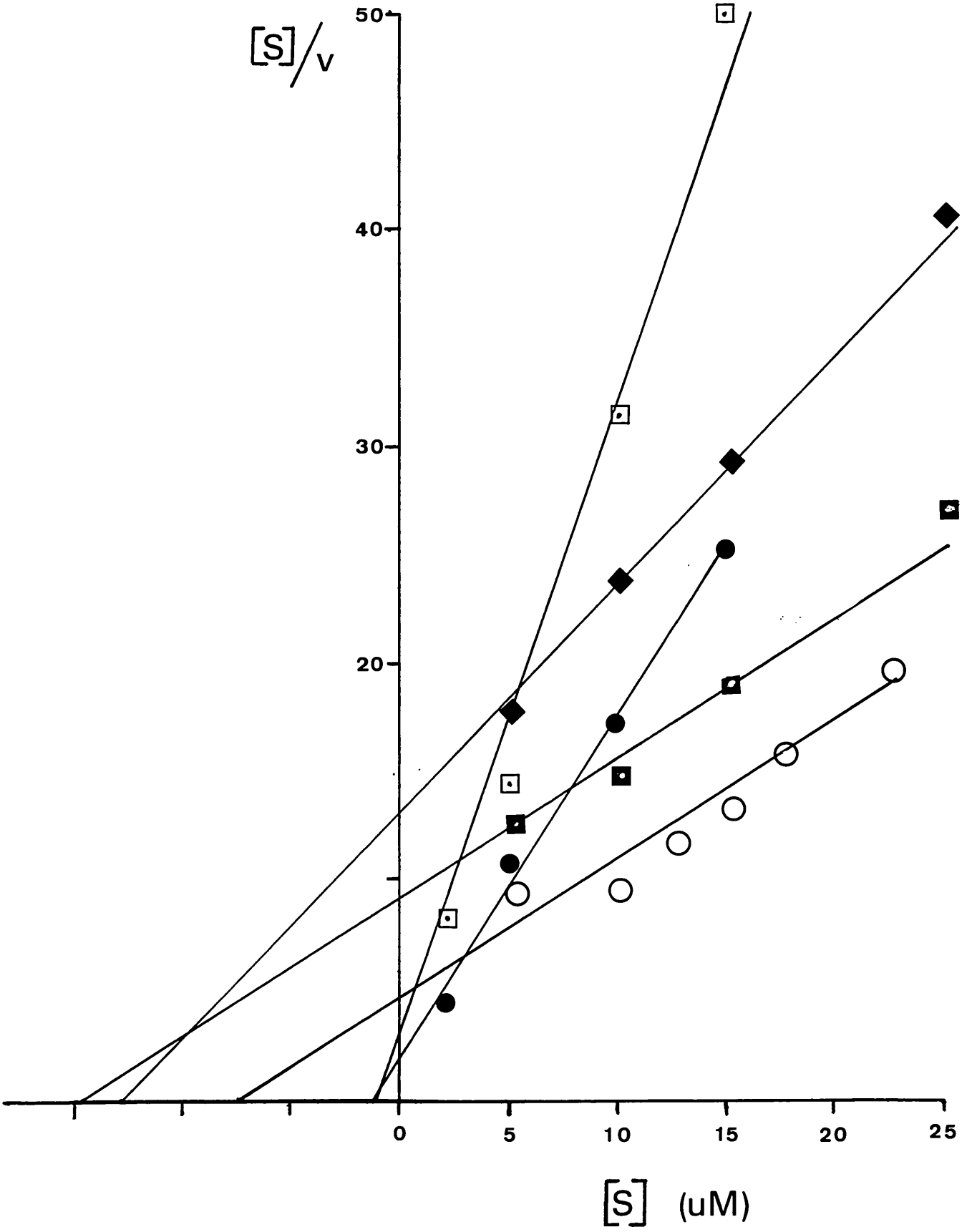


Figure 35. Substrate concentration versus initial velocity for 1% NP-40 extracts of *C.fasciculata*. The substrates used were Bz-arg-Nmec (■), Z-arg-arg-Nmec (⧈) and Z-phe-arg-Nmec (◆).

Figure 35. Linear kinetic plots for digestion of Bz-arg-Nmec (■), Z-arg-arg-Nmec (⊡), Z-phe-arg-Nmec (◆), leu-lys-arg-Nmec (●) and leu-gly-arg-Nmec (O), by *C. fasciculata* extracts.


[S]/v
50
40
30
20
0
5
10
15
20
25
[S] (uM)

Substrate	Km values (uM)
Bz-arg-pNan	75
Tos-gly-pro-arg-pNan	26
Tos-gly-pro-lys-pNan	45
Boc-val-gly-arg-pNan	60
Bz-arg-Nmec	15
Bz-arg-arg-Nmec	1
Bz-phe-arg-Nmec	13
Z-leu-lys-arg-Nmec	1
Z-leu-gly-arg-Nmec	8

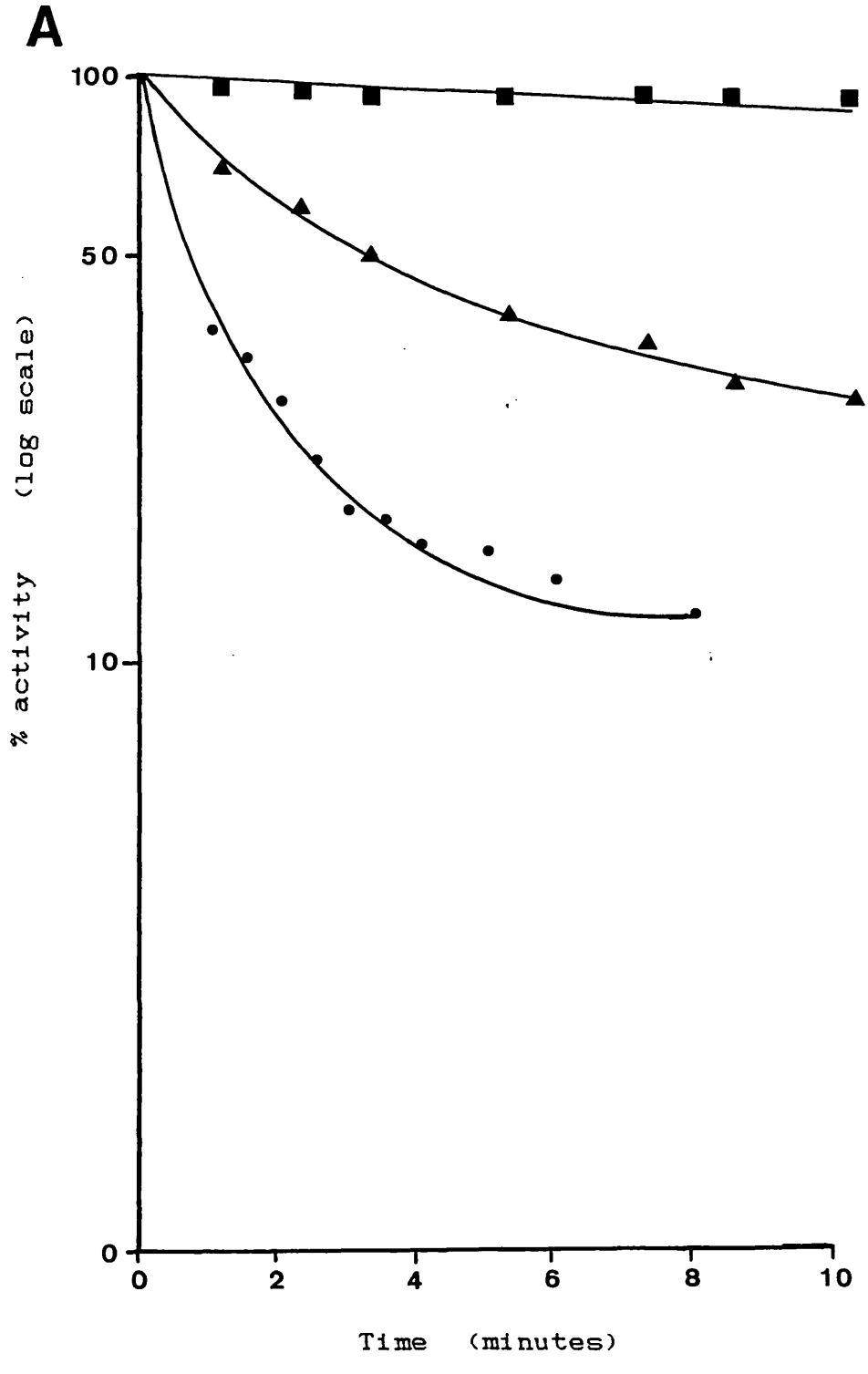
Table 15. Km values for nine substrates digested by 1% NP-40 extracts of *C. fasciculata*. The values for the p-nitroanilide substrates are from Figure 34. The values for the amidomethylcoumarin substrates are derived from the linear kinetic plots in Figure 36.

Peptidyl diazomethane inhibitors of the *C.fasciculata* alkaline peptidase.

The time courses of peptidase inactivation by three peptidyl diazomethanes were similar for extracts of both promastigotes and amastigotes of *C.fasciculata* (Figure 37). The two substrate analogue inhibitors with lysine in the P_1 position inhibited the enzyme strongly whereas the compound with methionine in this position gave no inhibition. This result is consistent with the activity in both promastigotes and amastigotes being homologous to the soluble alkaline peptidase described in *T.cruzi* (Ashall, 1989), and is consistent with the substrate-specificity of the enzyme for the substrate's P_1 position, namely that it prefers a basic amino acid at P_1.

Bz-arg-pNan hydrolysis by electrophoresed parasite extracts.

Detergent extracts of some trypanosomatids were subjected to SDS-PAGE and overlayed with a Bz-arg-pNan-containing agarose gel. This revealed a Bz-arg-pNan activity with an apparent molecular weight of about 200 kDa in all the trypanosomatids examined except *L.donovani* for which no activity could be detected by this method (Figure 38 A). In a separate experiment lanes from such SDS-PAGE gels were cut into transverse strips which were then incubated in cuvettes with Bz-arg-pNan. All the trypanosomatids tested, again except *L.donovani*, showed Bz-arg-pNan hydrolysing activity


A
100
50
10
0
% activity (log scale)
0
2
4
6
8
10
Time (minutes)

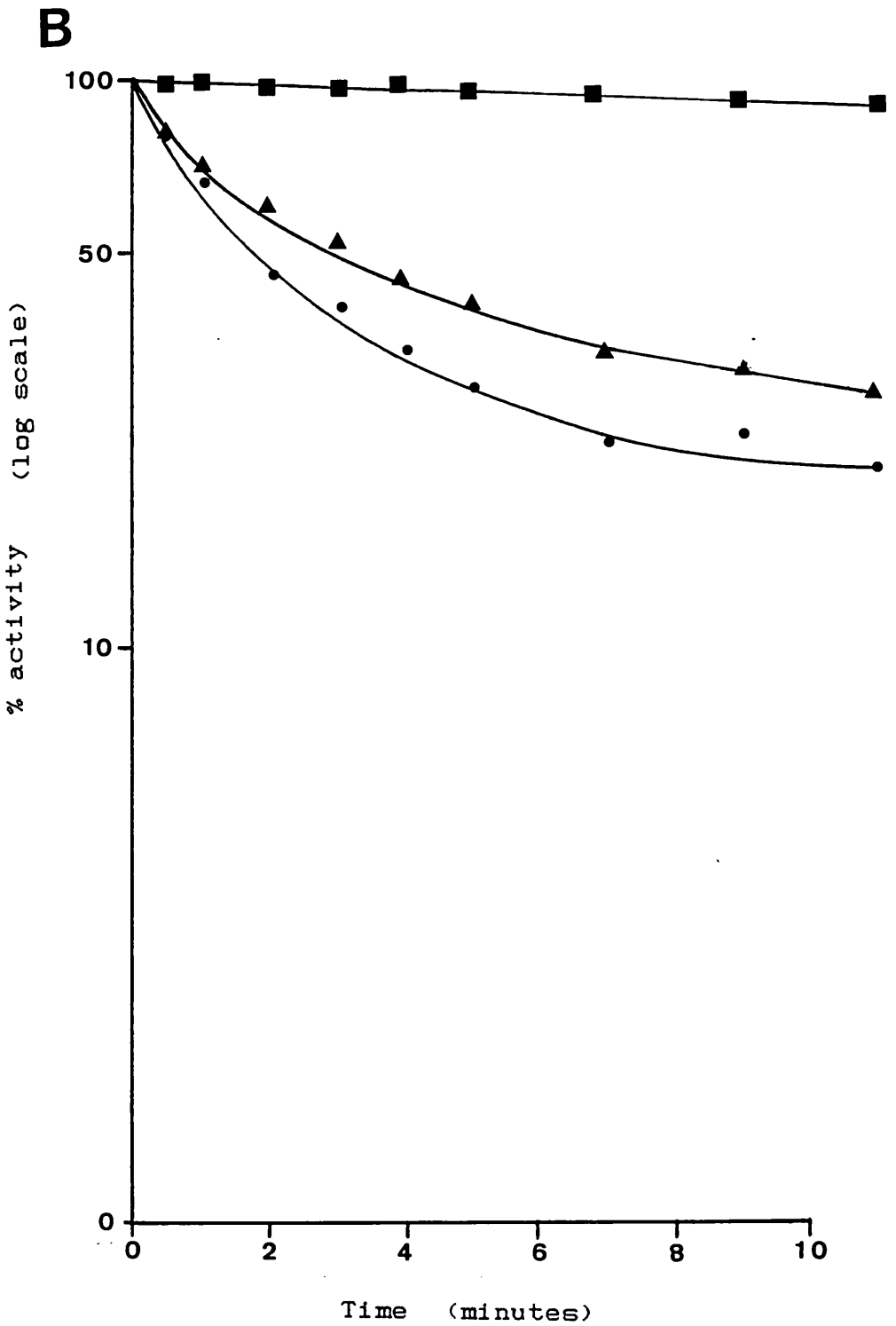
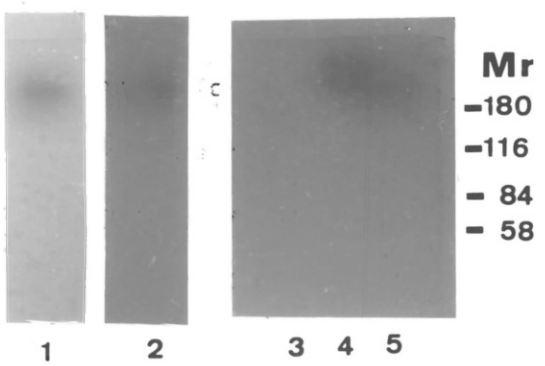

B
100
50
10
0
% activity (log scale)
0
2
4
6
8
10
Time (minutes)

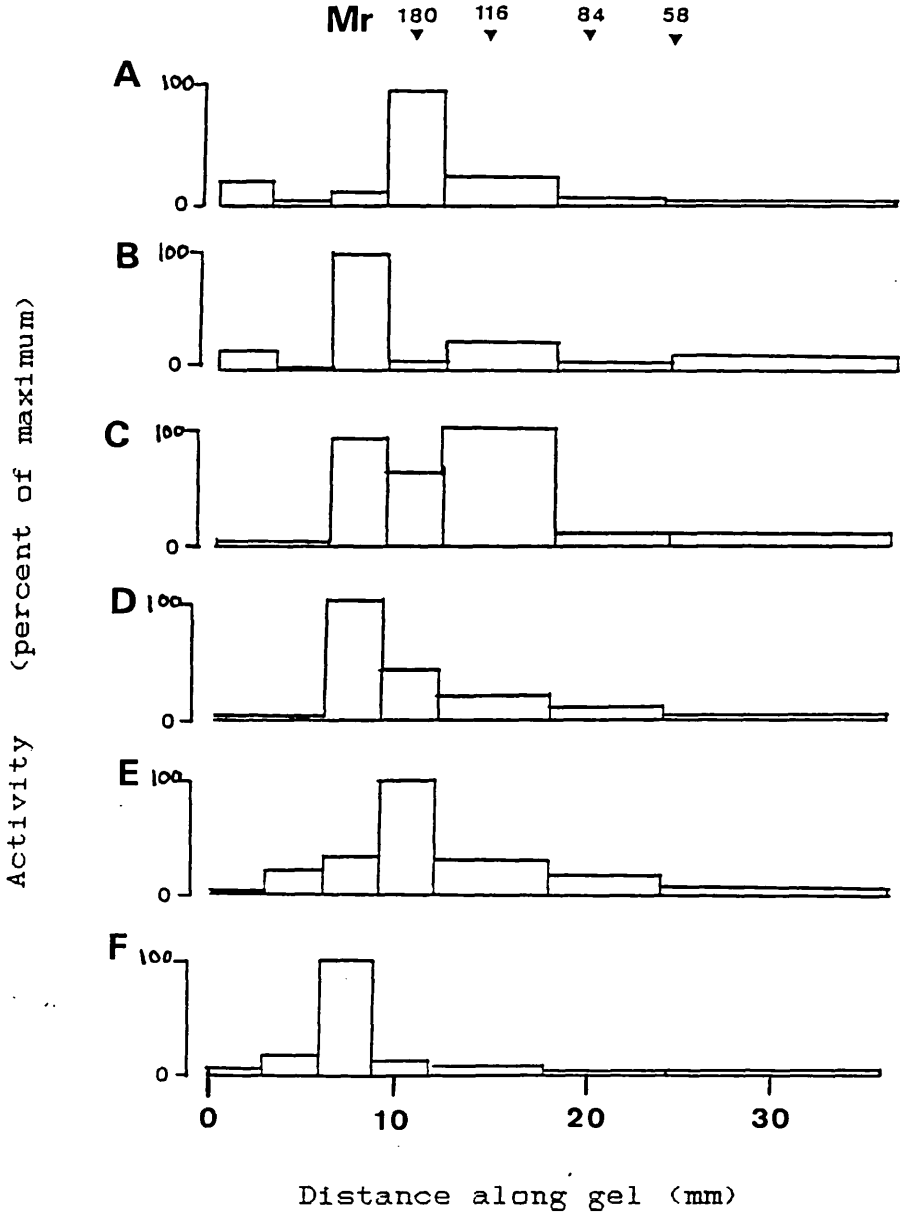
Figure 38. Bz-arg-pNan hydrolysing activity in gels after SDS-PAGE. A, activity detected *in situ* by layering the SDS-PAGE gel onto a substrate containing agarose gel. A yellow band of apparent approximate Mr 200 kDa became apparent after digestion (arrow). Lane 1, *T.cruzi (Y strain); Lane 2, C.fasciculata*; lane 3, *L.donovani*; lane 4, *L.major*; lane 5, *L.arabica*.

B, Activity detected in SDS-PAGE gels after slicing the gel longitudinally into strips and incubating these with substrate in cuvettes. Values are given relative to the maximum value for each species. A, *T.cruzi*; B, *L.hertigi*; C, *L.major*; D, *L.arabica*; E, *T.dionysii*; F, *C.fasciculata*.

A



B


Mr
180
116
84
58
A
B
C
D
E
F
100
0
Activity (percent of maximum)
0
10
20
30
Distance along gel (mm)

in the gel slice corresponding to an apparent molecular weight range of 200 to 270 kDa (Figure 38 B). *L.major* showed a doublet using the overlay method and gave rise to a second gel slice with Bz-Arg-pNan hydrolysing activity corresponding an apparent molecular weight range of 92 to 150 kDa (Figure 38 B).

Chapter Six

Discussion

Peptidase detection using substrate-SDS-PAGE.

Substrate-SDS-PAGE is a useful method for peptidase detection since it separates activities and also gives an indication of their molecular mass. However, there are a number of reports of variability in banding patterns between apparently identical experiments (for example, Pino-Heiss and Schubiger, 1989) and to some extent this problem was encountered in this study. It is probable that small variations in technique can have large effects on the activity or mobility of some peptidases. Some possible sources of variability are described below.

1. The dimensions of the gel system were shown to affect the number of bands produced by parasite extracts. Mini gels (LKB) were very much more sensitive than large gels (LKB). This could relate to the ratio of enzyme to substrate present; the rate of product removal from the gel matrix; the length of time required for electrophoresis or a combination of these and other factors.

2. The extent of heavy metal ion contamination of the solutions used for substrate gel electrophoresis may

be variable and this could explain some of the variation in the intensity of the peptidase activities detected by the technique. Circumstantial evidence for the presence of metal ion inhibitors in the digestion buffer used in this study came from the activation of the cysteine peptidase activities by the metal ion chelators, EDTA and o-phenanthroline. Heavy metal ions are known to inhibit many cysteine peptidases.

3. It is possible that changing the quantity of enzyme loaded may, under some conditions, give rise to qualitative variations in the banding pattern. For example an activity may be normally undetected at a particular pH that is non-optimal for the enzyme but if a larger amount of enzyme is used a band will be detected at this pH that may be as intense as the band seen at the optimal pH. This is because although the reaction rate may be slower at the non-optimal pH it may have time to reach completion, that is digest all the protein in the gel matrix where it is located.

The intensity of the band produced by the membrane-associated putative metallopeptidase was only reduced a small amount when 50-fold less extract was loaded. This, combined with the possibility that there is a threshold level of protein digestion at which a band becomes detectable, could lead to qualitative differences in banding patterns from quantitative variation in amounts of peptidase activity present.

4. If the peptidase is unstable then some activity may be lost during sample preparation and processing. It may be that variations in these procedures, for example the number of cells extracted in a given volume of detergent may influence the degree of peptidase inactivation and also peptidase breakdown into active fragments with lower molecular masses.

5. Latent peptidases may be activated to different degrees by small variations in sample preparation and processing. Such activation could be amplified if further activation is achieved through autocatalysis as has been shown for many peptidases.

6. Peptidase levels may vary with the growth phase or degree of parasite confluency and with different batches of serum used in the culture medium and this would of course be reflected in the intensity of bands detected by substrate-SDS-PAGE.

Of the explanations proposed above only 1 and 3 are related specifically to the substrate-SDS-PAGE technique. The comparative reproducibility of peptidase levels detected by the chromogenic and fluorogenic assays suggests that the other explanations may be less important. However they may contribute to variation which is amplified by the mechanisms outlined in explanations 1 and 3.

The strong activation of peptidase activities observed in some cases when 0.02% (w/v) SDS was included

during digestion was not understood but illustrates the potential for optimising activity through changes in technique. The amount of SDS present in the digestion buffer could vary due to the extent of removal of the electrophoresis running buffer [in which SDS is present at 0.1% (w/v)] when the gel is washed with 2% (v/v) Triton X-100. This could then cause variation in peptidase activities between experiments. By including 0.02% (w/v) in the digestion buffer this source of variablility is hopefully eliminated.

Other precautions taken to avoid variability included the following steps. The same number of cells were extracted in the same volume and percentage of detergent for different experiments. The same amount of material was loaded on to the gel each time and the dimensions of the gel were kept constant. Electrophoresis was carried out for the same length of time and the gel strips were incubated for the same length of time in incubation buffer before staining. For some experiments, however, the length of time for electrophoresis was increased so as to obtain greater resolution of bands.

The relationship between peptidase activities and enzymes.

The cathepsin L-like enzyme. It is difficult to unambiguously relate the activities detected by substrate-SDS-PAGE in this study to peptidase activities

detected in *T. cruzi* described elsewhere. However, the activities, designated as gel bands d and e in Figure 7, of apparent molecular masses 45 and 50 kDa share many properties with the enzyme described by Cazzulo et al. (1989) which has sequence homology to cathepsin L. Both enzymes are cysteine peptidases with acidic pH optima. In addition band e was the most intense of all the bands observed indicating that a highly active enzyme such as the cathepsin L homologue might be responsible for it.

When the purified cathepsin L-like enzyme from *T. cruzi* is run on large gelatin substrate-SDS-PAGE gels it gives rise to a band which comigrates with the only band detected in whole extracts of *T. cruzi* epimastigotes when digestion occurs at pH 5 (J.J. Cazzulo, personal communication). In this study, the only band detected in large gelatin substrate-SDS-PAGE using whole extracts had the same apparent molecular mass of 45 kDa as band e (Figure 7) using the mini gel system. This is close to the value of 50 kDa reported for the cathepsin L-like enzyme and suggests that gel band e may represent this enzyme. However, a less intense activity, band d of Figure 7, also migrates to this region of the gel.

The cysteine peptidase activity detected using azocasein as substrate at pH 5 is almost certainly due to the cathepsin L-like enzyme since azocasein was used as the substrate to follow enzyme activity during purification (Cazzulo et al., 1989). In addition the pH

and inhibitor profiles of the activities are similar. Digestion of Z-phe-arg-Nmec had the same inhibitor profile as the azocaseinase activity using both class specific and substrate analogue inhibitors of cysteine peptidases. This and the known preference of cathepsin L for substrates with phenylalanine in the P_2 position of the substrate suggests both azocasein and Z-phe-arg-Nmec may be digested by the same enzyme in extracts of *T. cruzi* epimastigotes.

A further similarity between the *T. cruzi* activities that hydrolyse Z-phe-arg-Nmec and azocasein and the mammalian cathepsins is the inhibition of the activities by Z-phe-ala-CH_2F but lack of inhibition by Z-ala-phe-CH_2F. These compounds are isomers, the only difference between them being the order of the two amino acid residues. That only Z-phe-ala-CH_2F inhibits digestion of Z-phe-arg-Nmec suggests a strong similarity between the subsite specificities of this enzyme and those of the cathepsins. Z-arg-arg-pNan, a substrate of cathepsin B at low pH (Barrett, 1981), is not cleaved by extracts of *T. cruzi* suggesting that a cathepsin B-like enzyme may not be present in *T. cruzi* epimastigotes: cathepsin L does not readily cleave this substrate.

The sensitivity of the activity against Z-phe-arg-Nmec to inhibition by SDS and urea contrasted with the enhancement of activity towards azocasein produced by the agents. It is possible that SDS and urea

may exert these differential effects through different interactions with the protein and small peptidyl substrates. The inhibitory effects of non-specific agents should certainly be interpreted with greater caution than those of class and substrate analogue inhibitors of peptidases.

Other cysteine peptidases detected by substrate gel electrophoresis. *T.cruzi* epimastigotes gave rise to four bands of activity at low pH on gelatin gels that were sensitive to inhibitors of cysteine peptidases. One of the bands, band b of Figure 7, appeared to have a more restricted range of pH values at which it was active. It was never observed at pH 7, for example, unlike the other activities. Apart from this the properties of all the bands were very similar which suggests that the low molecular mass bands could be break-down products of the high molecular mass form or alternatively the high molecular mass form could be due to an aggregation of the lower molecular mass peptidases. However, there is no direct evidence for this and it is also possible that each band represents a distinct enzyme.

The similarity in banding patterns on gelatin substrate-SDS-PAGE gels derived from NP-40 extracts of different strains of *T.cruzi* suggests that the functions of the peptidases are highly conserved within the species. The differences in the relative intensities of the bands comigrating with the Y strain cysteine

peptidase activities were within the range expected from the variablility of the technique and may well not represent true differences between the strains. However, it was noticed that the aqueous phase of Triton X-114 extracts gave rise to banding patterns that were reproducibly different between strains. In general the overall number and mobility of bands were the same for all the strains but the exact mobilities varied between strains.

The results presented here and examination of the literature on protozoa suggest that protein substrate-SDS-PAGE favours detection of cysteine peptidases. This may be due to this class of peptidase being more prevalent in protozoa. However, cysteine peptidases, in particular lysosomal ones, may be more active and have broader substrate specificities than many serine peptidases. The highly active mammalian lysosomal aspartic peptidase, cathepsin D, has not been detected in protozoa.

A major serine peptidase of *Dictyostelium* does not give rise to a band on substrate-SDS-PAGE, in contrast with many of this organism's cysteine peptidases (M.M.North, personal communication). Also, peptidases with limited substrate specificities, for example processing peptidases, would also fail to show up on these gels.

The membrane-associated putative metallopeptidase. This enzyme, of about 60 kDa, might be homologous to gp63 found in *Leishmania* which has a similar molecular mass and is a metallopeptidase. Gp63 is held in the membrane by a phophoinositol lipid anchor. However, in this study, in contrast to the results obtained with gp63 from *Leishmania*, treatment of Triton X-114 detergent phases of *T.cruzi* extracts with phospolipase C did not release the putative metallopeptidase into the aqueous phase on reextraction. Also, nucleic acid probes derived from gp63 of *Leishmania* did not hybridize to *T.cruzi* DNA at low stringency, although they did hybridize to DNA from *C.fasciculata* (D. Russell, personal communication).

The major component labelled by radioiodination of intact *Leishmania* parasites from a number of species has exactly the same mobility on SDS-PAGE as the gp63 peptidase for that species. In contrast, radioiodination of *T.cruzi* led to an intensely labelled component of 90 kDa and weaker labelling of a 45 kDa component. A band of similar mobility to the 60 kDa membrane-associated putative metallopeptidase was very faint and was not noticed in the reports on radioiodination of *T.cruzi* in the literature (Nogueira et al., 1981; Snary and Hudson, 1979). Thus there is evidence for dissimilarity between the membrane peptidase detected here and gp63 of *Leishmania*. This lack of iodination of the *T.cruzi* enzyme

may relate solely to the lack of an available tyrosine residue or to the enzyme being present in very small amounts. Alternatively, it might not be exposed extracellularly even though it is membrane-associated.

All the strains of *T.cruzi* tested gave rise to a band of gelatin-digesting activity that comigrated with the membrane-associated putative metallopeptidase activity characterized in the Y strain. In all cases tested the activity partitioned into the detergent phase of Triton X-114 extracts and was inhibited by o-phenanthroline. Thus the membrane-associated putative metallopeptidase is clearly conserved between strains of *T.cruzi* originating from a wide geographical area including both South and North America. A series of intense bands detected in the California strain also shared the same properties (apart from electrophoretic mobility) as the Y strain enzyme. It would be interesting to know if these activities are due to a homologous enzyme and what the functional significance of such increased activity could be for this strain.

Rangel et al. (1981) detected a peptidase in *T.cruzi* with a neutral pH optimum for which they provided some evidence of a surface location. However, this enzyme was sensitive to cysteine peptidase inhibitors and so is unlikely to be related to the membrane-associated peptidase detected in this study. Thus, the 60 kDa membrane-associated peptidase described here may be

unrelated to peptidases that have been detected in previous studies of *T.cruzi* and other trypanosomatids.

Although inhibitor profiles in some cases give a clear indication of the catalytic class of a peptidase, it has not unambiguosly been shown in this report that the membrane-associated peptidase is a metallopeptidase. This is partly due to the residual activity that was sometimes observed in the presence of 1mM o-phenanthroline or 5mM EDTA. Definitive evidence for the catalytic class could be obtained by sequencing the enzyme and noting homology to metallopeptidases, for example thermolysin, and by demonstration of the presence of zinc in its active site.

Ala-ala-pNan peptidase. This enzyme has the properties of a dipeptidyl aminopeptidase. A similar enzyme activity has not been reported in *T.cruzi* previously. The enzyme is unlikely to be represented by any of the bands seen in the substrate gel electrophoresis experiments since it was not active on substrates with blocked amino terminal groups such as boc-ala-ala-pNan. The latter substrate was digested by *T.cruzi* extracts but, unlike digestion of ala-ala-pNan, this was not sensitive to inhibition by E64 and IAAH indicating that a different enzyme is responsible.

The dipeptidyl aminopeptidase is specific for the amino acid residues in the P_1 and P_2 position of the substrate. Thus, changing the P_1 amino acid to proline as

in the substrate ala-pro-pNan abolished activity and changing the P_2 amino acid to valine as in the substrate val-ala-pNan also abolished activity.

That the enzyme is a dipeptidase is clear from the lack of p-nitroanilide liberation from the substrate, ala-ala-ala-pNan in the presence of o-phenanthroline. The latter inhibitor had no effect on the rate of digestion of ala-ala-pNan but it prevented digestion of ala-ala-ala-pNan presumably because ala-pNan is generated from the triple alanine substrate by the dipeptidyl aminopeptidase and the metalloaminopeptidase activity can then hydrolyse the ala-pNan (o-phenanthroline would also prevent the dipeptidyl aminopeptidase substrate, ala-ala-pNan, being generated from ala-ala-ala-pNan). Consistent with this, TLCK, which inhibits digestion of ala-ala-pNan, also inhibits ala-ala-ala-pNan digestion.

If the enzyme is a dipeptidyl aminopeptidase then the substrate ala-ala-ala-ala-pNan would be expected to be cleaved. Unfortunately this is not comercially available. *T.cruzi* extracts do not liberate p-nitroanilide from ala-ala-phe-ala-pNan and ala-ala-val-ala-pNan which is consistent with the specificity of the enzyme for alanine in the P_2 position of the substrate described above.

The function of the enzyme is unclear but the specificity for alanine in both the P_1 and P_2 site of the substrate suggests that it is not involved in general

degradation of peptides but may have a role in processing or enzyme activation where specificity of action may be required.

Boc-ala-ala-pNan peptidase. This activity, again, does not seem to have been described previously. It was strongly inhibited by the serine peptidase inhibitor, DFP, but not by any of the other commonly used class-specific inhibitors including PMSF, another serine peptidase inhibitor. The reliability of DFP as a specific inhibitor of serine peptidases is good evidence that the enzyme is, indeed, a serine peptidase. The Zn chelating inhibitors gave weak inhibition. The activity is unlikely to be related to the bands detected by substrate gel electrophoresis because of the lack of similarity in their inhibitor profiles.

The strong inhibition by DFP raises the possibility that this peptidase is one of the components specificaly labelled by [^{3}H]-DFP and detected in autoradiographs of SDS-PAGE gels. However, no further evidence as to the nature of the [^{3}H]-DFP binding proteins was obtained.

Metalloaminopeptidases. The pH profiles of arg-pNan, lys-pNan and leu-pNan were similar. They were effected by the same inhibitors as were ala-pNan and val-pNan. It is not clear, however, if a number of similar enzymes digested the different substrates or if a single enzyme with a broad substrate specificity was

responsible for digestion of all the substrates. Gly-pNan was the only substrate, of those tested, which was not digested.

It is probable that the activity detected here is the same as the ala-napthylamide digesting activity of *T. cruzi* extracts reported by Avila et al. (1979) which was also optimal at pH 7. However, the effects of inhibitors were not reported.

The major soluble alkaline peptidase of trypanosomatids. Bz-arg-pNan is digested very rapidly by extracts of *T. cruzi*. It is, in fact, by far the most readily hydrolysed of all the substrates examined. The inhibitor profile of this enzyme is unusual: a high concentration of DFP inhibits the activity in extracts of both *T. cruzi* and *C. fasciculata*, and a similar activity in *T. brucei* (Lonsdale Eccles et al. 1988). However, the extreme sensitivity of the enzyme from both species to micromolar and even nanomolar concentrations of certain peptidyl diazomethanes is good evidence that it is a cysteine peptidase, since the diazomethanes have been shown to be highly specific for cysteine peptidases (Shaw and Green, 1981). The catalytic class of the enzyme may become clear only when its sequence is known and any homology to, for example, the trypsin superfamily or the papain superfamily has been ascertained.

The kinetic analysis of the activity in the extracts of *L. donovani* and *C. fasciculata* reported here

leaves little doubt that these organisms share a very similar enzyme to that found in *Trypanosoma*. The data on the digestion of Bz-arg-pNan and Tos-gly-pro-arg-pNan and Tos-gly-pro-lys-pNan by a number of other trypanosomatid species, although less extensive than that obtained for *L.donovani* and *C.fasciculata*, suggests that the enzyme is also present in all these species. Thus, in all cases Bz-arg-pNan is cleaved the most readily and Tos-gly-pro-arg is preferred to Tos-gly-pro-lys-pNan.

Overlay of polyacrylamide gels of electrophoresed extracts from a variety of trypanosomatids onto agarose gels containing Bz-arg-pNan gave rise to digestion of this substrate in a single band with an apparent molecular mass of about 200 kDa except for *L.major* which gave two bands of activity in this region and *L.donovani* which did not give rise to a band. One of the cysteine peptidases detected by gelatin-SDS-PAGE of *T.cruzi*-extracts also had a molecular weight of about 200 kDa. However, the latter activity had an acidic pH optimum and was completely inhibited by E64 and IAAH in contrast to the soluble alkaline peptidase. The inability to detect the peptidase by gelatin, casein or haemoglobin substrate gel-SDS-PAGE is consistent with the lack of digestion of intact proteins by this enzyme reported by Ashall (1989).

Specific activities determined at one substrate concentration can be misleading as indicators of the substrate preference of an enzyme. In this study, for

example, extracts of *C.fasciculata* digested Bz-arg-Nmec more rapidly than Z-arg-arg-Nmec when both substates were present at concentrations over 5uM. Below this concentration the Z-arg-arg-Nmec substate was preferred. Since the natural substrate of the enzyme is likely to be present at very low concentrations (ie. below 1uM) the result at the lower concentration may have more biological significance. The probable reason for this concentration-dependence is that the enzyme undergoes substrate inhibition when Z-arg-arg-Nmec is used as a substrate, perhaps because the substrate can bind in an unproductive form with the arginine in the P_2 position of the substrate occupying the S_1 site of the enzyme. A similar kinetic analysis in *T.cruzi* showed that digestion of these substates is similarly influenced by their concentrations (Ashall et al., unpublished observations).

Comparative analysis of the soluble alkaline peptidase may be useful in determining its role in the parasite. For example, the presence of the enzyme in *C.fasciculata* suggests a function for the enzyme that is not related to parasitism of mammals since this parasite is limited to insect hosts. In different species new roles may be acquired by an enzyme but it is likely that the ancestral function may have been retained.

The function of the enzyme is not known at present. However, the lack of activity on intact proteins and the strong preference for arginine or lysine in the

P_1 and P_2 positions of the substrate suggest that the natural substrates may be limited to a small number of specific components rather than digestion of a broad range of substrates. By analogy with the substrate specificities of a large number of peptidases involved in specific processing of proteins it is possible that the soluble alkaline petidase has a similar function. However, until more data is available the function of this enzyme can only be speculated upon.

Aspartic peptidases.

Aspartic peptidases were not detected in this study. In these enzymes the nature of the substrate amino acids that bind to the S' sites of the enzyme are often important for productive interactions between the substrate and enzyme to occur (Barrett, 1977). In many synthetic substrates the non-amino acid chromogenic or fluorogenic group is normally in this position. For this reason a range of furylacrylolyl peptide substrates were tested. In these substrates cleavage between two amino acids alters the spectral properties of a chromogenic group attached to the N-terminus of the P_1 amino acid. A nitrophenylalanine-containing peptide in which the spectral properties of the modified amino acid, in the P_1 position of the substrate, change on cleavage of its C terminal peptide bond was also used [leu-ser-phe(NO_2)-leu-ala-leu-OMe). However, no digestion

of these substrates by extracts of *T.cruzi* epimastigotes was detected.

North (1982) in an extensive review cites no examples of aspartic peptidases identified amongst protozoans and it may be that they are not present in these lower eukaryotes. They have also not been found, to date, amongst prokaryotes, suggesting that aspartic peptidases evolved in eukaryotes after divergence of the protozoa.

Peptidase activities of different *T.cruzi* life cycle stages.

The infective, trypomastigote, stage of *T.cruzi* was produced in relatively small quantities from cultures of mammalian cells in contrast to the epimastigote stages which were easily grown in axenic culture. For this reason the activity of trypomastigote extracts for the chromogenic and fluorogenic substrates was not determined.

Gelatin substrate-SDS-PAGE of trypomastigote material gave a similar pattern of cysteine peptidase activities as that given by the epimastigote stage. This lack of developmental regulation between the stages suggests that the peptidases are engaged in a function common to both stages of the parasite. This would be consistent with a lysosomal location for the cysteine peptidases.

In contrast, the membrane-associated 60 kDa peptidase was not detected in NP-40 extracts or Triton X-114 detergent phases of *T.cruzi* trypomastigotes. This implies that the function of this enzyme is required only in the insect stage of the life cycle. However, the results presented here do not exclude a role for the enzyme in the infective metacyclic trypomastigotes produced in the hind gut of the insect vectors. This life cycle form is distinct from the infective trypomastigotes liberated from mammalian cells both in biochemical properties and in their preference for invading macrophages rather than nerve and muscle cells.

In view of the role of *Leishmania* gp63 in macrophage invasion and the possible similarity between the *T.cruzi* membrane-associated enzyme and gp63, it would be of interest to ascertain whether or not the *T.cruzi* enzyme is in metacyclic trypomastigotes. It is also possible that the function of the membrane associated peptidase is to generate amino acids externally from proteins in the gut of the insect which could then be used by the parasite for nutritional purposes. Consistent with this, *T.cruzi* epimastigotes cultured in the absence of amino acids, with the protein, catalase, as the only souce of nitrogen, grew and divided (Avila et al., 1983).

Secreted peptidases from *T.cruzi* were not detected.

Many invasive pathogens have been shown to secrete proteolytic enzymes and in some cases there is evidence that this facilitates the invasion process. It was therefore of interest to determine if *T.cruzi* parasites, particularly the tissue invasive trypomastigotes, secreted peptidases.

Gelatin substrate-SDS-PAGE of medium conditioned by *T.cruzi* epimastigotes and trypomastigotes did not reveal any peptidase activities. Gelatin is composed of denatured extracellular matrix collagen and is therefore a good substrate for detection of peptidases that might be involved in invasion of tissues. Medium conditioned by *T.cruzi*-infected Veros containing the amastigote stage of the parasite also failed to show any peptidase activity.

Again, due to the difficulty of obtaining large amounts of trypomastigote material, chromogenic and fluorogenic substrates were not used to detect putative shed peptidases. The major soluble alkaline peptidase of *T.cruzi* is not shed from either epimastigotes or trypomastigotes (Ashall et al., unpublished observations) and there have been no reports of peptidases secreted by or shed from *T.cruzi*.

Perspective: Parasite peptidases.

Peptidases are an interesting class of enzymes in that, although they all share the same substrate, that is

a peptide bond, there is tremendous diversity in the specificity for the nature of the amino acids surrounding this bond. In many cases specificity has been imposed on these highly conserved enzymes through small changes in the substrate-binding region of the active site.

These contrasting properties allow, on the one hand, knowledge and insight gained from the study of a particular peptidase to be usefully transferred to the study of other peptidases and, on the other hand, highly specific reagents to be developed that are capable of inhibiting a single enzyme. This and the importance of peptidases in the life cycles of parasites suggests that they may well represent a fruitful area of investigation in the search for chemotherapeutic and immunolgical reagents designed to cure and prevent parasitic infections. Knowledge of the biochemistry of parasite peptidases may also lead to a greater understanding of the biology and biochemistry of the parasites themselves opening new avenues to the control of the diseases that they cause.

This study has identified a mumber of peptidase activities in *T. cruzi* and investigated some of their properties. The major activities that were detected are summarized in Table 16.

Substrate digested	Mr(kDa)	Class	Notes
Gelatin (SDS-PAGE)	200	cysteine	soluble,
Gelatin (SDS-PAGE)	120	cysteine	soluble
Gelatin (SDS-PAGE)	60	Metallo-*	membrane-associated, epimastigotes only
Gelatin (SDS-PAGE)	55	cysteine	soluble
Gelatin (SDS-PAGE)	45	cysteine	soluble
Bz-arg-pNan	200	cysteine*	soluble, conserved in trypanosomatids
Ala-ala-pNan	ND	cysteine	soluble, dipeptidyl aminopeptidase
Boc-ala-ala-pNan	ND	serine	soluble
Z-phe-arg-Nmec (pH3)	ND	cysteine	soluble
Azocasein	ND	cysteine	soluble
arg-, leu-, lys-ala- and val-pNan	ND	metallo-	soluble, aminopeptidase

Table 16. Major peptidase activities detected in the study reported here. * indicates a putative assignment; ND = not determined.

Appendix.

Detection of Trypanosomatids Using Total Parasite DNA Probes.

Summary.

A radiolabelled total *Trypanosoma cruzi* DNA probe has been used to detect as few as five *T.cruzi* parasites under conditions where no signal was detected from up to 20,000 *T.brucei*, *T.rangeli*, *C.fasciculata* or *Leishmania* parasites (Ashall and Miles, 1988; Ashall et al, 1988). These authors also showed that the *T.cruzi* total DNA probe could be used to detect parasites present in mammalian blood applied directly to nylon membranes.

The suitability of this technique for *T.cruzi* and *T.rangeli* detection, and discrimination between these two organisms, in *Rhodnius prolixus* was investigated and is presented here together with the results of a small field trial using *Rhodnius prolixus* collected from dwellings in a rural area near Bogota in Colombia. Of forty five insects tested five had *T.cruzi* infections and two had *T.rangeli* infections. There were no mixed infections. Reports describing this work (Greig et al, 1987 and in press) are included in the folder in the inside back cover of this thesis.

A *Leishmania donovani* total parasite DNA probe did not discriminate between different *Leishmania* species and it was concluded that *Leishmania* total DNA probes would not be useful for species-specific detection of parasites from this genus. A brief report of this work (Greig et al, 1989) is included in the folder in the inside back cover of this thesis.

Introduction.

Total DNA probes.

DNA probes are increasingly being used for the detection of infectious parasites in man and in disease vectors (Gibson et al. 1988). In general cloned DNA has been used for the probes, although there are several reasons for thinking that for some applications total DNA probes may be advantageous because of their high sensitivity and ease of preparation.

Highly repetitive DNA sequences have been estimated to constitute about 9% of the nuclear DNA of *T.cruzi* (Lanar et al, 1981; Castro et al, 1981). The kinetoplast also contains large amounts of repetitive DNA in the form of about 5,000 to 20,000 catenated minicircles, each about 1.4 kb in length. The basis for specificity of detection with total DNA probes is firstly, that denatured repetitive sequences re-hybridize

more rapidly than less highly represented sequences and secondly, that repetitive DNA sequences are evolutionarily divergent between different eukaryotic species and are often species-specific (Dover, 1982). The greater amount of target DNA available for hybridization in the sample when total DNA probes, as opposed to cloned DNA probes, are used is a theoretical reason for expecting high sensitivity with total DNA probes.

Detection of Trypanosomes.

Epidemiological and diagnostic work on Chagas' disease is complicated by *T.rangeli* (Guhl et al, 1987) due to its similar morphology, host-range, vector species and serological cross-reactivity. *T.rangeli*, however, is non-pathogenic in man. Examination of the morphology of the forms found in mammalian blood and insects' faeces is used to differentiate between these two species of parasite. The morphological similarity between the two species and the presence of morpholgical heterogeneity within each species makes identification difficult and potentially unreliable.

Sanchez et al (1984) used purified kinetoplast DNA from *T.cruzi* for the specific detection of *T.cruzi* isolates. The authors suggested that the isolate-specificity of detection was due to the presence of isolate-specific DNA sequences in the kinetoplasts.

Gonzalez et al (1984) showed that a repetitive

nuclear DNA probe cloned from *T.cruzi* identified strains of *T.cruzi* from diverse origins in South America. The probe detected DNA from as few as thirty *T.cruzi* parasites. The method employed purification of the target DNA and it is therefore difficult to access how the sensitivity would be affected if viable parasites were used as samples.

The polymerase chain reaction (PCR) has recently been applied to the detection of *T.cruzi* DNA sequences (Sturm et al. 1989). This method potentially has exquisite sensitivity, that is it can detect a single molecule of a DNA sequence in a vast excess of DNA of different sequences. However, the technique requires more work per sample than other DNA detection methods, although sample automation may reduce this problem. There are also considerable problems encountered with false positives due to contamination of samples to be tested with previously amplified DNA. It will be necessary to use sequences for PCR primers whose distribution and sequence conservation amongst *T.cruzi* strains and related species is well known if interpretable results from epidemiological studies are to be obtained.

T.cruzi total DNA detected as few as five *T.cruzi* parasites in a hybridization assay under conditions where no signal was given by up to 10,000 *T.rangeli* or *Leishmania* parasites (Ashall et al, 1988). In the same study *T.cruzi* parasites in mammalian blood were detected

by the total DNA probe suggesting a possible method of diagnosis. Such a method might be used in preference to xenodiagnosis, in which *T.cruzi* vectors are fed on patients, since it would be possible to avoid many of the disadvantages of xenodiagnosis. These are the long time taken for diagnosis (the parasites take up to a month to multiply in the insect vector to levels where they can reliably be detected) and the psychological and physiological unpleasantness of the technique for the patient (many patients show a marked immunological reaction to the insect bites).

In this study total parasite DNA hybridization was applied to *T.cruzi* and *T.rangeli* detection in field samples of *Rhodnius prolixus* from Colombia in South America.

Detection of *Leishmania* species.

Specific detection of *Leishmania* parasites, which are believed to infect about 20 million people (WHO, 1984), is important in view of the pathologies caused by infection with members of this genus and the different therapeutic regimens required depending on the infecting species (Ridley, 1980). Epidemiological studies, including parasite detection in sandfly vectors, might also benefit from a simple, reliable, sensitive and specific reagent to differentiate between species of *Leishmania*. In many areas more than one pathogenic

species of *Leishmania* are found together and both may produce similar symptoms. For example *L.braziliensis braziliensis* (the causative agent of mucocutaneous leishmaniasis) and *L.donovani* (the causative agent of Kala Azar) may both present with a single cutaneous lesion, even though the subsequent pathologies may turn out to be very different. Accurate diagnosis is therefore difficult on the basis of early symptoms at the time when decisions concerning chemotherapy are being made by the clinician.

In this study data is presented which suggests that discrimination between *Leishmania* and other, related species using total DNA probes is not feasible. In contrast to the situation with *Trypanosoma* it therefore appears that cloned, species-specific, DNA sequences will be required for the specific detection of *Leishmania* (Barker, 1987).

Results.

Detection of Trypanosomes using total DNA probes.

R.prolixus collected in a rural area near Bogota in Colombia were tested for the presence of trypanosomatid parasites in their faeces and salivary glands by microscopy and DNA detection. Table 16 shows that the DNA hybridization method detected all the

Table 16. Results of microscopic examination and DNA probing of *R.prolixus* faeces. The number of plusses indicates the number of parasites seen (+ = less than 1 parasite per field; ++ = about 1 or 2 parasites per field and +++ = more than 5 parasites per field) or the intensity of the autoradiographic signal shown in Figure 39.

Sample	Visual	Probe	
		T. cruzi	*T. rangeli*
A1	–	–	–
A3	–	–	–
A5	+	–	+
A7	++	+	–
A9	–	–	–
B1	–	–	–
B3	+	++	–
B5	–	–	–
B7	–	–	–
B9	–	–	–
C1	–	–	–
C3	–	–	–
C5	–	–	–
C7	–	–	–
C9	–	–	–
D1	–	–	–
D3	–	–	–
D5	+++	+++	–
D7	–	–	–
D9	–	–	–
E1	–	–	–
E3	–	–	–
E5	–	–	–
E7	–	–	–
E9	–	–	–
F1	–	–	–
F3	–	–	–
F5	–	–	–
F7	–	–	–
F9	–	–	–
G1	–	–	–
G3	–	–	–
G5	–	–	–
G7	–	–	–
G9	–	–	–
H1	–	–	–
H3	–	–	+
H5	–	–	–
H7	–	–	–
H9	–	–	–
I1	–	–	–
I3	+	+	–
I5	++	+	–
I7	–	–	–
I9	–	–	–

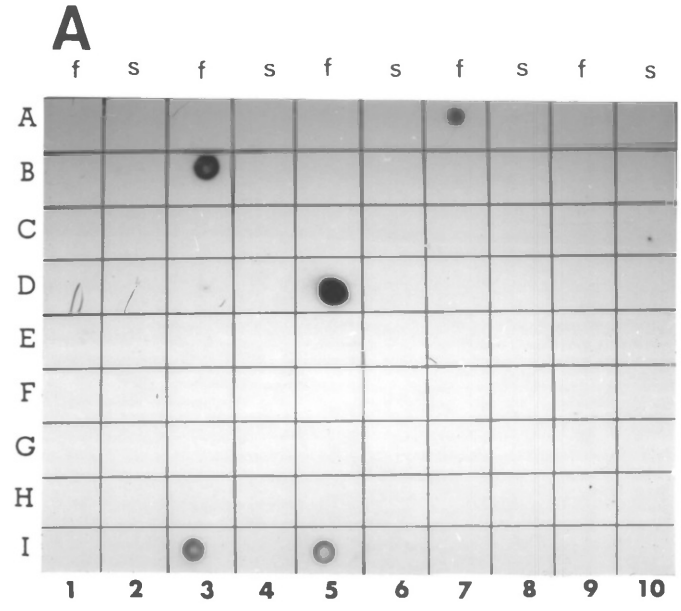
samples in which parasites were seen under the microscope. In one case the DNA hybridization method gave a positive signal for a sample which was negative by microscopy. None of the insects had salivary gland infections according to both methods of detection. The autoradiographs in Figure 39 A and B show that, of the 45 insects tested, five insect faeces were positive for *T.cruzi* and two for *T.rangeli*. There were no mixed infections.

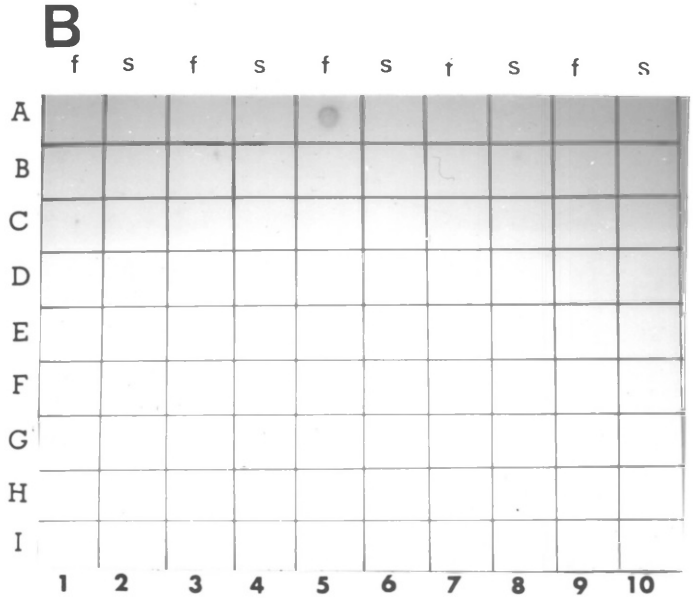
Control membranes with known numbers of tissue culture-derived *T.cruzi* and *T.rangeli* epimastigotes were processed together with the insect sample membranes and these show the sensitivity and specificity achieved in this experiment (Figure 39 C). From these results an approximate estimation can be made of the numbers of parasites in the samples since mixing known numbers of parasites with uninfected *R.prolixus* did not alter the sensitivity of parasite detection.

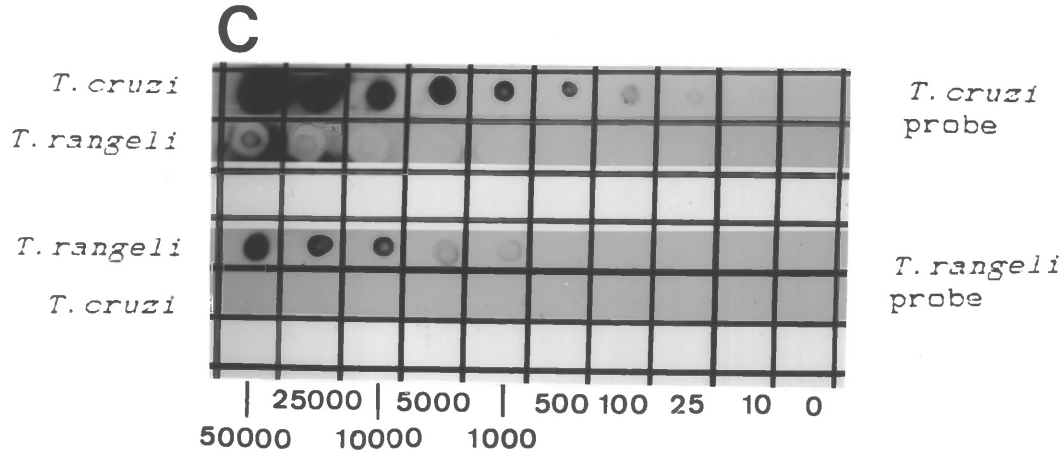
Trypanosome detection at low stringency.

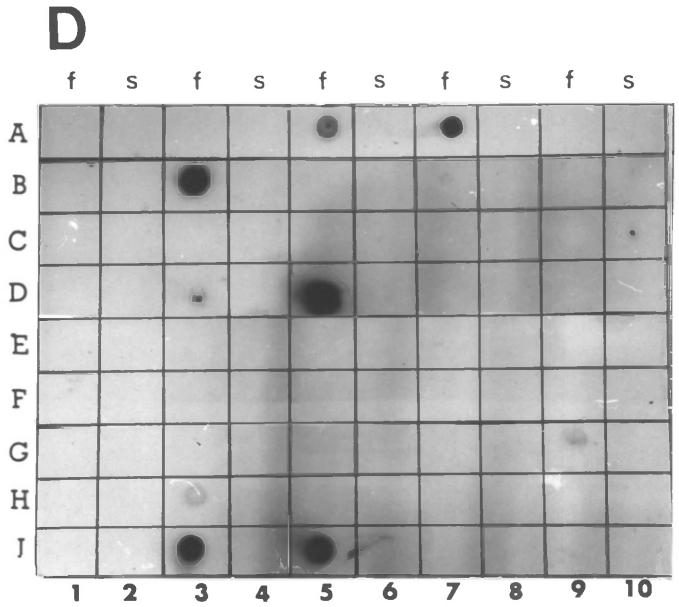
Before washing the membranes at high stringency, a low stringency wash was performed and followed by autoradiography overnight. In this case both probes recognised the same set of seven positive faeces, there being no distinction between the two species' DNA. The results of the *T.cruzi* DNA-probed membrane washed at low stringency are shown in Figure 39 D. Washing at high

Figure 39. Autoradiographs of [^{32}P]-labelled DNA-probed membranes. The squares of the grids represent the original location of the samples on the nylon membrane. A) after probing with total *T.cruzi* DNA and B) a duplicate membrane after probing with total *T.rangeli* DNA. Columns headed with an "s" represent salivary gland material; columns headed with an "f" represent faeces. Microscopic examination of the faeces and salivary glands showed trypanosomes in samples A5, A7, B3, D5, I3 and I5 (Table 16). No parasites were seen microscopically in sample H3 which is weakly positive with the *T.rangeli* probe. C) Autoradiographs of membranes spotted with known numbers of tissue culture-derived *T.cruzi* and *T.rangeli* epimastigotes. The top two strips were probed with *T.cruzi* DNA and the lower two strips with total *T.rangeli* DNA. The numbers below the squares represent the number of parasites applied to the membrane. D) The same membrane as in A) but autoradiographed after an initial low stringency wash. A), B) and C) were washed with 0.1 X SSC, D) with 0.2 X SSC.


A
f s f s f s f s f s
A
B
C
D
E
F
G
H
I
1 2 3 4 5 6 7 8 9 10


B
f s f s f s f s f s
A
B
C
D
E
F
G
H
I
1 2 3 4 5 6 7 8 9 10


C
T. cruzi
T. rangeli
T. rangeli
T. cruzi
T. cruzi probe
T. rangeli probe
50000 25000 10000 5000 1000 500 100 25 10 0


D
f s f s f s f s f s
A
B
C
D
E
F
G
H
J
1 2 3 4 5 6 7 8 9 10

stringency removed signals from the two *T.rangeli* positives which cross-reacted with the *T.cruzi* probe at the lower stringency (Figure 39 A). Washing at high stringency also removed a third signal (from sample G9 see Figure 39 D) which hybridised to the *T.cruzi* DNA probe at low stringency but did not hybridize to the *T.rangeli* probe at low or high stringency. This signal may represent a very low number of *T.cruzi* parasites or possibly a different species of cross-reacting parasite altogether.

Analysis of *Trypanosoma* total DNA hybridization to DNA from diverse parasites.

T.cruzi (strain Esmeraldo) and *T.rangeli* (strain San Augustin) total DNA probes were also hybridized to a panel of DNA samples from a variety of organisms (Figure 40 A and B). The *T.cruzi* probe hybridized strongly to itself and to three other *T.cruzi* isolates. It hybridized weakly to 1ug of the *T.rangeli* DNA. In general *T.rangeli* total DNA tended to be less sensitive and specific than *T.cruzi* total DNA when used as a probe (Figure 40 B). No cross-hybridization with either of the probes was seen with 1ug of DNA from *Crithidia fasciculata*. This parasite is found in a number of Chagas' disease vectors and therefore the lack of cross-reactivity with these probes is important for application of the technique to field studies.

Figure 40. Species-specificity of total *T.cruzi* and *T.rangeli* DNA probes. DNA from various sources was hybridized to equivalent amounts of radiolabelled total DNA from either *T.cruzi* (Esmeraldo strain) (A), T.rangeli (San Augustin strain) (B) or *L.donovani* (HOM/IN/82/NAND3) (C). Left track, 1ug; centre track 10ng; right track, 100pg of DNA from the sources indicated below.

a. *L.donovani* (HOM/IN/82/NANDI 3).

b. *L.braziliensis braziliensis (MHOM/BR/75/M2903).*

c. *Sauroleishmania adleri (WA/KE/57/SKINK 7) (previously L.adleri*, see Killick-Kendrick et al., 1986).

d. *T.platydactyli* (previously *L.tarantole*, see Wallbanks et al., 1985).

e. *T.rangeli* (strain 1625).

f. *Endotrypanum* sp.

g. *L.mexicana amazonensis (IFLA/BR/67/PH8).*

h. Giardia lamblia.

i. *Crithidia fasciculata* (Molteno strain).

j. *Mammalian DNA (Vero cells).*

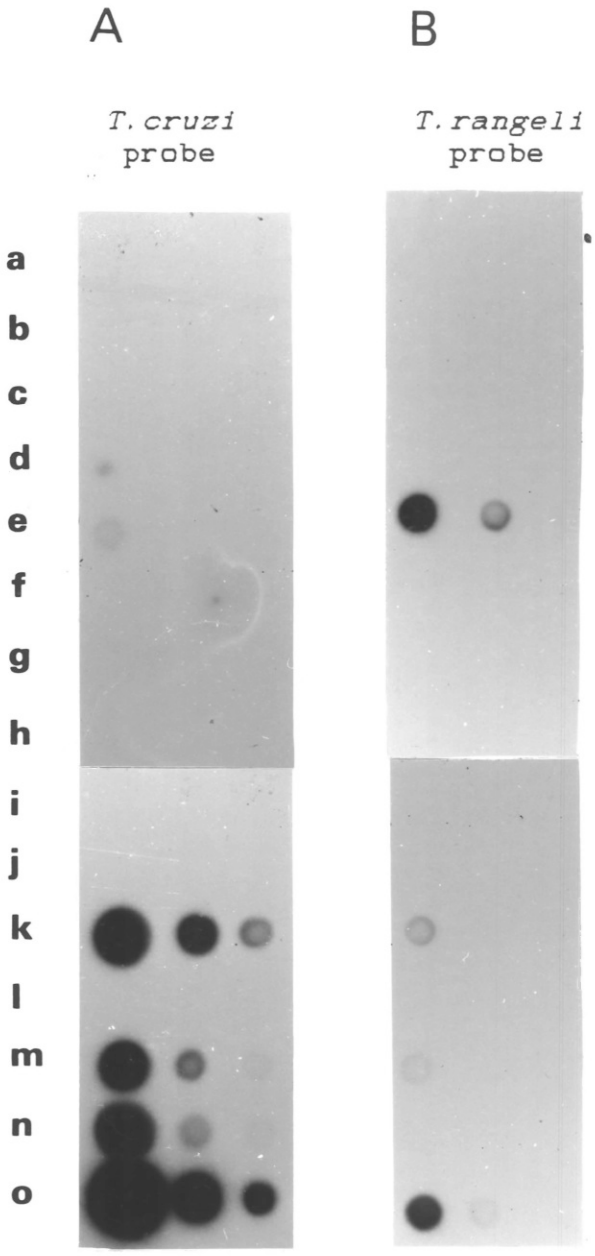
k. T.cruzi (strain 92/80).

l. *L.donovani* (LV9) (MHOM/ET/67/HU3).

m. *T.cruzi* (strain Armadillo).

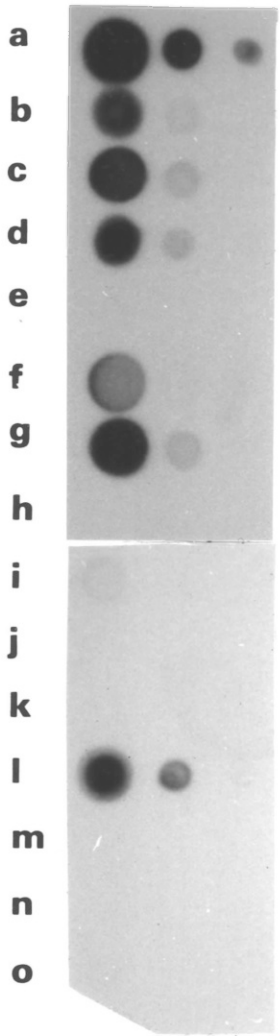
n. *T.cruzi* (strain Vinc C68).

o. *T.cruzi* (strain Esmeraldo).


A
B
T. cruzi
probe
T. rangeli
probe
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o

C

L. donovani
probe



Analysis of *Leishmania* total DNA hybridization to DNA from diverse parasites.

Total DNA from *L.donovani* (HOM/IN/82/NANDI 3) hybridized to 10ng of DNA from all strains and species of *Leishmani* that were examined. This probe also hybridized to 10ng of *Sauroleishmania adleri* and *T.platydactyli*. Both these species have recently been removed from the genus *Leishmania* (Killick-Kendrick, 1986; Wallbanks et al. 1985). Weak hybridization of the probe to 1ug of *Endotrypanum* and *C.fasciculata* DNA was also observed (Figure 40).

Discussion.

Detection of *T.cruzi* parasites in insects is important both for the epidemiology and xenodiagnosis of Chagas' disease. The results obtained here show the feasibility of detecting *T.cruzi* and *T.rangeli* in vectors using total DNA probes. The rapidity of the technique would allow far larger numbers of insects to be screened than could be achieved with microscopy. Such work may be useful for understanding the way in which *T.rangeli* interacts with *T.cruzi* in areas where their distribution overlaps. It may also facilitate the discovery of previously unknown insect vectors of these parasites.

A monoclonal antibody has recently been used for fluorescent detection of *T. cruzi* parasites in insect faeces. It did not bind strongly to *T. rangeli* in these faeces (Hudson et al. 1988). *T. vespertilionis* is considered morphologically identical to *T. cruzi* and both are members of the same subgenus but can also be distinguished from *T. cruzi* with monoclonal antibodies and lectins. It would be of interest to examine the cross-reactivity between DNA from these species. However, the mammalian hosts of *T. vespertilionis* are bats and by definition they do not infect humans. The status as regards their vectors is less clear and it is possible that *T. vespertilionis* may share some vectors with *T. cruzi* (Hoare, 1972).

Comparative studies using monoclonal antibodies, lectins and total and cloned DNA probes will be necessary to asses their relative advantages for various applications. The DNA detection method has the advantage that large numbers of samples can rapidly be applied to the same membrane and these then processed simultaneously.

Methods for identification of *Leishmania* species include microscopy, differential binding of monoclonal antibodies, isozyme analysis, differential binding of lectins, fatty acid typing, radiorespirometry and growth experiments (references given in Barker, 1987). Despite this there is still a requirement for rapid and accurate

methods to identify *Leishmania* parasites (Chance and Walton, 1982). The unsuitability of total DNA probes for this, demonstrated in this study, suggests that purified kDNA and cloned repetitive sequences may be necessary (Barker, 1987).

Phylogenetic relationships amongst the *Leishmania* and *Trypanosoma* are currently not well understood. This is largely due to a lack of suitable characteristics on which to base phylogenies. Morphological differences between species are often subtle or absent and like the other characters used, such as host preferences, reactivity with certain monoclonal antibodies and clinical features of infection, they may not correlate with phylogenetic relationships in an obvious way.

The results of total DNA hybridization studies together with DNA sequence data may be a useful addition to the characters used for the determination of phylogenies. It is interesting to note that *T.platydactyli*, which has recently been removed from the genus *Leishmania* (Wallbanks, 1985), hybridized to *L.donovani* total DNA but not at all to *T.cruzi* or *T.rangeli* DNA under the conditions used in this study. Although this result suggests that the parasite might more correctly be placed in the *Leishmania* genus, a greater understanding of the relationship between the extent of DNA hybridization between the DNA of different species and their phylogenies is needed. It would also be

interesting to know the result of the reciprocal experiment in which radiolabelled *T.platydactyli* total DNA is hybridized to unlabelled DNA of various *Leishmania* and *Trypanosoma* species.

In conclusion, the specificity and sensitivity of *T.cruzi*, *T.rangeli* and *Leishmania donovani* total DNA probes was evaluated using a panel of DNA samples from various organisms. The *Leishmania donovani* total DNA probe was found not to be specific enough for discrimination between *Leishmania* species. The *T.rangeli* and *T.cruzi* total DNA probes were found to be useful for specific detection of parasites. The results of a small field survey of wild caught insects illustrating the potential use of the *Trypanosoma* probes for epidemiological studies are also presented.

REFERENCES.

Andrade SG and Andrade ZA. Estudo histopatalogico comparativo das lesoes produzidas por cepas do *Tripanosoma cruzi*. O Hospital **70**, 1267-1278 (1966).

Andrade ZA. Mechanisms of myocardial damage in *Trypanosoma cruzi* infection. Pp 214-233 in: "Cytopathology of parasitic diseases". (Eds. Evered D and Collins GM) Ciba Foundation Symposium **99**. Pitman Books (1983).

Andreeva NS, Zdanov AS, Gustchina AE and Fedorov AA. Structure of ethanol-inhibited porcine pepsin at 2-A resolution and binding of the methyl ester of phenylalanyl diiodotyrosine to the enzyme. J. Biol. Chem. **259**, 11353-11365 (1984).

Araujo FG, Chiari E and Dias JCP. Demonstration of *T.cruzi* antigen in serum from patients with Chagas disease. Lancet **1**, 246-249 (1981).

Araujo FG, Sharma SD, Tsai V, Cox P and Remington JS. Monoclonal antibodies to stages of *T.cruzi*: characterization and use for antigen detection. Infect. Immun. **37**, 344-349 (1982).

Arfin SM and Bradshaw RA. Cotranslational processing and protein turnover in eukaryotic cells. Biochem. **27**, 7979-7984 (1988).

Ashall F. Characterization of an alkaline peptidase of *Trypanosoma cruzi* and other trypanosomatids. Mol. Biochem. Parasit. (In press).

Ashall F and Miles MA. Diagnosis of parasitic diseases using DNA-to-DNA hybridization. Trans. Roy. Soc. Trop. Med. Hyg. **82**, 235-236 (1988).

Ashall F, Yip-Chuck DAM, Luquetti AA and Miles MA. Radiolabelled total parasitic DNA probe specifically detects Trypanosomes in mammmalian blood. J. Clin. Microbiol. **26**, 576-578 (1988).

Auriault CMA, Ouassi G, Torpier H, Eisen H and Capron A. Proteolytic cleavage of IgG bound to the Fc receptor of *Schistosoma mansoni* schistosomule. Parasite Immunol. **3**, 33-44 (1981).

Avila JL, Casanova MA, Avila A and Bretana A. Acid and neutral hydrolases in *Trypanosoma cruzi*. Characterization and assay. J. Protozool. **26**, 304-311 (1979).

Avila JL, Perez-Kepp N and Bretana A. A minimal medium for the cultivation of infective *Trypanosoma cruzi* epimastigotes. J. Gen. Microbiol. **129**, 285-291 (1983).

Baker JR. The evolution of parasitic protozoa. Pp 1-27 in: "Evolution of parasites". (Ed. Taylor AER). Blackwell Scientific Publications, Oxford (1965).

Banyal HS, Misra GC, Gupta CM and Dutta GP. Involvement of malarial proteases in the interaction between the parasite and host erythrocyte in *Plasmodium knowelsi* infections. J. Parasitol. **67**, 623-626 (1981).

Barker DC. DNA diagnosis of human leishmanias. Parasitol. Today **3**, 177-184 (1987).

Barrett AJ. Proteinases in mammalian cells and tissues. In: Research Monographs in Cell and Tissue Physiology. Vol 2. North Holland, (1977).

Barrett AJ. The classes of proteolytic enzymes. Pp 1-16 in: "Plant proteolytic enzymes". Vol 1. (Ed. Dalling MJ). CRC Press. (1986).

Barrett AJ, kembhavi AA, Kirschke H, Knight CG, Tamai M and Hanada K. L-trans-epoxysuccinyl-leucylamido-(4-guanidino)butane (E64) and its analogues as inhibitors of cysteine proteases including cathepsins B, H and L. Biochem. J. **201**, 189-198 (1982).

Barrett AJ and McDonald JK. Nomenclature; a possible solution to the "peptidase anomaly". Biochem. J. **231**, 807 (1985).

Barrett AJ and McDonald JK. Nomenclature: protease, proteinase and peptidase. Biochem. J. **237**, 935 (1986

Bender ML and Philipp M. Subtilisin catalysis of nonspecific anilide hydrolases. J. Am. Chem. Soc. **95**, 1665-1666 (1973).

Bergmann M and Ross WF. On proteolytic enzymes. X. The enzymes of papain and their activation. J. Biol. Chem. **114**, 717-726 (1936).

Bernard F and Schrevel J. Purification of a *Plasmodium berghei* neutral endopeptidase and its localization in the merozoite. Mol. Biochem. Parasitol. **26**, 167-174 (1987).

Birktoft JJ, Kraut J and Freer ST. Biocem. **15**, 4481-4494 (1978).

Bjorck L, Akesson P, Bohus M, Trojnar J, Abrahamson M, Olafsson I and Grubb A. Bacterial growth blocked by a synthetic peptide based on the structure of a human proteinase inhibitor. Nature **337**, 385-386 (1989).

Blackwell JM. Receptors and recognition mechanisms of *Leishmania* species. Trans. Roy. Soc. Trop. Med. Hyg. **79**, 606-610 (1985).

Blundell T, Jenkins J, Pearl L and Sevell T. Pp 151-161 in: "Aspartic proteinases and their inhibitors". (Ed. Kostka V). Walter de Gruyter, Berlin (1985).

Bohni PC, Daum G and Schatz G. Import of proteins into mitochondria. Partial purification of a matrix-located protease involved in cleavage of mitochondrial precursor polypeptides. J. Biol. Chem. **258**, 4937-4943 (1983).

Bone R, Silen JL and Agard DA. Structural plasticity broadens the specificity of an engineered protease. Nature **339**, 191-195 (1989).

Bongertz V and Hungerer KD. *Trypanosoma cruzi*: isolation and characterization of a protease. Exp. Parasitol. **45**, 8-18 (1978).

Bontempi E, Martinez J and Cazzulo JJ. Subcellular localization of a cysteine proteinase from *Trypanosoma cruzi*. Mol. Biochem. Parasitol. **33**, 43-47 (1989).

Bordier C. Phase separation of integral membrane proteins in Triton X-114 solution. J. Biol. Chem. **256**, 1604-1607 (1981)

Bordier C, Bouvier J, Schneider P and Shaw E. Zinc signature in the promastigote surface protease of *Leishmania*. J. Cell. Biochem. Supp. **13E**, 152 (1989).

Bott R, Subramanian E and Davies DR. Three-dimensional structure of the complex of the *Rhizopus chinensis* carboxyl proteinase and pepstatin at 2.5-A resolution. Biochem. **21**, 6956-6962 (1982).

Bouvier J, Etges R and Bordier C. Identification of the promastigote surface protease in seven species of *Leishmania*. Mol. Biochem. Parasitol. **24**, 73-79 (1987).

Bradford MM. A rapid method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**, 248-254 (1976).

Brown JR. Trypanosomiasis and leishmaniasis. Pp 72-101 in: "Chemotherapy of tropical diseases". (Ed. Hooper M). Clnical Reports on Applied Chemistry **21**, 72-101. John Wiley and Sons (1987).

Brumpt E. Le *Trypanosoma cruzi* evolve chez *Conorhinus megistus*, *Cimex lecturlarius*, *Cimex boueti* et *Ornithodorus moubata*. Cycle evolutif de ce parasite. Bull. Soc. Pathol. Exot. **5**, 360-367 (1912).

Budzko DB and Kierszenbaum F. Isolation of *T.cruzi* from blood. J. Parasitol. **60**, 1037-1038 (1974).

Burger MM. Proteolytic enzymes initiating cell division and escape from contact inhibition of growth. Nature **227**, 170-171 (1970).

Cancado JR, Salgado AA, Cordoso dos Santos JF, Batista SM and Chiari C. Clinical trials in Chagas disease. Pp 266-272 in: "New approaches in American Trypanosomiasis research". Proceedings of an International Symposium, Belo Horizonte, Minas Gerais, Brazil. Pan American Health Organizaton, Scientific Publicaiton Number 318, 266-272 (1976).

Castro C, Craig SP and Castaneda M. Mol. Biochem. Parasitol. **4**, 273-282 (1981).

Cazzulo JJ, Couso R, Raimondi A, Wernstedt C and Hellman U. Further characterization and partial amino acid sequence of a cysteine proteinase from *Trypanosoma cruzi*. Mol. Biochem. Parasitol. (1989).

Cedillos RA. Chagas' disease in El Salvador. Pp 135-141 in Pan American Health Organization Bulletin Vol IX (2) (1975).

Chagas C. Nova tripanosomiase humana. Estudos sobre a morfologia e o ciclo evolutivo do *Schizotrypanum cruzi* n.g., n.sp., agente etiologico de nova entidade morbida no homem. Memorias do Instituto Oswaldo Cruz **1**, 159-218 (1909).

Chance ML and Walton BC. Biochemical characterization of *Leishmania*. Pp 263-267 in: Report of a study group. WHO Report Series Number 174 (1982).

Chappell CL and Dresden MH. *Schistosoma mansoni*: proteinase activity of "hemoglobinase" from the digestive tract of adult worms. Exp. Parasitol. **61**, 160-167 (1986).

Chasan R and Anderson KV. The role of easter, an apparent serine protease, in organizing the dorsal-ventral pattern of the *Drosophila* embryo. Cell **56**, 391-400 (1989).

Chaudhuri G and Chang K-P. Acid protease activity of a major surface membrane glycoprotein (gp63) from *Leishmania mexicana* promastigotes. Mol. Biochem. Parasitol. **27**, 43-52 (1988).

Chen KCS and Tang J. ,Amino acid sequence around the epoxide-reactive residues in pepsin. J. Biol. Chem. **247**, 2566-2574 (1972).

Chen LB and Buchanan JM. Mitogenic activity of blood components. I. thrombin and prothrombin. Proc. Natl. Acad. Sci. USA **72**, 131-135 (1975).

Cornish-Bowden A and Wharton CW. Enzyme kinetics. (Ed. Rickwood D). IRL Press, Oxford and Washington DC. (1988)

Craik CS, Roscznіak S, Sprang S, Fletternick R and Rutter C. Redesigning trypsin via genetic engineering. J. Cell. Biochem. **33**, 199-211 (1987).

Cromlish JA, Seidah NG and Chretien MA. Novel serine protease (IRCM-serine protease 1) from porcine neurointermediate and anterior pituitary lobes. J. Biol. Chem. **261**, 10850-10858 (1986).

Davis AH, Nanduri J and Watson DC. Cloning and gene expression of *Schistosoma mansoni* protease. J. Biol. Chem. **262**, 12851-5 (1987).

DeLotto R and Spierer R. A gene required for the specification of dorsal-ventral pattern in *Drosophila* appears to encode a serine protease. Nature **323**, 688-692 (1986).

Dias JCP. Control of Chagas disease in Brazil. Parasitol. Today **3**, 336-341 (1987).

Dluzewski AR, Rangachari K, Wilson RJM and Gratzer WB. *Plasmodium falciparum*: protease inhibitors and inhibition of erythrocyte invasion. Exp. Parasitol. **62**, 416-422 (1986).

Doring G, Dalhoff A, Vogel C, Brunner H, Droge U and Botzenhart K. In vivo activity of proteases of *Pseudomonas aeruginosa* in a rat model. J. infect. Dis. **149**, 532-537 (1984).

Dorsey C. *Schistosoma mansoni*: development of acetabular glands of cercaria at ultrastructural level. Exp. Parasitol. **37**, 37-59 (1975).

Dover G. Molecular drive: a cohesive mode of species evolution. Nature **299**, 111-117 (1982).

Dvorak JA. New in vitro approach to quantitation of *Trypanosoma cruzi* vertebrate cell interactions. Pp 109-120 in: "Pan American Health Organization International Symposium on New Approaches in American Trypanosomiasis Research". PAHO, Belo Horizonte, Brazil, Washington DC (1976).

Dvorak JA. The natural heterogeneity of *Trypanosoma cruzi*: Biological and medical implications. J. Cell. Biochem. **24**, 357-371 (1984).

Dvorak JA, Hartman DL and Miles MA. *Trypanosoma cruzi*: correlation of growth kinetics to zymodeme type in clones derived from various sources. J. Protozool. **27**, 472-474 (1980).

Enzyme Nomenclature, Academic Press (1984)

Erlanger BF, Kokowsky N and Cohen W. The preparation and properties of two new chromogenic substrates of trypsin. Arch. Biochem. Biophys. **95**, 271-278 (1961).

Etges R, Bouvier J and Bordier c. The major surface protein of *Leishmania* promastigotes is a protease. J. Biol. Chem. **261**, 9098-9101 (1986 a).

Etges R, Bouvier J and Bordier C. The major surface protein of *Leishmania* promastigotes is anchored in the membrane by a myristic acid-labelled phopholipid. EMBO **5**, 597-601 (1986 b).

Fastrez J and Fersht AR. Demonstration of the acyl-enzyme mechanism for the hydrolysis of peptides and anilides by chymotrypsin. Biochem. **12**, 2025-2034 (1973).

Fricker LD and Snyder SH. Enkephalin convertase: purification and characterization of a specific, enkephalin-syntehsizing carboxypeptidase localized to adrenal chromaffin granules. Proc. Natl. Acad. Sci. USA **79**, 3886-3890 (1982).

Fujimura S and Nakamura T. Isolation and characterization of a protease from *Bacterioides gingivalis*. Infect. Immun. **55**, 716-720 (1987).

Gibson WC, Dukes P and Gashumba JK. Species-specific DNA probes for the identification of African trypanosomes in tsetse flies. Parasitol. **97**, 63-73 (1988).

Gonzalez A. Prediger E. Huecas ME. Nogueira N and Lizardi PM. Minichromosomal repetitive DNA in *T.cruzi*: its use in a high-sensitivity parasite detection assay. Proc. Natl. Acad. Sci. USA **81**, 3356-3360 (1984).

Grassmann W and Dyckerhoff H. Divers activites that digest protein and peptidic substrates. Hoppe-Seyler's Z. Physiol.Chem **179**, 41-78 (1928).

Greig S, Akinsehinwa FA, Ashall F, Lainson R, Shaw JJ, Miles MA and Barker DC. The feasibility of discrimination between *Leishmania* and *Endotrypanum* using total parasite DNA probes. Trans. Roy. Soc. Trop. Med. Hyg. **83**, 196 (1989)

Greig S and Ashall F. Direct detection of South American trypanosome infections in field samples using total parasite DNA probes. Parasitol. Today **3**, 375-376 (1987).

Greig S, Hudson L and Ashall F. Detection of *Trypanosoma cruzi* and *Trypanosoma rangeli* in field caught *Rhodnius prolixus* using total DNA probes. Trans. Roy. Soc. Trop. Med. Hyg. (In press).

Grimm F, Jenni L, Bouvier J, Etges R and Bordier C. The promastigote surface protease of *Leishmania donovani* infantum in the midgut of *Phlebotamus perniciosus*. Acta Tropica **44**, 375-377 (1987).

Guhl F, Hudson L, Marinkelle CJ, Jaramillo CA and Bridge D. Clinical *Trypanosoma rangeli* infection as a complication of Chagas' disease. Parasitol. **94**, 475-484 (1987).

Gumbiner B and Kelly RB. Secretory granules of an anterior pituitary cell line, AtT-120, contain only mature forms of corticotropin and B-lipotropin. Proc. Natl. Acad. Sci. USA **78**, 318-322 (1981).

Hadley TJ, Aikawa M and Miller L. *Plasmodium knowlesi*: studies in invasion of rhesus erythrocytes by merozoites in the presence of protease inhibitors. Exp. Parasitol. **55**, 306-311 (1986).

Hatcher VB and Norin AJ. Expression of protease and protease-inhibiting activity in human mononuclear leukocyte cultures. Effect of Con A. Exp. Cell Res. **142**, 471-476 (1982).

Heck LW, Morihara K and Abrahamson DR. Degradation of soluble laminin and depletion of tissue-associated basement membrane laminin by *Pseudomonas aeruginosa* elastase and alkaline protease. Infect. Immun. **54**, 149-153 (1986).

Heck LW, Morihara K, McRae WB and Miller EJ. Specific cleavage of human type III and IV collagens by *Pseudomonas elastase*. Infect. Immun. **51**, 115-118 (1986).

Henderson R. Structure of crystalline a-chymotrypsin. IV. The structure of indoleacryloyl-a-chymotrypsin and its relevance to the hydrolytic mechanism of the enzyme. J. Mol. Biol. **54**, 341-354 (1970).

Hess GP, McConn J, Ku E and McConkey G. Studies of the activity of chymotrypsin. Phil. Trans. Roy. Soc. Lond. B **257**, 89-104 (1970).

Higgins DG, McConnell DJ and Sharp PM. Malarial proteinase? Nature **340**, 604 (1989).

Hoare CA. Morphological and taxonomic strudies on mammalian trypanosomes. X. Revision of the systematics. J. Protozool. **11**, 200-207 (1964).

Hoare CA. The trypanosomes of mammals. Oxford, Blackwell (1972).

Hoare CA and Wallace FG. Developmental stages of trypanosomatid flagellates: a new terminology. Nature **212**, 1385-1386 (1966).

Hoff R. A method for counting and concentrating living *Trypanosoma cruzi* in blood lysed with ammonium chloride. J. Parasitol. **60**, 527-528 (1974).

Hook VYH and Peng Loh Y. Carboxypeptidase B-like converting enzyme activity in secretory granules of rat pituitary. Proc. Natl. Acad. Sci. USA **81**, 2776-2780 (1984).

Hsu I-N, Delbaere CTJ, James MNG and Hofmann T. Penicillopepsin from *Penicillium janthinellum* crystal structure at 2.8 A and sequence homology with porcine pepsin. Nature **266**, 140-145 (1977).

Hudson L. Autoimmune phenomena in chronic Chagas' cardiopathy. Parasitol. Today **1**, 6-9 (1985).

Hudson L, Guhl F, Marinkelle CJ and Rodriques J. Use of monoclonal antibodies for the differential detection of *Trypanosoma cruzi* in epidemiological studies and xenodiagnosis. Acta Tropica **44**, 387-394 (1988).

Itow S and Camargo EP. Proteolytic activities in cell extracts of *Trypanosoma cruzi*. J. Protozool. **24**, 591-595 (1977).

Jackson CM and Nemerson Y. Blood coagulation. Ann. Revs. Biochem. **49**, 765-811 (1980).

James MNG and Sielecki A. Structure and refinement of penicillopepsin at 1.8 A resolution. J. Mol. Biol. **163**, 299-361 (1983).

Jaynes JM, Burton CA, Barr SB, Jeffers GW, Julian GR, White KL, Enright FM, Klei TR and Laine RA. *In vitro* cytocidal effect of novel lytic peptides on *Plasmodium falciparum* and *Trypanosoma cruzi*. FASEB 2, 2878-2883 (1988).

Kasschar MR and Dresden MH. *Schistosoma mansoni*: characterization of hemolytic activity from adult worms. Exp. Parasitol. **61**, 201-209 (1986).

Kester WR and Matthews BW. Crystallographic study of the binding of dipeptide inhibitors to thermolysin: implications for the mechanism of catalysis. Biochem. **16**, 2506-2516 (1977).

Kierszenbaum F. Is there autoimmunity in Chagas' disease? Parasitol. Today 1, 4-6 (1985).

Kilian M, Thomsen B, Petersen TE and Bleeg H. Molecular biology of *Haemophilus influenzae* IgA1 proteases. Mol. Immunol. **20**, 1051-1058 (1983).

Killick-Kendrick R, Lainson R, Rioux J-A and Safjanova VM. The taxonomy of *Leishmania*-like parasites of reptiles. Pp 143-148 in: "Leishmania: Taxonomie-Phylogenese. Applications Eco-epidemiologiques. Montpellier, France: Colloque International CMRS/INSERM/OMSIMEE, (1986).

Klinkert M, Felleisen R, Link G, Ruppel A and Beck E. Primary structures of Sm 31/32 diagnostic proteins of *Schistosoma mansoni* and their identification as proteases. Mol. Biochem. Parasitol. **33**, 113-122 (1989)

Koberle F. Chagas' disease and Chagas' syndromes: the pathology of American trypanosomiasis. Adv. Parasitol. **6**, 63-116 (1968).

Koberle F. Pathogenesis of Chagas disease. Pp 137-152 in: "Trypanosomiasis and Leishmaniasis with special reference to Chagas' disease". Ciba Foundation 20 (new series) (1974).

Kramer MD and Justus C. The antiproteolytic compound alpha-2-macroglobulin in human skin. Arch. Derm. Res. **280**, 93-96 (1988).

Kraut J. Serine proteases: structure and mechanism of catalysis. Ann. Rev. Biochem. **46**, 331-358 (1977)

Kuettner KE, Soble L, Croxen RL and Marczynska B. Tumor cell collagenase and its inhibition by a cartilage-derived protease inhibitor. Science **196**, 653-654 (1977).

Lacks SA and Springhorn SS. Renaturation of enzymes after polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate. J. Biol. Chem. **255**, 7467-7473 (1980).

Laemli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**, 680-685 (1970).

Lanar DE, Levy LS and Manning. Mol. Biochem. Parasit. **3**, 327-341 (1981).

Landsperger WJ, Stirewalt MA and Dresden MH. Purification and properties of a proteolytic enzyme from the cercariae of the human trematode parasite *Schistosoma mansoni*. Biochem. J. **201**, 137-144 (1982).

Laughon BE, Syed SA and Loesche WJ. Rapid identification of *Bacteroides gingivalis*. J. Clin. Microbiol. **15**, 345-346 (1982).

Levine AE and Walsh KA. Involvement of an acrosin-like enzyme in the acrosome reaction of sea urchins. Dev. Biol. **72**, 126-137 (1979).

Lewart RM and Lee CL. Studies on the passage of helminth larvae through host tissues. J. Infect. Dis. **95**, 13-51 (1954).

Lindquist RN, Senft AW, Petit M and McKerrow JH. *Schistosoma mansoni*: purification and characterization of the major acidic proteinase from adult worms. Exp. Parasitol. **61**, 398-404 (1986).

Liotta LA, Lee CW and Morakis DJ. New direct method for preparing large surfaces of intact human basement membrane for tumor invasion studies. Cancer Lett. **11**, 141-152 (1980).

Lipscomb WN, Reeke GN Jr, Hartsuck JA, Quiocho FA and Bethge PH. The structure of carboxypeptidase A. VIII. Atomic interpretation at 0.2 nm resolution, a new study of the complex of glycyl-L-tyrosine with CPA, and mechanistic deductions. Phil. Trans. Roy. Soc. Lond. B **257**, 177-214 (1970).

Lockwood BC. North MJ, Scott KI, Bremmer AF and Coombs GH. The use of a highly sensitive electrophoretic method to compare the proteinases of trichomonads. Mol. Biochem. Parasitol. **24**, 89-95 (1987).

Lonsdale-Eccles JD and Grab DJ. Lysosomal and non-lysosomal peptidyl hydrolases of the bloodstream forms of *Trypanosoma brucei brucei*. Eur. J. Biochem. **169**, 467-475 (1987).

Lonsdale-Eccles JD and Mpimbaza GWN. Thiol-dependent proteases of african trypanosomes. Analysis by electrophoresis in sodium dodecylsulfate / polyacrylamide gels copolymerized with fibrinogen. European J. of Biochemistry **155**, 469-473 (1986).

Madison EL, Goldsmith EJ, Gerard RD, Gething M-JH and Sambrook JF. Serpin-resistant mutants of human tissue-type plasminogen activator. Nature **339**, 721-724 (1989).

Maingon R, Elson L, Crampton J, Hommel M and Chance M. Multiple distinct gp63 genes in *L.donovani*. J. Cell. Biochem. Supp. **13E**, 110 (1989).

Marikovsky M, Arnon R and Fishelson Z. Proteases secreted by transforming schistosomula of *Schistosoma mansoni* promote resistance to killing by complement. J. Immunol. **141**, 273-278 (1988).

Marikovsky M, Fishelson Z and Arnon R. Purification and characterization of proteases secreted by transforming schistosomula of *Schistosoma mansoni*. Mol. Biochem. Parasitol. **30**, 45-54 (1988).

Martins MS, Hudson L, Krettli AV, Cancado JR and Brener Z. Human and mouse sera recognize the same polypeptide associated with immunological resistance to *Trypanosoma cruzi* infection. J. Exp. Immunol. **61**, 343-350 (1985).

Matthews DA, Alden RA, Birktoft JJ, Freer ST and Kraut J. Re-examination of the charge relay system in subtilisin and comparison with other serine proteases. J. Biol. Chem. **252**, 8875-8883 (1977).

Matthews DA, Alden RA, Birktoft JJ, Freer ST and Kraut J. X-ray crystallographic study of boronic acid adducts with subtilisin BPN' (Novo). A model for the catalytic transition state. J. Biol. Chem. **250**, 7120-7126 (1975).

Mazzei D, Novi C and Bazzi C. Mitogenic action of trypsin and chymotrypsin. Lancet **11**, 232 (1966).

McKerrow JH and Doenhoff MJ. Schistosome proteases. Parasitol. Today **4**, 334-340 (1988).

McKerrow JH, Jones P, Sage H and Pino-Heiss S. Proteinases of invasive larvae of the trematode parasite *Schistosoma mansoni* degrade connective tissue and basement membrane macromolecules. Biochem. J. **231**, 47-51 (1985 a).

McKerrow JH, Pino-Heiss S, Lindquist R and Werb Z. Purification and characterization of an elastinolytic proteinase secreted by cercariae of *Schistosoma mansoni*. J. Biol. Chem. **260**, 3703-3707 (1985 b).

McMaster WR, Button L, Wallis A, Frommel T, Fujikura Y, Webb J and Reiner NE. Molecular genetics of the major glycoprotein and repetitive antigens of *Leishmania*. J. Cell Biochem. Supp. **13E**, 69 (1989).

Medina-Acosta E, Russel DG and Karess RE. Organization of the gene family encoding the major surface glycoprotein (gp63) of *Leishmania mexicana mexicana*. J. Cell Biochem. Supp. **13E**, 111 (1989).

Mignatti P, Robbins E and Rifkin DB. Tumor invasion through the human amniotic membrane: requirement for a proteinase cascade. Cell 47, 487-498 (1986).

Miles MA. Transmission cycles and the heterogeneity of *Trypanosoma cruzi*. Pp 117-196 in: "Biology of the kinetoplastidia". (Eds: Lumsden WHR and Evans DA). Vol 2, Academic Press, London, New York and San Francisco (1979).

Miles MA, Povoa M, Prata A, Cedillos RA, De Souza AA and Macedo. Do radically dissimilar *Trypanosoma cruzi* strains (zymodemes) cause Venezuelan and Brazilian forms of Chagas' disease? Lancet **1**, 1338-1340 (1981).

Miller RA, Parsons M and Reed SG. Cloning of the gp63 gene of *Leishmania donovani chagasi*. J. Cell Biochem. Supp. **13E**, 112 (1989).

Minter-Goedbloed E, Minter DM and Marshall TF de C. Quantitative comparison between xenodiagnosis and haemoculture in the detection of *Trypanosoma (Schizotrypanum) cruzi* in experimental and natural chronic infections. Trans. Roy. Soc. Trop. Med. Hyg. **72**, 217-220 (1978).

Morel C, Chiari E, Plessman Camargo E, Mattei SM, Romanha AJ and Simpson L. Strains and clones of *T. cruzi* can be characterized by patterns of restriction endonuclease products of kinetoplast DNA minicircles. Proc. Natl. Acad. Sci. USA **77**, 6810-6814 (1980).

Mori M, Miura S, Tatibana M and Cohen PP. Characterization of a protease apparently involved in processing of pre-ornithine transcarbomylase in rat liver. Proc. Natl. Acad. Sci. USA **77**, 7044-7048 (1980).

Morihara KH, Tsuzuki H and Oda K. Protease and elastase of *Pseudomonas aeruginosa*: inactivation of human plasma alpha-1-proteinase inhibitor. Infect. Immun. **24**, 188-193 (1979).

Mosser DM and Edelson PJ. The mouse macrophage receptor for C3bi (CR3) is a major mechanism in the phagocytosis of *Leishmania promastigotes*. J. Immunol. **135**, 2785-2789 (1985).

Mumford RA, Strauss A, Powers JC, Pieizchala PA, Nichino N and Zimmerman MA. Zinc metalloendopeptidase associated with dog pancreatic membranes. J. Biol. Chem. **255**, 2227-2230 (1980).

Murray AW and Kirschner MW. Cyclin synthesis drives the early embryonic cell cycle. Nature **339**, 275-280 (1989).

Murray AW, Solomon MJ and Kirschner MW. The role of cyclin synthesis and degradation in the control of maturation promoting factor activity. Nature **339**, 280-286 (1989).

Navia MA et al. Three-dimensional structure of aspartyl protease from human immunodeficiency virus HIV-1. Nature **337**, 615-620 (1989).

Neurath H. Evolution of proteolytic enzymes. Science **224**, 350-357 (1984).

Newport GR, McKerrow JH, Hedstrom R, Petitt M, McGarrigle L, Barr PJ and Agabian N. Cloning of the proteinase that facilitates infection by schistosome parasites. J. Biol. Chem. **263**, 13179-13184 (1988).

Newton BA. Biochemical approaches to the taxonomy of kinetoplastid flagellates. Pp 405-434 in: "Biology of the kinetoplastida". (Eds. Lumsden WHR and Evans DA). Vol 1, Academic Press, London, New York, San Francisco (1979).

Nogueira N. Host and parasite factors affecting the invasion of mononuclear phagocytes of *Trypanosoma cruzi*. Pp52-73 in: "Cytopathology of parasitic diseases". (Eds. Evered D and Collins GM). Ciba Foundation Symposium 99. Pitman Books (1988).

Nogueira N, Chaplan S, Tydings JD, Unkeless J and Cohn Z. *Trypanosoma cruzi*. Surface antigens of blood and culture forms. J. Exp. Med. **153**, 629-639 (1981)

North MJ and Coombs GH. Proteinases of *Leishmania mexicana* amastigotes and promastigotes: analysis by gel electrophoresis. Mol. Biochem. Parasitol. 3, 293-300 (1981).

North MJ, Coombs GH and Barry JD. A comparative study of the proteolytic enzymes of *Trypanosoma brucei*, *T.evansi*, *T.vivax*, *Leishmania tarentolae* and *Crithidia fasciculata*. Mol. Biochem. Parasitol. **9**, 161-180 (1983).

O'Connor SP and Cleary PP. Localization of the streptococcal C5a peptidase to the surface of group A streptococci. Infect. Immun. **53**, 432-434 (1986).

O'Connor SP and Cleary PP. *In vivo Streptococcus pyogenes* C5a peptidase activity: analysis using transposon- and nitrosoguanidine- induced mutants. J. Infect. Dis. **156**, 495-504 (1987).

Ouaissi MA. Role of the RGD sequence in parasite adhesion to host cells. Parasitol. Today **4**, 169-173 (1988).

Pamer EG, So M and Davis CE. Identification of a developmentally regulated cysteine protease of *Trypanosoma brucei*. Mol. Biochem. Parasitol. **33**, 27-32 (1989).

Pearl LH. The catalytic mechanism of aspartic proteinases. FEBS Lett. **214**, 8-12 (1987).

Pearl LH and Blundell TL. The active site of aspartic proteinases. FEBS Lett. **174**, 96-101 (1984).

Petana WB. American Trypanosomiasis (Chagas' Disease) in the Caribbean. Bull. PAHO **12**, 45-50 (1978).

Pino-Heiss S and Schubiger G. Extracellular protease production by *Drosophila* imaginal discs. Dev. Biol. **132**, 282-291 (1989).

Piras R, Piras MM and Henriquez D. *Trypanosoma cruzi*-fibroblastic cell interactions necessary for cellular invasion. Pp 31-51 in: "Cytopathology of parasitic diseases". (Eds. Evered D and Collins GM). Ciba Foundation Symposium 99, Pitman Books (1983).

Piras MM, Henriquez D and Piras R. The effect of proteolytic enzymes and protease inhibitors on the interaction *Trypanosoma cruzi*-fibroblasts. Mol. Biochem. Parasitol. **14**, 151-163 (1985).

Plaut AG. The IgA1 proteases of pathogenic bacteria. Ann. Revs. Microbiol. **37**, 603-622 (1983).

Pohjanpelto P. Proteases stimulate proliferation of human fibroblasts. J. Cell Physiol. **91**, 387-392 (1977).

Polgar L, Halasz P. Current problems in mechanistic studies of serine and cysteine proteinases. Biochem. J. **207**, 1-10 (1982).

Porter WH, Cunningham LW and Mitchell WM. Studies on the active site of Clostripain. The specific inactivation by the chloromethyl ketone derived from α-N-tosyl-L-Lysine. J. Biol. Chem. **246**, 7675-7682 (1971).

Poulos TL, Alden RA, Freer ST, Birktoft JJ and Kraut J. Polypeptidylhalomethyl ketones bind to serine proteases as analogs of the tetrahedral intermediate. J. Biol. Chem. **251**, 1097-1103 (1976).

Pupkis MF and Coombs GH. Purification and characterization of proteolytic enzymes of *Leishmania mexicana mexicana* amastigotes and promastigotes. J. Gen. Microbiol. **130**, 2375-2383 (1984).

Prata AR. Natural history of chagasic cardiomyopathy. Pp 191-194 in: Pan American Health Organization Scientific Publication Number 318. PAHO, Washington DC, (1976).

Ragagopolan TG, Stein WH and Moore S. The inactivation of pepsin by diazoacetylnorleucine methyl ester. J. Biol. Chem. **241**, 4295-4297 (1966)

Rangel HA, Araujo PMF, Camargo IJB, Bonfitto M, Repka D, Sakurada J and Atta AM. Detection of a proteinase common to epimastigote, trypomastigote and amastigote of different strains of *Trypanosoma cruzi*. Tropenmed. Parasit. **32**, 87-92 (1981).

Rauber P, Walker B, Stone S and Shaw E. Synthesis of lysine-containing sulphonium salts and their properties as proteinase inhibitors. Biochem. J. **250**, 871-876 (1988).

Ready PO and Miles MA. Delimitation of *Trypanosoma cruzi* zymodemes by numerical taxonomy. Trans. Roy. Soc. Trop. Med. Hyg. **74**, 238-242 (1980).

Reid KBM and Porter RR. Proteolytic systems of complement. Ann. Revs. Biochem. **50**, 433-464 (1981).

Rezende JFM de. Chagasic megasyndromes and regional differences. Pp 195-205 in: "New approaches in American trypanosomiasis research". Pan American Health Organization Scientific Publication Number 318. PAHO, Washington DC (1976).

Ridley DS. Trans. Roy. Soc. Trop. Med. Hyg. **74**, 515-521 (1980)

Robertus JD, Alden RA, Birktoft JJ, Kraut J, Powers JC and Wilcox PE. An X-ray crystallographic study of the binding of peptide chloromethyl ketone inhibitors to subtilisin BPN'. Biochem. **11**, 2439-2449 (1972 a).

Robertus JD, Kraut J, Alden RA and Birktoft J. Subtilisin; a stereochemical mechanism involving transition-state stabilization. Biochem. **11**, 4293-4303 (1972 b).

Ruderman J. Mitotic cyclins and regulation of the cell cycle in early embryos. Pp 140-144 in: "Cell cycle control in eukaryotes". Current Communications in Molecular Biology. (Eds. Beach D, Basilico C and Newport J). Cold Spring Harbor (1988).

Russell DG and Wilhelm H. The involvement of gp63, the major surface glycoprotein, in the attachment of *Leishmania* promastigotes to macrophages. J. Immunol. **136**, 2613-2620 (1986).

Russell DG and Wright SD. Complement receptor type 3 binds to an arg-gly-asp containing region of *Leishmania* gp63. J. Cell Biochem. Supp. **13E**, 157 (1989).

Salazar Schettino PM, de Haro Arteaga I and Uribarren Berrueta T. Chagas disease in Mexico. Parasitol. Today **4**, 348-352 (1988).

Sanchez DO, Madrid D, Engel JC and Frasch ACC. Rapid identification of *Trypanosoma cruzi* by "dot-spot" hybridization. FEBS Lett. **168**, 139-142 (1984).

Scharfstein J, Luquetti A, Murta ACM, Senna M, Rezende JM, Rassi A and Mendonca-Previato L. Chagas' disease: serodiagnosis with purified Gp25 antigen. Am. J. Trop. Med. Hyg. **34**, 1153-1160 (1985).

Schechter M, Flint JE, Voller A, Guhl F, Marinkelle CJ and Miles MA. Purified *T.cruzi* specific glycoprotein for discriminative serological diagnosis of South American trypanosomiasis (Chagas' disease). Lancet **2**, 939-941 (1983).

Schechter I and Berger A. On the size of the active site in proteases. I. Papain. Biochem. Biophys. Res. Comm. **27**, 157-162 (1967).

Schenkman S, Andrews NW, Nussenswieg V and Robbins ES. *Trypanosoma cruzi* invade a mammalian epithelial cell in a polarized manner. Cell **55**, 157-165 (1988).

Schiffler RJ, Mansur GP, Nauin TR and Limpakarnjanarat K. Indiginous Chagas' disease (American trypanosomiasis) in California. J. Am. Med. Ass. **251**, 2983-2984 (1984).

Schwartz TW. The processing of peptide precursors. Proline-directed arginyl cleavage and other monobasic processing mechanisms. FEBS Lett. **200**, 1-10 (1986).

Sefton BM and Rubin H. Stimulation of glucose transport in cultures of density inhibited chick embryo cells. Proc. Natl. Acad. Sci. USA **68**, 3154-3157 (1971).

Segal DM, Powers JC, Cohen JC, Davies GH and Wilcox PE. Substrate binding site in bovine chymotrypsin A. A crystallographic study using peptide chloromethyl ketones as site specific inhibitors. Biochem. **10**, 3728-3738 (1971).

Shaw E and Green GDJ. Inactivation of thiol proteases with diazomethylketones. Methods Enzymol. **80**, 820-826 (1981).

Shaw E, Wilkstrom P and Ruscica J. An exploration of the primary specificity site of cathepsin B. Arch. Biochem. Biophys. **222**, 424-431 (1983).

Silva LHP and Nussenzweig V. Sobre uma cepa de *T. cruzi* altamente virulenta para o camundongo branco. Folia. Clin. Biol. **20**, 191-207 (1953).

Snary D and Hudson L. *Trypanosoma cruzi* cell surface proteins: identification of one major glycoprotein. FEBS Lett. **100**, 166-170 (1979).

Sodeinde OA and Goguen JD. Nucleotide sequence of the plasminogen activator gene of *Yersina pestis*: relationship to ompT of *Escherichia coli* and Gene E of *Salmonella typhimurium*. Infect. Immun. **57**, 1517-1523 (1989).

Steiger RF, Van Hoof F, Bontemps J, Nyssens-Jadin M and Druetz J-E. Acid hydrolases of trypanosomatid flagellates. Acta Tropica **36**, 335-341 (1979).

Steitz TA, Henderson R and Blow DM. Structure of crystalline K-chymotrypsin. III. Crystallographic studies of substrates and inhibitors bound to the active site of -chymotrypsin. J. Mol. Biol. **46**, 337-348 (1969).

Stirewalt MA. *Schistosoma mansoni*: cercariae to schistosomule. Adv. Parasitol. **12**, 115-182 (1974).

Sturm NR, Degrave W, Morel C and Simpson L. Sensitive detection and schizodeme classification of *Trypanosoma cruzi* cells by amplification of kinetoplast minicircle DNA sequences: use in diagnosis of Chagas' disease. Mol. Biochem. Parasitol. **33**, 205-214 (1989).

Takio K, Towatari T, Katunuma N, Teller D and Titani K. Homology of amino acid sequences of rat liver cathepsins B and H with that of papain. Proc. Natl. Acad. Sci. USA **80**, 3666-3670 (1983).

Takle GB and Hudson L. Autoimmunity and Chagas' disease. Curr. Top. Micro. Immunol. **145**, 79-92 (1989)

Thomas DJ, Richards AD and Kay J. Inhibition of aspartic proteinases by α_2-macroglobulin. Biochem. J. **259**, 905-907 (1989).

Thompson RC. Use of peptide aldehydes to generate transition-state analogs of elastase. Biochem. **12**, 47-51 (1973).

Tibayrenc M and Ayala FJ. Isoenzyme variability in *Trypanosoma cruzi*, the agent of Chagas' disease: genetical, taxonomical and epidemiological significance. Evolution **42**, 277-292 (1988).

Timms AR and Bueding E. Studies of a proteolytic enzyme from *Schistosoma mansoni*. British J. Pharmacology and Chemotherapy **14**, 68-73 (1959).

Torpier G, Capron A and Ouaissi MA. Receptor for IgG (Fc) and human α_2-macroglobulin on *S. mansoni* schistosomula. Nature **278**, 447-449 (1979).

Torruella M, Franke de Cazzulo BM, Engel JC, Ruiz AM, Segura EL and Cuzzulo JJ. *Trypanosoma cruzi* and *Trypanosoma rangeli*: glutamate dehydrogenases and proteolytic activities. Comp. Biochem. Physiol. **70B**, 463-468 (1981).

Toy L. et al. Pp 85-103 in: "Molecular Paradigms for eradicating Helminthic parasites". Alan R. Liss (1987).

Tzeng S, McKerrow JH, Fukuyama K, Jeong K and Epstein WL. Degradation of purified skin keratin by a proteinase secreted from *Schistosoma mansoni* cercariae. J. Parasitol. **69**, 992-994 (1983).

Uitto V-J, Larjava H, Heino J and Sorsa T. A protease of *Bacteroides gingivalis* degrades cell surface and matrix glycoproteins of cultured gingival fibroblasts and induces secretions of collagenase and plasminogen activator. Infect. Immun. **57**, 213-218 (1989).

Uitto V-J and Raeste A-M. Activation of latent collagenase of human leukocytes and gingival fluid by bacterial plaque. J. Dent. Res. **57**, 844-851 (1978).

Umezawa H and Aoyagi T. Activities of proteinase inhibitors of microbial origin. Pp 637-688 in: "Proteinases of mammalian cells and tissues". (Ed. Barrett AJ) North Holland (1977).

Utsunomiya N and Nakanishi M. A serine protease triggers the initial step of transmembrane signalling in cytotoxic T cells. J. Biol. Chem. **261**, 16514-16517 (1986).

Valinsky JE, Reich E and Le Douarin NM. Plasminogen activator in the bursa of Fabricius: correlations with mophogenetic remodelling and cell migrations. Cell **25**, 471-476 (1981).

Vianna G. Contribuicoes para o estudo da anatomia patologica da "molestia de Carlos Chagas". Memorias de Instituto Oswaldo Cruz Rio de Janeiro **3**, 276-294 (1911).

Wallace FG. The trypanosomatid parasites of insects and arachnids. Exp. Parasitolol. **18**, 124-193 (1966).

Wallbanks KR, Maazoum R, Canning E and Rioux J-A. The identity of *Leishmania tarentolae* Wenyon, 1921. Parasitol. **90**, 67-78 (1985).

Webb JR, Button LL and McMaster WR. Comparison of the genomic DNA sequence encoding the cell surface glycoprotein gp63 of *Leishmania major* and *L.donovani*. J. Cell Biochem. Supp. **13E**, 126 (1989).

Weissmann G, Zurier RB and Hoffstein S. Leukocytic proteases and the immunologic release of lysosomal enzymes. Am. J. Pathol. **68**, 539-563 (1972).

Wexler DE, Chenworth DE and Cleary PP. Mechanism of action of the group A streptococcal C5a inactivator. Proc. Natl. Acad. Sci. USA **82**, 8144-8148 (1985).

Wexler DE, Nelson RD and Cleary PP. Human neutophil chemotactic response to group A streptococci: bacterial-mediated interference with complement-derived factors. Infect. Immun. **39**, 239-246 (1983).

Wood JN, Hudson L, Jessell TM and Yamamoto M. A monoclonal antibody defining determinants on subpopulations of mammalian neurones and *Trypanosoma cruzi* parasites. Nature **296**, 34-38 (1982)

World Health Organization. Chagas' disease, Report of a study group. WHO Technical Report series Number 202 (1960).

World Health Organization. The Leishmanias. WHO Technical Report Series 701, (1984).

Wulf RJ and Mertz ET. Studies on plasminogen. VIII, Species specificity of streptokinase. Can. J. Biochem. **47**, 927-931 (1968).

Zeledon R, Solano G, Burstin L and Swartzwelder JC. Epidemiological pattern of Chagas' disease in an endemic area of Costa Rica. Am. J. Trop. Med. Hyg. **24**, 214-225 (1975).