

Proteinases in Trichomonads and Trichomoniasis

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ABBREVIATIONS

ADP	: adenosine 5'-diphosphate
AZCase	: proteinase activity towards azocasein
BAPA	: α -L-benzoyl-D-L-arginine <i>p</i> -nitroanilide
Bicine	: <i>N,N</i> -Bis(2-hydroxyethyl)glycine
Con A	: concanavalin A
CTP	: cytidine 5'-triphosphate
DEAE	: diethylaminoethyl
DFMO	: α -difluoromethylornithine
DMSO	: dimethylsulphoxide
DNA	: deoxyribonucleic acid
DTT	: dithiothreitol
EDTA	: ethylenediamine tetraacetate
GDP	: guanosine 5'-diphosphate
HPAase	: proteinase activity towards hide powder azure
NDM	: modified Diamond's medium
NES	: 2-(<i>N</i> -Morpholino)ethanesulphonic acid
NADH	: nicotinamide adenine dinucleotide (reduced)
NADP	: nicotinamide adenine dinucleotide phosphate
NADPH	: nicotinamide adenine dinucleotide phosphate (reduced)
PAGE	: polyacrylamide gel electrophoresis
PBS	: phosphate buffered saline
PMSF	: phenylmethylsulphonylfluoride
PNA	: peanut agglutinin
SAM	: S-adenosyl-L-methionine
SDS	: sodium dodecyl sulphate
TLCK	: <i>N</i> - α -tosyl L-lysine chloromethyl ketone
TPCK	: L-tosylamido-2 phenylethyl chloromethyl ketone
Tris	: 2-amino 2(hydroxymethyl)1.3-propandiol
UTP	: uridine 5'-triphosphate

ABSTRACT

Multiple proteolytic activities were detected in the cell lysates of each of *Trichomonas vaginalis*, *Tritrichomonas foetus*, *Trichomitus batrachorum* and *Pentatrichomonas hominis*. Analysis of the enzymes responsible using electrophoretic techniques, however, demonstrated differences between the four species with respect to number, molecular weight and specific activity of the proteinases. Differences were also demonstrated in the proteinases present in a range of isolates of *Trichomonas vaginalis*.

Subcellular fractionation studies showed that most of the proteolytic activity in *T. vaginalis* and *Tritrichomonas foetus* was associated with the particulate fractions and appeared to be located, along with a number of other hydrolases, in lysosome-like organelles. The results suggested that a heterogeneous population of lysosomes was present in *Trichomonas vaginalis*.

Two proteinases were partially purified from the cell lysates of *T. vaginalis* by a combination of gel filtration, ion-exchange chromatography and affinity chromatography. Both activities were shown to be due to cysteine proteinases although they differed with respect to molecular weight, pH optima, isoelectric point and sensitivity to inhibitors.

An electrophoretic technique for the detection and separation of proteinases on gels containing co-polymerised gelatin (gelatin-PAGE) was used to demonstrate extracellular proteinase in cell cultures of *T. vaginalis* and *Tritrichomonas foetus*. A number of other hydrolase activities were also found to be present extracellularly. Low molecular

weight extracellular proteinases that were not found in cell lysates were purified from the medium in which *T. vaginalis* had been grown and ~~were~~ used to raise antiserum. The antiserum was shown to bind both intra- and extracellular proteinases of *T. vaginalis* and also intracellular proteinases in *Tritrichomonas foetus*.

The gelatin-PAGE technique was also used to investigate the proteinases of a number of other species of protozoa, in particular *Leishmania* species. A number of differences were found not only between the species investigated but also between developmental forms of the same species.

INTRODUCTION

1.1. Introduction.

Protozoa are unicellular (or noncellular) eukaryotic microorganisms that occur singly or as colonies. Each protozoan is a complete unit capable of performing all the physiological functions necessary for its survival. For the most part they are free living but some are parasitic, having become adapted to an altered existence in association with a specific host or hosts. Although the complete classification of the protozoa remains a subject of much debate the most widely accepted scheme is that proposed by Baker (1973) which divides the protozoa into four subphyla, the Sarcostigophora, the Sporozoa, the Ciliophora and the Cnidospora. The flagellates (superclass Mastigophora of the subphylum Sarcostigophora) have been said to surpass all other protozoa both in terms of numbers of individuals and in the variety of environments in which they survive. Free living species can be found from the 'red snows' of alpine summits to the depths of the oceans and the parasitic species are found in the bodies of the majority of species of animals and plants where few tissues are immune to invasion.

There are five genera of parasitic flagellates that are of special importance in that they possess species that infect and are pathogenic to man and domestic animals; namely *Giardia*, *Histomonas*, *Leishmania*, *Trypanosoma* and *Trichomonas*. All members of the genus *Giardia* are intestinal parasites of vertebrates and one species *Giardia intestinalis* is a pathogenic parasite of man. Symptoms of human giardiasis include diarrheal disease, malabsorption of fats and infection of the bile tract and liver. *Giardia* infections have been reported in a range of animals including dogs, cats, mice, calves and sheep but most are asymptomatic

and the existence of pathogenic strains has not been demonstrated conclusively (Kulda and Hohnkova 1978).

Histomonas meleagridis parasitises the caecum and liver of chickens and domestic turkeys and was at one time responsible for losses of up to 45% in turkey production in the United States. More recently, improved management techniques and the development of chemotherapeutic agents have led to the decline in prevalence of histomoniasis in poultry (McDougald and Reid 1978).

Species of the genus *Trypanosoma* are classified into salivarian (mainly African) and stercorarian species. The salivarian species include *T. rhodesiense* and *T. gambiense* which cause sleeping sickness in man and *T. brucei*, *T. congolense* and *T. vivax* which cause nagana and similar diseases in cattle. The life cycle of trypanosomes is complex, transmission in most species involves an insect vector, usually the tsetse fly. In the mammalian host, salivarian trypanosomes live in the body fluids including the blood and lymphatic system. The stercorarian species *T. cruzi* causes Chagas' disease in man. The disease is endemic in areas of the South American subcontinent. The parasite is transmitted by reduviid bugs and infects the blood and muscle of the heart and gut in the host (Gutteridge and Coombs 1977).

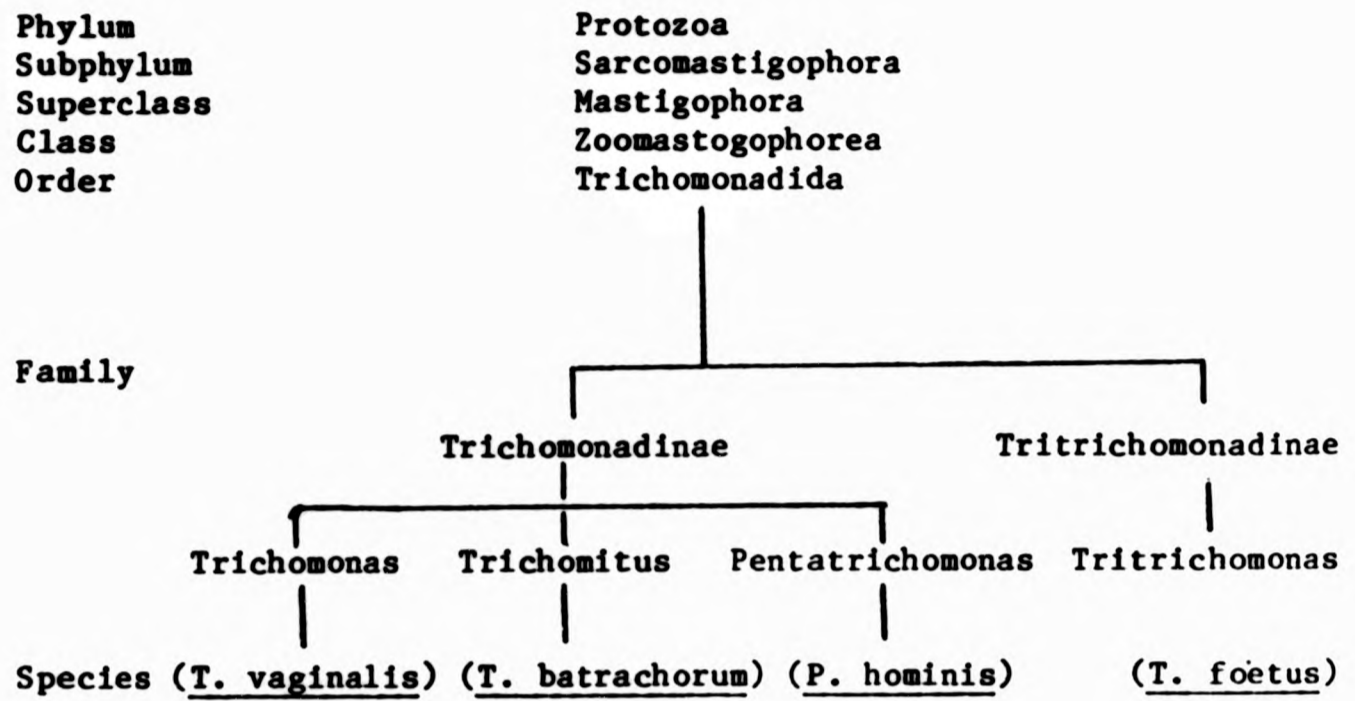
Leishmaniasis is a general term used to describe a range of diseases of humans caused by a number of species of *Leishmania*. The three main types are visceral leishmaniasis or kala-azar caused by, for example, *Leishmania donovani*, cutaneous leishmaniasis or oriental sore caused by, for example *L. tropica* and mucocutaneous leishmaniasis caused by, for example, *L. braziliensis*. Leishmanias grow intracellularly in the macrophages of the mammalian body, infected cells occur in all tissues

including the blood. The life cycle of leishmania is complex, as with trypanosomes, and it includes the sand fly as the insect vector (Chang and Bray 1985).

The complete classification of trichomonads is shown in Table 1. There are certainly three and possibly four species of trichomonad that infect man. *Trichomonas vaginalis* (Donne 1836) which inhabits the urogenital tract is the only species which has strains of proven pathogenic potential. *Pentatrichomonas hominis* (Davaine 1860), which inhabits the large intestine and *Trichomonas tenax* (Muller 1773), which is found in the mouth, are generally considered to be harmless commensals although *T. tenax* has recently been reported to cause pulmonary trichomoniasis. The infection is, however, thought to have been opportunistic and no truly pathogenic strains have been identified (Hersh 1985). The fourth species of trichomonad found in man, *Trichomitus fecalis* (Cleveland 1928) was isolated repeatedly from the faeces of one individual but has not been reported since.

Very few species of trichomonad are proved pathogens of mammals and birds. *Tritrichomonas foetus* (Riedmuller 1928) which parasitises the urogenital tract of cattle is of significant veterinary importance. *Tritrichomonas suis* (Gruby and Delafond 1843) which inhabits the nasal passages and the large intestine of swine has been demonstrated to be morphologically (Hibler et al. 1960), physiologically (Doran 1957) and immunologically (Stepkowski 1966; De Carli and Guerrero 1975) similar to *T. foetus*. *Trichomonas gallinae* and *T. gallinarum* are both parasites of birds. *T. gallinae* usually inhabits the upper digestive tract of birds but can also invade the viscera and central nervous system. Similarly,

Table 1. Classification of Trichomonads.



Taken from Honigberg (1963).

T. gallinarum which normally inhabits the caecum can also invade the liver.

A large number of other species of trichomonads have been isolated from animal hosts and in particular from the digestive tracts of birds but none have been shown to be pathogenic.

1.2. Biology of trichomonads.

Trichomonads are aerotolerant, anaerobic flagellate protozoa. They are actively motile. Only one development stage is known and they are not generally considered to produce cysts, although 'cyst' (Holz 1953) 'pseudocyst' (Mattern and Wendell 1980) 'giant' and 'non-motile' (Fari et al. 1985) forms have all been reported. The significance of these forms is uncertain and they are generally considered to be dying or degenerate forms although it has been suggested that they may play a role in transmission.

The shape and size of trichomonads varies between species and also between cells of the same species. In general cells grown axenically have a relatively uniform shape and size (Table 2). Although trichomonad species differ in shape, size, locomotion and in the morphology of subcellular structures they have many features in common. The most readily identifiable features of trichomonad cells are the anterior flagella, the recurrent flagellum associated with the undulating membrane and a non-motile axostyle that extends from the posterior end of the cell body as a long thin projection that tapers to a narrow terminal segment (Fig. 1). Electron microscopy has been used to study the complex cytoskeleton and intracellular organisation.

Table 2 Characteristics of the live trichomonads observed by phase contrast microscopy.

Species	Shape	Size (length x width, μm)	Locomotion [†]	'protruding' axostyle	Undulating membrane	Miscellaneous
<u>Trichomonas vaginalis</u>	ovoid or pyriform	(4.5-19 x 2.5-12.5)	Slow with little jerky movements	prominent, long (about half body length)	half body length, with no free posterior flagellum	
<u>Pentatrichomonas hominis</u>	ellipsoidal or pyriform	(6-20 x 4-7)	fast jerky movements	prominent, long (about half body length)	full body length, with a free posterior flagellum	
<u>Tritrichomonas foetus</u>	elongate	(9-25 x 3-15)	fast jerky movements, twisting or rolling of the cell on its axis	less prominent, short	full body length, with a free posterior flagellum	large bright vesicles/vacuoles usually visible at the posterior end of the body
<u>Trichomitus batrachorum</u>	elongate or pyriform	(8-18 x 4-10)	fast jerky movements, twisting or rolling of the cell on its axis	less prominent, short	full body length, with a free posterior flagellum	form pseudocysts which occasionally display pseudopodia

[†] in all cases, whip-like actions of flagella, wave-like movements of undulating membrane and rotation of the cells on their axes can be seen. Table taken from Thong (1986).

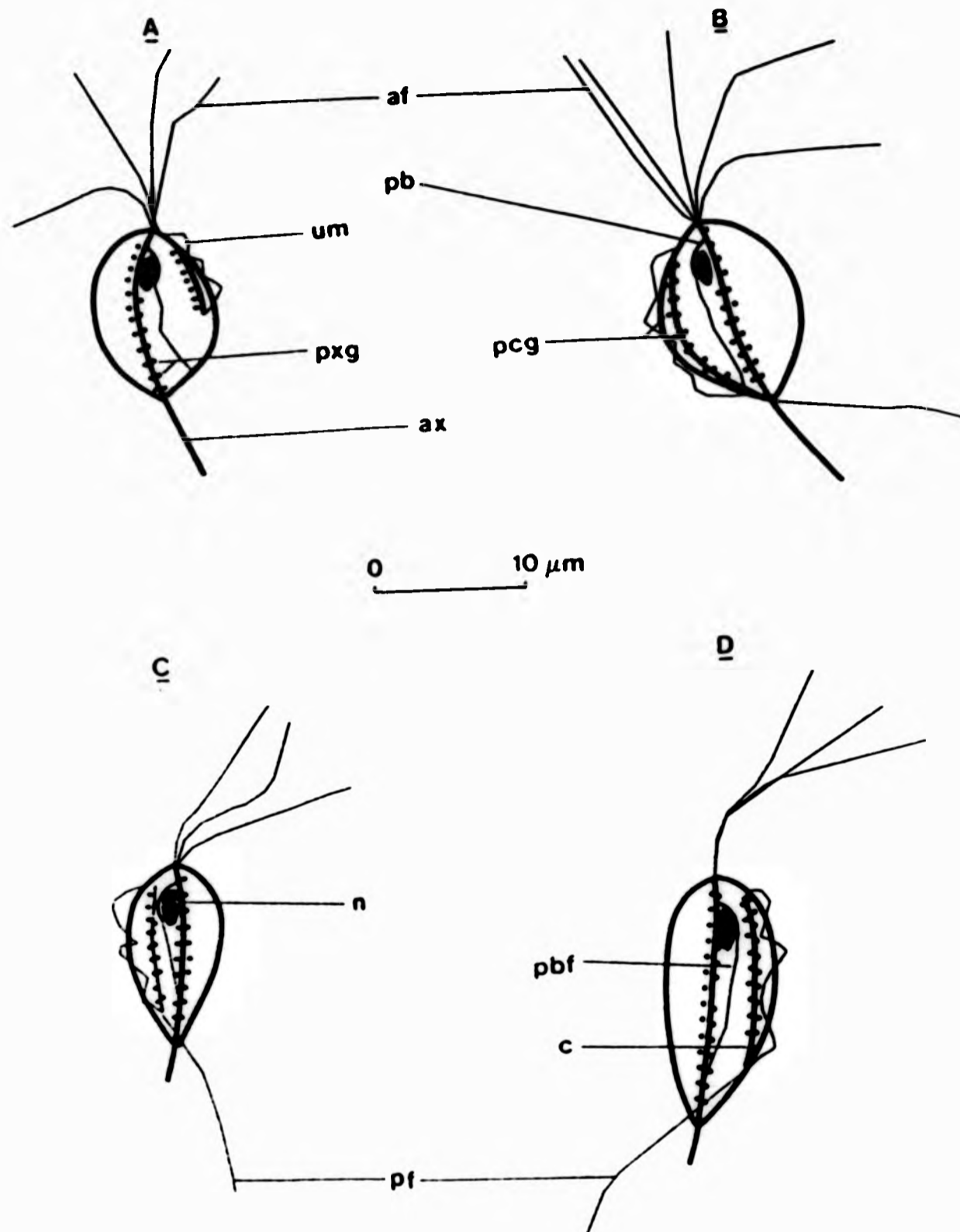


Fig. 1. Line diagrams of *Trichomonas vaginalis* (A), *Pentatrichomonas hominis* (B), *Tritrichomonas foetus* (C), and *Trichomitus batrachorum* (D). (Modified from Honigberg 1978a,b; Farmer 1980). Key: af, anterior flagella; pb, parabasal body; um, undulating membrane; pxg, paraxostylar granules; pcg, paracostal granules; ax, axostyle; n, nucleus; pbf, parabasal filament; c, costa; pf, posterior flagellum.

The prominent nucleus is situated towards the anterior end of the organism between the parabasal body (Golgi) and the capitulum of the axostyle. Rough endoplasmic reticulum surrounds the nucleus. Parabasal filaments are also associated with the kinetosomal complex which is situated at the anterior end of the cell and is where the anterior flagella originate. In *Pentatrichomonas hominis* the fifth anterior flagellum originates from another kinetosome located ventrally to the base of the bundle of the typical anterior flagella. The costa also originates in the kinetosomal complex and extends posteriorly beneath the undulating membrane. The pelta, a large sheet of microtubules, courses upward from the capitulum and around the kinetosomal complex terminating in the periflagellar lip bordering the periflagellar canal, a shallow indentation from which the anterior flagella project. Associated with the costa and axostyle are the paracostal and paraxostylar granules which are spherical, membrane bound organelles called hydrogenosomes (Honigberg 1978 a,b).

Significantly, no morphologically identifiable mitochondria are present in trichomonads and no evidence for mitochondrial metabolism has ever been found (Wellerson et al. 1959; Nielson et al. 1966; Lloyd et al. 1979; Muller 1980).

Trichomonads feed by pinocytosis or both pinocytosis and phagocytosis (Honigberg 1978a,b). There are no structurally differentiated organelles of feeding comparable to cytosomes. Endocytic processes have been demonstrated by means of different tracers and primary and secondary lysosomes have been identified by electron microscope cytochemistry (Kulda et al. 1985a).

1.3. Human trichomoniasis.

Trichomonas vaginalis is the causative agent of human urogenital trichomoniasis or trichomonal vaginitis. It is thought to infect up to 200 million women worldwide (Brown 1972) and between 1-70% of people in particular areas (Diamond 1983). It is transmitted primarily by sexual intercourse, although neonates may become infected during passage through the birth canal. It has been suggested that *T. vaginalis* can cause pneumonia in babies infected in this way (Hiemstra et al. 1984). In contrast, *Trichomonas tenax* and *Pentatrichomonas hominis* are usually transmitted through indirect contact via contaminated food and drinking water (Honigberg 1978b).

The primary site of infection of *T. vaginalis* in women is the vagina, although in latent trichomoniasis the infection may extend to the cervix. The parasite has also been found to infect the bladder and Skene's ducts (Grys 1973) and urinary passages (Soszka et al. 1973). Trichomonads are not generally considered to infect the upper reproductive tract, owing to the cervical mucus forming an impenetrable barrier (Grys 1966). Recently, however, there have been a number of reports that suggest that trichomonads can penetrate the cervical mucus and furthermore, that *T. vaginalis* may be implicated in tubal infertility in some patients (Larsen 1985; Keith et al. 1986). In men the parasite is usually confined to the urethra, prostate and epididymis (Gardner et al. 1986).

The symptoms of trichomoniasis vary significantly between individuals and range in severity from completely asymptomatic to causing severe morbidity. More infected men are asymptomatic than women.

The infection in women has two stages: the acute stage is often characterized by copious, frothy, foul smelling vaginal discharge, inflammation and even ulceration of the genitalia leading to severe irritation and itching (Rein and Chapel 1974; Honigberg 1978a; Spence *et al.* 1980; Kreiger 1981). The chronic stage of the infection usually results in few symptoms other than an atypical discharge.

In men, the primary infection can take three forms (Jira 1958). The most frequently encountered is the latent form which is usually asymptomatic. The other two forms are both symptomatic; the acute form is of rapid onset accompanied by copious urethral discharge. In contrast, the subchronic form is of slower onset and accompanied by a relatively slight discharge. All three primary forms can lead to a secondary latent or chronic stage, characterised by a slight itching sensation inside the penis and slight moisture at its tip. In other cases, purulent discharge may be observed before passing early morning urine or else scanty secretion may be present throughout the day. Acute trichomonal urethritis and prostatitis are usually associated with purulent urethral discharge and inflammation, occasionally leading to ulceration of the external meatus.

1.3.1. Diagnosis.

The most commonly used method of diagnosis is direct microscopic observation (wet mount) of vaginal secretions which, although rapid and inexpensive, is only 50 to 70% effective (Fouts and Kraus 1980; Schmid and Larsen 1984). The sensitivity is markedly increased by additional *in vitro* cultivation of the parasite (Diamond 1983). Diagnosis by serological identification systems (Ackers *et al.* 1975; Honigberg 1978a,b)

have not proved to be satisfactory because the body's immune response to *T. vaginalis* is poor and variable. Recently, an enzyme-linked immunosorbent assay (ELISA) for the detection of *T. vaginalis* antigens present in vaginal secretions has been described (Watt et al. 1986). The authors reported it to be twice as sensitive as the wet mount procedure but not as sensitive as *in vitro* cultivation, although considerably more rapid. Its usefulness has yet to be established.

1.3.2. Treatment.

The drugs of choice for the treatment of human trichomoniasis are the 5'-nitroimidazoles. The most commonly used is metronidazole or Flagyl (May and Baker Ltd. Dagenham, Essex, England) (Fig. 2), which has cure rates of 90 to 95%. Other derivatives including tinidazole, ornidazole, econazole and nimorazole have also been used successfully (Bloch and Smyth 1985; Valent et al. 1985; Ogunbanjo et al. 1985; Zrubek and Szymanski 1986). It is usually necessary to treat both the infected person and their sexual partner(s).

There are, however, a number of disadvantages associated with the use of these drugs including i) the existence of resistant strains of *T. vaginalis*, ii) the contraindication of the drug during pregnancy which may result in the infection being transmitted to the neonate and iii) reports of the drug having carcinogenic (Rustica and Shubik 1972) and mutagenic (Voogd et al. 1974, 1975; Speck et al. 1976) effects. There are no other effective drugs available.

A recently introduced vaccine, SolcoTrichovac (Basel Solco Ltd., Switzerland), produced from killed variants of *Lactobacillus acidophilus* isolated from the vaginal secretions of patients with trichomoniasis is

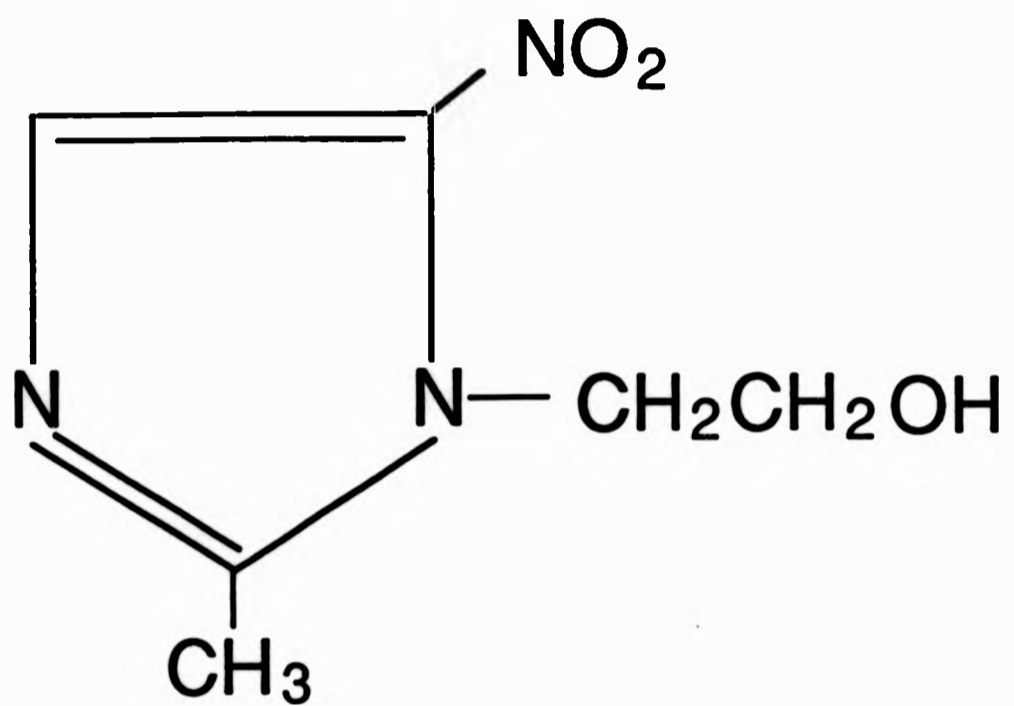


Fig. 2. Structure of metronidazole.

claimed to be highly successful in treating non-specific vaginitis and trichomoniasis (see 'SolcoTrichovac: a new concept for the treatment and prophylaxis of trichomoniasis and non-specific vaginitis.' Published by Solco Basle Ltd.). Although there is no clear cut explanation for the mechanism of action, the manufacturers suggest that it is due to antigenic cross-reactivity between strains of *L. acidophilus* and *T. vaginalis*. In an independent study of this cross reactivity, however, Gombosova et al. (1986) failed to show any such antigenic relationship. If the remarkable clinical effects of SolcoTrichovac, claimed by the manufacturers are confirmed, a different explanation for the mode of action of the vaccine will have to be sought by further investigation of the microbial interactions in the vagina as well as of the antigenic relationships of the individual micro-organisms present.

1.4. Bovine trichomoniasis

Bovine trichomoniasis, caused by the urogenital parasite *Tritrichomonas foetus*, is distributed worldwide and ranked third after leptospirosis and brucellosis as the disease most responsible for abortions in cattle. The main clinical features of the disease are early foetal death and infertility which have been reported to lead to losses of £millions in the beef industry (Honigberg 1978a). Transmission of the parasite is during coitus. The main site of infection in the cow is the uterus although in some cases the parasite may remain in the vagina causing low grade inflammation accompanied by vaginal catarrh (Morgan 1946). Infection of the uterus usually results in endometritis and increased uterine and cervical secretion which is believed to prevent

fertilization. Severe endometritis can lead to permanent sterility. The infection in cows often subsides without treatment but reinfection can occur although the consequences may be less severe (Clark et al. 1986). Abortion usually occurs following conception in an infected cow. In complete abortion, the foetus and placental membrane are both expelled, and the cow will normally recover and can conceive again. If incomplete abortion occurs, the placenta is retained in the uterus often leading to chronic endometritis and permanent sterility.

The usual site of infection in the bull is the prepuccial cavity although the parasite may also be found in the urethra or deeper in the urogenital tract (Hammond and Bartlett 1943). Without treatment the bull normally harbours the parasite for life. The primary stage of infection is characterised by acute inflammation, swelling of the prepuce and mucopurulent discharge. After about two weeks the bull usually becomes asymptomatic although in some bulls chronic infection may develop leading to orchiditis.

Diagnosis in the bull is often made by identification of the characteristic swelling and discharge from the prepuce. Confirmation requires the collection and culturing of prepuccial smegma for the presence of *T. foetus*. Diagnosis of bovine vaginitis requires identification of the parasite.

There is no fully effective treatment available for the treatment of bovine trichomoniasis, although a large number of different therapies have been tested. Topically applied drugs such as boroflavine-saalble (Bartlett 1948), acriflavine and berenil (Fitzgerald et al. 1963) were found to be ineffective. More success was achieved with oral dimetronidazole (McLaughlin 1963), intravenous dimetridazole (McLaughlin

1968) and intramuscular ipronidazole with pretreatment of procaine penicillin (Skirrow et al. 1985).

1.5. Pathogenicity of trichomonads.

Although accounts of the pathological changes accompanying trichomonad infections have been described in some detail (Levine 1973; Honigberg 1979) little is known about the early stages of infection or the mechanisms of pathogenicity. The different consequences of *T. foetus* and *Trichomonas vaginalis* infections suggest that the pathogenic mechanisms of the two parasites are likely to differ at least in some aspects. Furthermore, symptoms of trichomoniasis vary significantly between individuals, which may be due either to variation in the response of the host or different strains of the parasites having different pathogenic potentials.

The adverse effect of *Trichomonas vaginalis* on tissue culture cells was first demonstrated by Hogue (1943) using chicken embryo explants. Since then numerous investigations involving different cell types and different strains of *T. vaginalis* have been carried out (see Pindak et al. 1986 for references) and the cytopathogenic effects well characterised. The cause of these changes remains a subject of much debate. When freshly isolated strains of *T. vaginalis* are added to monolayers of various mammalian cell types or human cancer cells the trichomonads adhere to the cells and produce lesions resulting in the destruction and detachment of the cells (Farris and Honigberg 1970; Heath 1981; Alderete and Pearlman 1984; Rasmussen et al. 1986). The various authors concluded that the cytopathogenic effects were mediated by a direct cell contact

mechanism. Two possibilities were suggested; either that some mechanical process was involved or that the changes in the cells were due to 'membrane-bound lytic factors' present on the surface of the trichomonads. A report on the haemolytic activity of trichomonads supports the latter proposition (Kreiger et al. 1983). Furthermore, Alderete and Pearlman (1984) concluded that the release of a toxin responsible for the destruction of the cell monolayers was unlikely for two reasons. Firstly, the effect of the trichomonad was always focal with some apparently normal cells always present, and secondly, cell free supernatants of strains of *T. vaginalis* shown to be cytopathogenic had no effect on the mammalian cells. If a toxin were produced it was either at a very low level, acted locally or was inhibited in the medium. In contrast, there have been a number of reports supporting the release of a cytotoxin. Nielsen and Nielsen (1975) examined the interaction between trichomonads and the vaginal epithelium. They reported clusters of protozoa overlying mucosal microulcerations in biopsies from women with trichomoniasis. Since trichomonads were not present in every area of epithelial disruption they concluded that most cytopathology resulted from cell free cytotoxins and that contact dependent destruction of epithelial cells was a secondary phenomenon. Pindak et al. (1986) supported this view. Although confirming the observation that the trichomonads attach to the cell monolayer and produce a focal lesion, they reported that the lesion expanded until the entire monolayer was destroyed and that when a fresh suspension of McCoy or HeLa cells was added at this stage the cells did not form a monolayer. They concluded that a substance that interfered with cell attachment was present in the medium. This effect was repeated using a cell free supernatant and it

was suggested that some substance released by the trichomonads may be the cause of some of the pathogenic changes previously seen only in the presence of live parasites.

A third mechanism proposed for the cytopathogenic effects of trichomonads involved cytophagocytosis. Ovcinikov et al. (1975) described a change in trichomonad morphology from spherical to amoeboid associated with the development of surface projections directed towards the epithelial cells. These observations were interpreted as active phagocytosis of the epithelial cells. Kreiger et al. (1985), however, used radiolabelled target cells and ruled out this possibility.

Since no naturally occurring model for *T. vaginalis* occurs in mammals, a number of experimental systems have been developed both for assessing the pathogenicity of different strains of *T. vaginalis* and for screening antitrichomonal compounds. In addition to the cell culture techniques described above, intravaginal (Honigberg 1978b), subcutaneous and intraperitoneal (Honigberg 1979) models have been described which allow the level of pathogenicity of a particular strain to be evaluated and inherent pathogenic mechanisms can be investigated in the absence of other factors known or thought to influence pathogenicity e.g. immunocompetence, bacterial flora.

Numerous investigations have been carried out to seek correlations between various biological, biochemical and immunological characteristics of strains of *T. vaginalis* and virulence. These have included studies of protein composition (Ivey 1975), polysaccharides (Warton and Honigberg 1983), isoenzyme profiles (Soliman et al. 1982) and phenotypic variation (Alderete et al. 1986).

1.6. Immunological aspects of trichomonad infections.

The immunogenic and antigenic properties of *T. vaginalis* have been studied in some detail. Infected individuals produce humoral, secretory and cellular immune responses to the parasite (Vos and Watt 1986) and this has led to a large number of investigations with the aim of developing immunologically based tests as diagnostic tools. Reactivity to *T. vaginalis* antigens has been demonstrated by passive haemagglutination (Yano et al. 1983), complement fixation (Jaakmees et al. 1966), enzyme-linked immunoabsorbent assays (Alderete et al. 1985; Watt et al. 1986)), immunofluorescence (Torian et al. 1984) and lymphocyte transformation assays (Rein and Chapel 1975).

Antigenic heterogeneity has been demonstrated in *T. vaginalis* and the relationship between antigenic composition and pathogenicity has also been investigated (see Honigberg 1979). Alderete et al. (1985) demonstrated that sera from patients with trichomoniasis or from experimentally infected mice discriminated among *T. vaginalis* isolates and subpopulations. A group of high molecular weight surface proteins were thought to be responsible for the antigenic heterogeneity based on the antibody recognition of the trichomonads. Recently monoclonal antibodies prepared against *T. vaginalis* have been described by a number of authors (Connelly et al. 1985; Alderete and Kasmala 1986, Alderete et al. 1986; Chang et al. 1986) and a range of highly immunogenic proteins and glycoproteins have been identified. Alderete and Kasmala (1986) demonstrated that a monoclonal antibody which reacted with a highly immunogenic trichomonad membrane glycoprotein, with a molecular weight of approximately 270 kDa, produced complement-independent cytolysis of *T. vaginalis*. Organisms that did not possess the antigen were resistant

to cytolysis. The authors suggested that trichomonads that undergo variation of surface antigen composition during growth may be capable of evading humoral immune mechanisms in their host.

Little is known of the immune response to *Tritrichomonas foetus* or the nature of its antigenic components. Early reports indicated that some degree of acquired resistance to *T. foetus* develops in the cow after infection or parental immunization (Andrews 1938; Morgan 1947). More recently, Clark et al. (1984) demonstrated successful immunization of bulls with crude antigen and membrane glycoprotein preparations of *T. foetus*. No data were presented on the mechanism of resistance or the target antigens of the immune response in the vaccinated animals. Burgess (1986) described the preparation of monoclonal antibodies against a *T. foetus* isolate, some of which were demonstrated to bind to the surface of the organism and mediate adherence of the parasite to cells of the immune system such as monocytes.

1.7. Biochemistry of trichomonads.

Despite the fact that trichomonads can be grown with comparative ease in axenic culture and large quantities of uncontaminated material are readily available for biochemical investigations, information on this aspect of trichomonads remains limited. *Trichomonas vaginalis* and *Tritrichomonas foetus* have been most widely investigated although there are a few reports on biochemical aspects of other species of trichomonad.

The area of trichomonad biochemistry most extensively studied is carbohydrate metabolism and energy production, in particular with respect

to hydrogenosomal metabolism. Other areas investigated include nucleic acid, protein and amino acid, polyamine and lipid biochemistry.

1.7.1. Carbohydrate metabolism and energy generation.

Trichomonads are considered to be basically anaerobic organisms dependent on the reactions of the glycolytic pathway for most of their energy requirements. Both endogenous and exogenous carbohydrates are utilised by the parasites (Honigberg 1978a,b). Glycogen is the main endogenous substrate, and is synthesised to an appreciable degree in the presence of exogenous substrates and present in abundance in trichomonad cells (10-30% of the dry weight). A variety of exogenous carbohydrates is utilised by trichomonads although the range differs with species. Glucose and maltose are the most frequently used in axenic growth media. Little is known of the transport of these substrates into the cell.

Typically, trichomonads possess a similar glycolytic pathway to that found in most prokaryotes and eukaryotes. Activities of all the enzymes of the Embden-Meyerhof sequence have been either demonstrated directly or inferred on the basis of presumptive evidence. The most studied include glycogen phosphorylase, phosphoglucomutase, phosphohexisomerase, hexokinase, phosphofructokinase, aldolase, triosephosphate isomerase, triosephosphate dehydrogenase, phosphoglyceromutase, enolase, pyruvate kinase and lactate dehydrogenase (Honigberg 1978a,b). Very few of the enzymes have been purified or characterised in detail.

Glycogen phosphorylase is responsible for the conversion of glycogen into glucose-1-phosphate for entry into the glycolytic pathway. of *Tritrichomonas foetus*. This enzyme is absent from *Trichomonas*

vaginalis and an alternative route for the cleaving of glycogen has been suggested for this species (Wellerson and Kupferberg 1962).

Until recently the major anaerobic end-products of glucose fermentation were thought to be lactate, acetate H_2 and CO_2 in *T. vaginalis* (Mack and Muller 1980) and succinate, acetate H_2 and CO_2 in *Tritrichomonas foetus* (Ryley 1955). A large number of minor products had also been detected including traces of butyrate, propionate and isovalerate (Saeki et al. 1984) and various gases including methane, ethane, propane, ethylene, carbon monoxide, carbon dioxide and other unidentified gases (Ishiguro 1985). Quantitative analysis of glucose fermentation by whole trichomonad cells however, revealed that the known end products were not sufficient to account for all the reducing equivalents produced. Recent reports have demonstrated that glycerol is an end-product of glucose metabolism in *Trichomonas vaginalis* and *Tritrichomonas foetus* and that the amounts of glycerol detected compensated exactly for the deficits in fermentation products previously reported (Chapman et al. 1985a). Glycerol is formed from the glycolytically derived dihydroxyacetone by two enzymes, an NADP dependent glycerol-3-phosphate dehydrogenase and a Mg^{2+} -dependent glycerol-3-phosphatase (Steinbuechel and Muller 1986a). The metabolic pathways of *Trichomonas vaginalis* and *Tritrichomonas foetus* are shown in Figs. 3 and 4.

Phosphoenolpyruvate is carboxylated to oxaloacetate in both species of trichomonad but it is only reduced further to succinate in *T. foetus*. The remainder of the phosphoenolpyruvate is converted to pyruvate. In both species pyruvate enters the hydrogenosomes but some is reduced to lactate in *Trichomonas vaginalis*. The results of some studies suggest

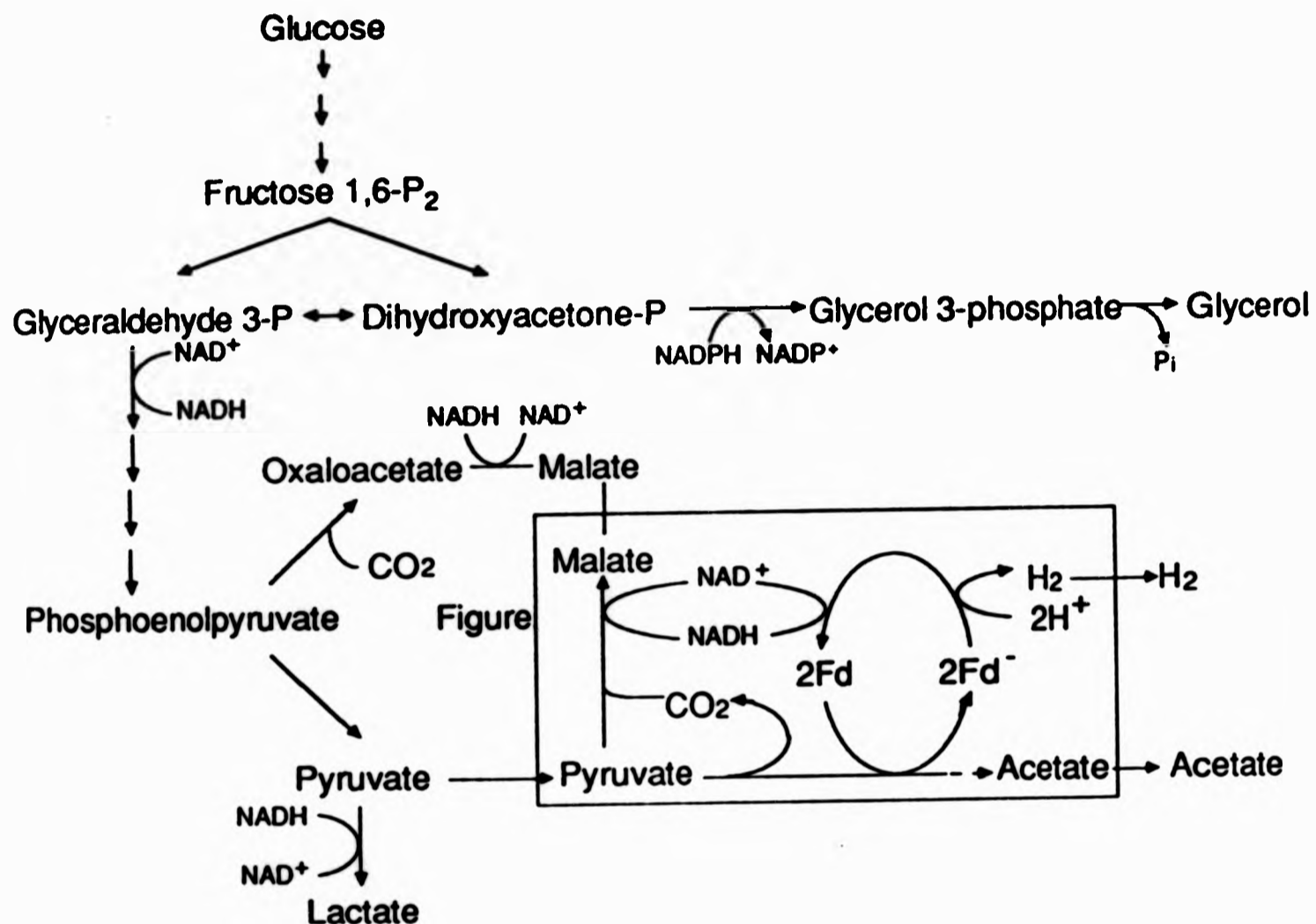


Fig. 3. Metabolic map for *Trichomonas vaginalis*. Hydrogenosomal reactions involved in the formation of acetate from acetyl CoA are simplified. For detailed information see Muller 1980; Steinbuchel and Muller 1986b; section 1.7.2. Arrows indicate the assumed physiological direction *in vivo*. The direction of flow is not indicated where uncertain. (Figure obtained from Steinbuchel and Muller 1986a).

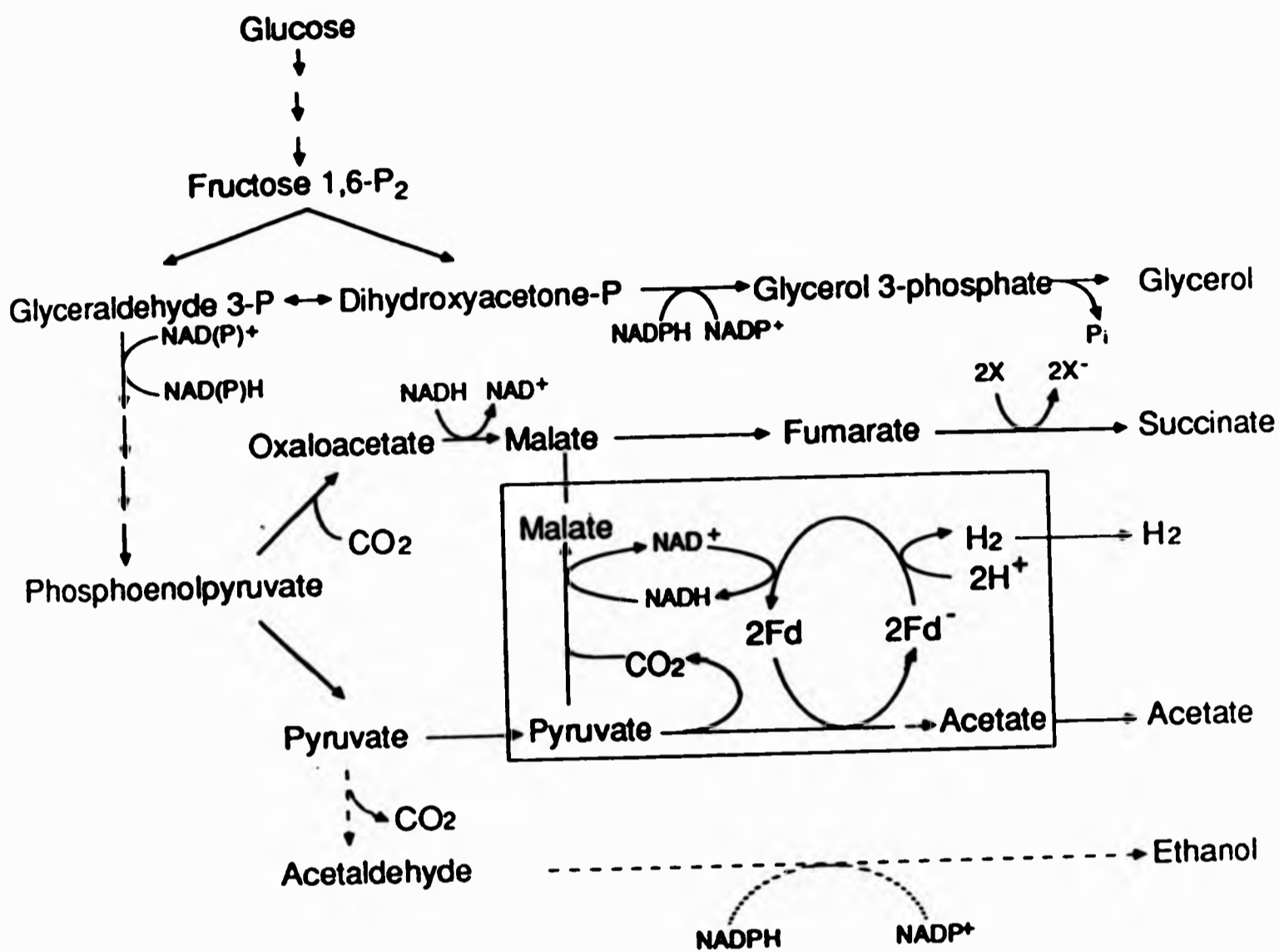


Fig. 4. Metabolic map for *Tritrichomonas foetus*. Hydrogenosomal reactions involved in the formation of acetate from acetyl CoA are simplified. For detailed information see Muller 1980; Steinbuchel and Muller 1986b; section 1.7.2. Pathway only active in *T. foetus* strain KV,-1MR-100. Arrows indicate the assumed physiological direction *in vivo*. The direction of flow is not indicated where uncertain. (Figure obtained from Steinbuchel and Muller 1986a).

almost complete homolactic fermentation whereas others report lactate only as a minor end product.

The presence of oxygen significantly affects the energy metabolism of trichomonads with respect to both the formation of end-products and energy production. In *T. foetus*, oxygen decreases the production of succinate but stimulates the production of acetate with a concomitant cessation of hydrogen production (Muller 1976). The decrease in succinate formation may be accounted for by the diversion of the reducing equivalents to oxygen, the electron acceptor for NADH oxidase, such that less oxaloacetate is reduced (see section 1.7.2).

The increase in acetate production and hence substrate level phosphorylation would appear to be advantageous to the parasite. Trichomonad growth *in vitro*, however, is adversely affected by high oxygen concentrations (see Diamond 1957) which suggests that other factors are also involved. The inhibition of hydrogen production is probably due to oxygen scavenging all the electrons from the reduced ferredoxin.

A significant feature of trichomonads in aerobic conditions is the intense respiration (O_2 consumption) that occurs (Muller 1976; Mack and Muller 1980). The enzymes responsible for metabolising or scavenging oxygen and its metabolites are NADH oxidase, NADPH oxidase, catalase and superoxide dismutase (see Honigberg 1978a,b). These enzymes are thought to be essential in maintaining the low redox potential that is optimal for the cells.

Recently some attention has been focused on the study of carbohydrate metabolism in *Pentatrachomonas hominis*. Lo et al. (1984) demonstrated that *P. hominis* is capable of utilizing glucose, fructose,

galactose, maltose, sucrose, ribose, glycogen and amylopectin. Glucose and galactose were shown to be transported in to the cells by specific transport mechanisms and are catabolised via glycolysis. The end products of glucose catabolism in *P. hominis* were reported to be lactate, acetate and succinate. Although the information available is still relatively sparse the proposed pathway for the metabolism of carbohydrates in this species is illustrated in Fig 5.

1.7.2. Hydrogenosomal metabolism.

A number of anaerobic protozoa including trichomonads and hypermastiginid flagellates, holotrich and entodinomorphid ciliates of the rumen and certain ciliates living in anaerobic marine and fresh water sediments contain membrane bounded micro-body like organelles called hydrogenosomes (Muller 1980). Although hydrogenosomes are involved in energy metabolism, they resemble neither mitochondria nor peroxisomes (Muller 1973; Lindmark and Muller 1973). The properties of hydrogenosomes in different groups of protozoa are known to different extents, however those of trichomonads have been studied in some detail. Typically, trichomonad hydrogenosomes are round bodies with a diameter of about 0.5 μm . They have a granular matrix and are surrounded by two closely associated membranes 6 nm thick (Benchimol and De Souza 1983). Their function is described from studies of enzymatic composition and metabolism of whole cells or from investigations of isolated intact hydrogenosomes. Anaerobic metabolism has only received attention relatively recently.

The main metabolic function of hydrogenosomes is the oxidative decarboxylation of pyruvate with acetate as the major end-product. The

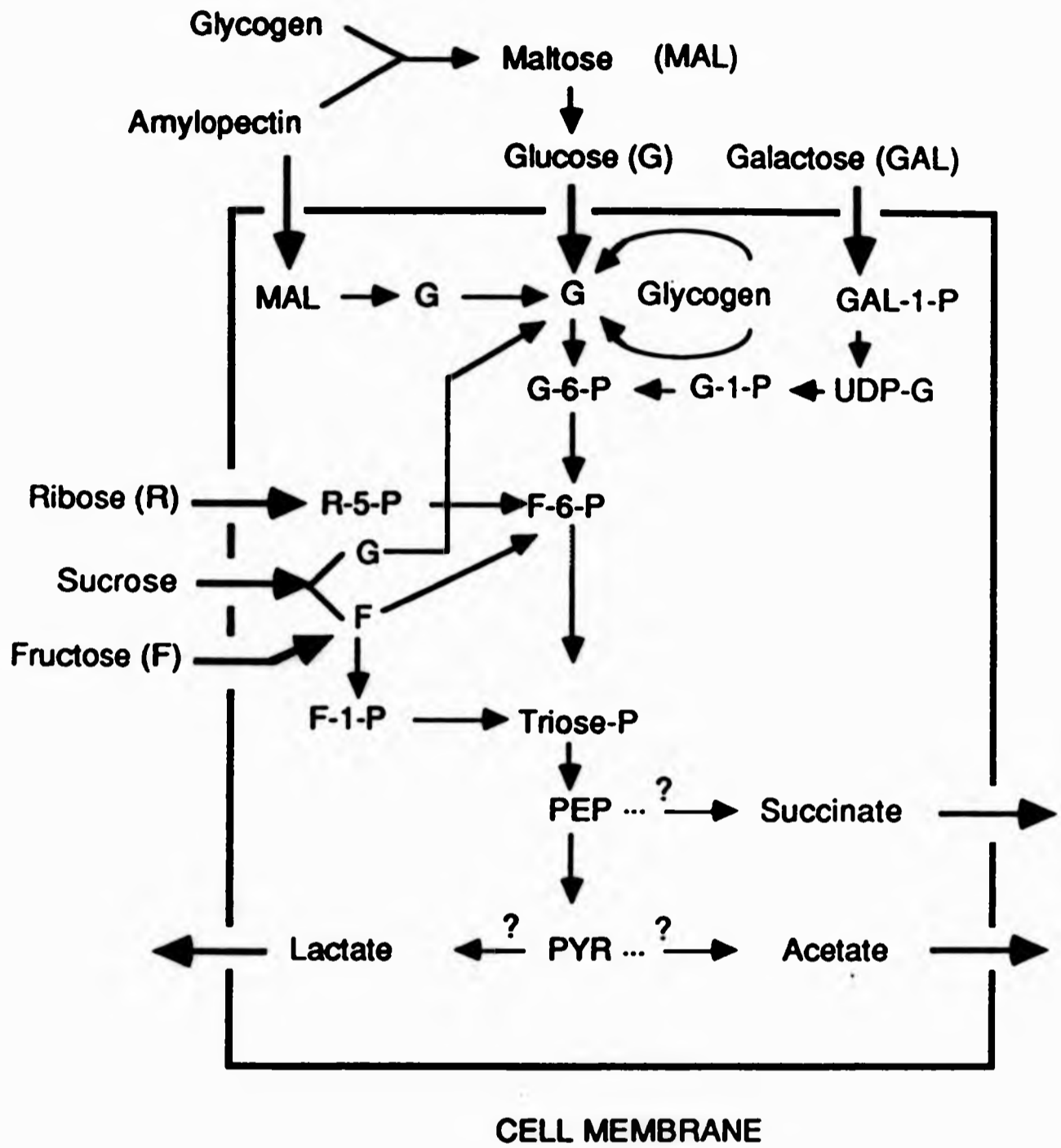


Fig. 5. Pathways of carbohydrate metabolism in *Pentatrichomonas hominis*. Arrows indicate the assumed physiological directions *in vivo*. The box represents the whole cell. Abbreviations used are: UDP-G, uridine diphosphate-glucose; G-1-P, glucose 1-phosphate; G-6-P, glucose 6-phosphate; F-6-P, fructose 6-phosphate; F-1-P, fructose 1-phosphate; triose-P, triose phosphate; PEP, phosphoenolpyruvate; PYR, pyruvate; R-5-P, ribose 5-phosphate. (Figure obtained from Lo et al. 1984).

metabolic reactions known to occur in the hydrogenosomes are illustrated in Fig 6. This process is accompanied by substrate level phosphorylation, involving acetate thiokinase (ADP, GDP), acetate, succinate CoA transferase and succinate thiokinase (ADP, GDP), the last of which does not occur in mammals. The reducing equivalents generated in the hydrogenosome are removed under anaerobic conditions as hydrogen. Under aerobic conditions oxygen is reduced via a non-haem terminal oxidase of high oxygen affinity, with the concomitant cessation of hydrogen production. It is thought that for the organelle to function effectively anaerobic conditions are required (Muller 1976). Recently an NAD⁺: ferredoxin oxidoreductase activity detected in *Trichomonas vaginalis* and *Tritrichomonas foetus* has been described in some detail (Steinbuchel and Muller 1986a). Both trichomonads were found to contain similar activities, although the precise role of the enzyme is not known. It was suggested that the enzyme is involved in the recycling of NADH required for malate dehydrogenase (decarboxylating) activity and/or the reoxidisation of ferredoxin.

Iron-sulphur proteins play a major role in hydrogenosomal electron transport, in particular ferredoxin is thought to be a physiological carrier in hydrogenosomal pyruvate oxidation (Marczak et al. 1983). This protein contains a [2Fe-2S] cluster, has a low molecular weight (approximately 12 kDa) and has been isolated from the hydrogenosomes of both *Tritrichomonas foetus* (Marczak et al. 1983) and *Trichomonas vaginalis* (Gorrell and Muller 1985). The amino acid sequences of the two trichomonad ferredoxins were found to be similar but they differ from mitochondrial and chloroplast ferredoxins. Using electron paramagnetic resonance spectroscopy techniques Chapman et al. (1986) demonstrated that

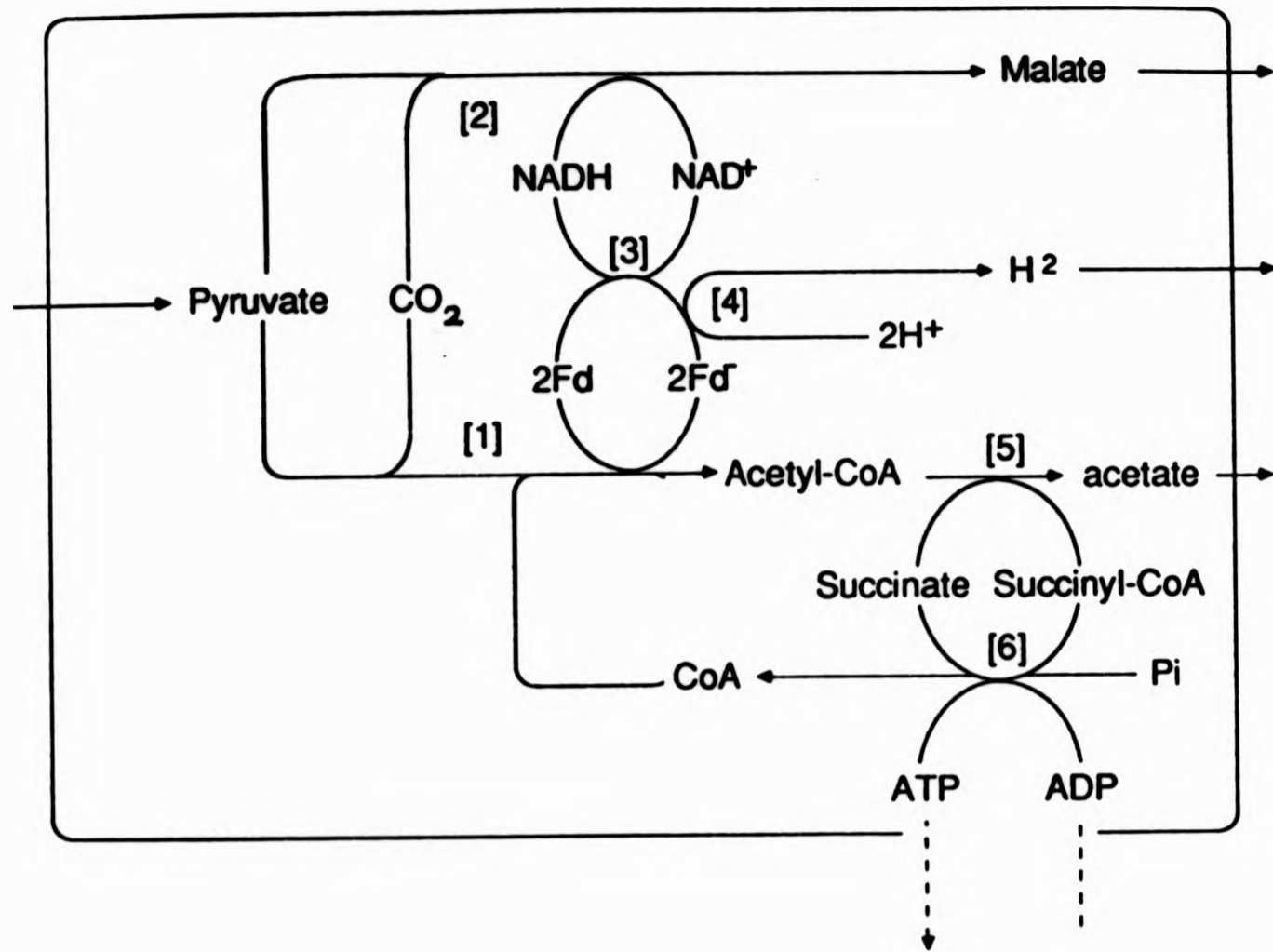


Fig. 6. Map of hydrogenosomal metabolism of *Tritrichomonas foetus* and *Trichomonas vaginalis*. Key to enzymes: [1], pyruvate: ferredoxin oxidoreductase; [2], malate dehydrogenase (decarboxylating) (NAD); [3], NAD⁺: ferredoxin oxidoreductase; [4], hydrogenase; [5], acetate: succinate CoA transferase; [6], succinate thiokinase. Arrows indicate the assumed physiological direction *in vivo*. Dashed arrows indicate a postulated adenyly nucleotide transfer. The box (hydrogenosome) shows only the organellar localisation of the enzyme activities and does not reflect their relationship to the organelle envelope. (Diagram obtained from Steinbuchel and Muller 1986b)

the pattern of iron clusters in *T. vaginalis* ferredoxin was more complex than in *Tritrichomonas foetus* ferredoxin. The synthesis of the two trichomonad ferredoxins *in vitro* on free polysomes has been studied using a combination of antibody, SDS-PAGE and fluorographic procedures (Weiss and Muller 1986). The results indicated that both ferredoxins are synthesised as larger precursors.

Hydrogenosomes play a significant role in the reductive activation of antitrichomonad 5'-nitroimidazole derivatives (Muller and Gorrell 1983; Cerkasovova *et al.* 1984; Kulda *et al.* 1984; Chapman *et al.* 1985b; Lloyd and Pedersen 1985; Lloyd and Kristensen 1985; Yarlett *et al.* 1985, 1986). *T. foetus* strains lacking hydrogenosomal functions exhibit low sensitivity to these compounds. It is now believed that reduced ferredoxin is the major electron donor involved in the reduction of metronidazole.

A number of other enzymic activities have been detected in trichomonad hydrogenosomes. They include NADH oxidase, NADPH oxidase, adenylate kinase and superoxide dismutase, whereas there is no evidence for the presence of acetyl phosphate transferase, acetate kinase, catalase or any of the enzymes of the Krebs or glyoxylate pathways (see Muller 1973; Cerkasov *et al.* 1978; Takeuchi and Kobayashi 1982). Thus, although there are some similarities, hydrogenosomes clearly differ from other micro-bodies such as mammalian peroxisomes, mitochondria and glycosomes. Other aerotolerant anaerobic protozoa including *Entamoeba histolytica* and *Giardia lamblia* which also lack mitochondria do possess typical hydrogenosomal enzymes but the enzymes in these two parasites are located free in the cytoplasm and hydrogenosomes appear to be absent

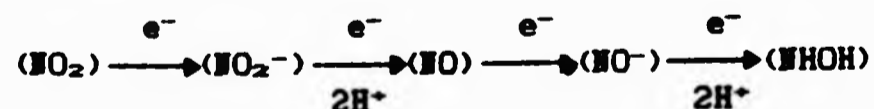
(Reeves et al. 1977; Lindmark 1980). At present the evolutionary and cellular origin of the organelles remains unknown.

1.7.3. Reductive activation of 5'-nitroimidazoles and mechanisms of resistance.

Reductive activation of the antitrichomonal 5'-nitroimidazoles such as metronidazole occurs in the hydrogenosome (Chapman et al. 1985b; Lloyd and Pedersen 1985; Yarlett et al. 1985). Pyruvate: ferredoxin oxidoreductase is the major source of reduced ferredoxin which is the electron donor for the reduction of metronidazole and its metabolites (Fig. 7)

Metronidazole was found to inhibit the production of hydrogen and carbon dioxide in *T. vaginalis* under both aerobic and anaerobic conditions. It was proposed not only that the drug competes successfully with protons for the available electrons but also the drug or a product of its reduction, actively inhibits some hydrogenosomal enzyme or electron carrier, involved in hydrogen and carbon dioxide production (Lloyd and Kristensen 1985).

The reduction of the nitro group of metronidazole under anaerobic conditions produces cytotoxic intermediates which are generated according to the scheme



proposed by Muller (1983).

The mechanism of cytotoxicity is not well understood but hypothetical schemes involving binding of these radicals to macromolecules have been proposed (Edwards 1979). The one-electron nitro radical (NO_2^-), the first product in the proposed metronidazole reduction

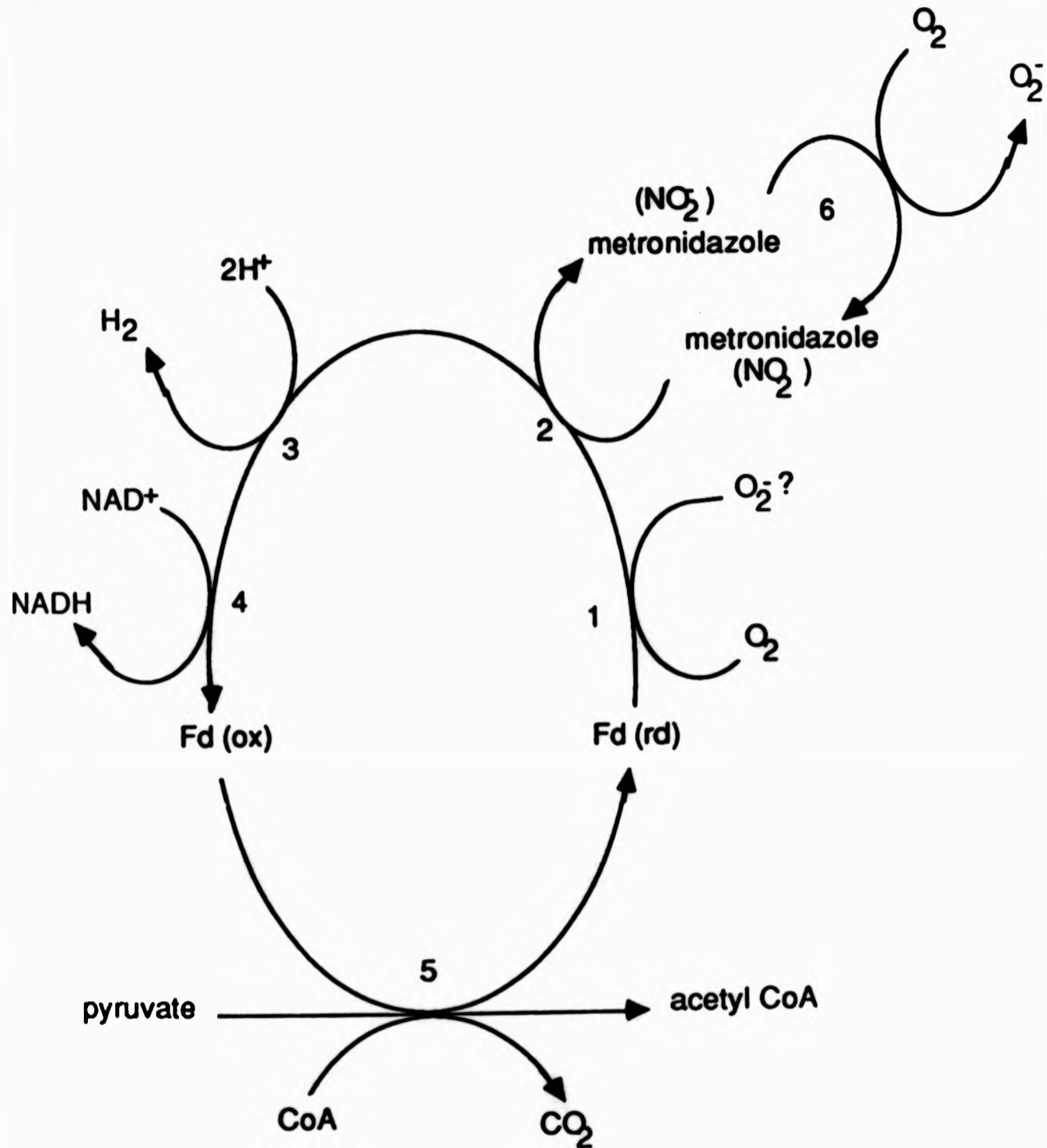


Fig. 7. Reductive activation of metronidazole and reoxidation of the nitro-free radical (NO_2^-) by oxygen. Key: rd, reduced; ox, oxidised; Fd, ferredoxin; 1, oxygen reduction; 2, metronidazole reduction; 3, hydrogenase; 4, NAD^+ :ferredoxin oxidoreductase; 5, pyruvate:ferredoxin oxidoreductase; 6, reoxidation of the one-electron nitro-free radical (NO_2^-) of metronidazole. (Diagram obtained from Thong 1986).

scheme, can be reoxidised by oxygen to yield the parent compound and superoxide (Muller 1973). This is thought to account, at least in part, for the lack of activity of 5'-nitroimidazoles against aerobes. The process of metronidazole reduction decreases the cellular level of the drug, thereby increasing the gradient of diffusion of the drug into the cell; metronidazole uptake occurs by passive diffusion.

Resistant strains of clinically isolated *T. vaginalis* have been reported (Meingassner et al. 1978; Lossick et al. 1986). In *in vitro* studies, however, resistance was only apparent in assays performed under aerobic conditions. Under anaerobic conditions the resistant strains show similar sensitivity to metronidazole as non-resistant strains and drug resistance appears to be directly related to aerobiosis. Laboratory induced anaerobic resistance to metronidazole *in vitro* of strains of *Trichomonas foetus* (Cerkasovova et al. 1984; Kulda et al. 1984; Cerkasovova 1985a; Kabickova et al. 1985) and strains of *Trichomonas vaginalis* (Cerkasovova 1985b; Demes et al. 1985; Kulda et al. 1985b) has been described. Anaerobic resistance is characterised by marked alterations in carbohydrate metabolism, including the elimination of hydrogenosomal functions (Cerkasovova et al. 1984).

In contrast very little is known of the mechanism of aerobic resistance. Comparisons of drug-resistant and drug-sensitive strains of *T. vaginalis* demonstrated decreased metabolic activation and lowered NADH oxidase activity in drug resistant lines. A later study however, failed to show any correlation between the level of drug sensitivity and aerobic and anaerobic fermentation, respiration, anaerobic intracellular accumulation of [¹⁴C] metronidazole and activities of pyruvate: ferredoxin oxidoreductase, NADH oxidase or NADPH oxidase (Muller and Gorrell 1983).

Similarly, studies on the enzymology of drug activation in *Tritrichomonas foetus* failed to show differences between susceptible and resistant strains (Cerkasovova et al. 1980). More recent studies have indicated that resistant strains of *Trichomonas vaginalis* possess defective oxygen scavenging systems within the hydrogenosomes (Lloyd and Pedersen 1985; Yarlett et al. 1986). It was suggested that unidentified oxygen scavengers normally present in the hydrogenosomes but modified in drug-resistant lines are implicit in 5'-nitroimidazole resistance in trichomonads. Thus, although the precise mechanism is still unknown, the observation that oxygen plays an important role in resistance of clinical *T. vaginalis* infections to metronidazole therapy is in agreement with the results of biochemical investigations of the parasite *in vitro*.

1.7.4. Polyamine metabolism.

Polyamines probably occur in all living organisms, the three most commonly found are putrescine (1,4-diaminobutane), spermidine and spermine (Raina and Janne 1975; Tabor and Tabor 1984, 1985). Although these compounds have been associated with a number of cellular processes including nucleic acid and protein biosynthesis, membrane and cell wall stability and as cofactors for enzymes, their precise functions are not fully understood. The importance of polyamines in normal cell growth is, however, indicated by the severe effects brought about by inhibitors and mutations which interfere with polyamine biosynthesis.

In general, eukaryotes contain both spermine and spermidine but have only trace amounts of putrescine, whereas prokaryotes possess higher concentrations of putrescine than spermidine and lack spermine (Tabor and Tabor 1976; Stevens and Winther 1979). Protozoa differ from this

generalization; the trypanosomatids apparently have a higher concentration of spermidine than putrescine while spermine is either absent or present in only small amounts (Bacchi 1981; White et al. 1983; North et al. 1986). Trichomonads, however, contain high concentrations of putrescine and lower concentrations of spermidine and spermine (White et al. 1983; North et al. 1986). Interest in polyamines in trichomonads has arisen owing to a possible link between polyamines and infection suggested by the presence of putrescine in the vaginal fluid of patients with trichomoniasis (Sanderson et al. 1983).

Ornithine is the direct precursor of putrescine. The enzyme that catalyses the reaction, ornithine decarboxylase has been detected in *Trichomonas vaginalis*, *Tritrichomonas foetus* and *Trichomitus batrachorum* (North et al. 1986). Trichomonads grown in the presence of α -difluoro-methylornithine (DFMO), a specific inhibitor of ornithine decarboxylase, and thus unable to synthesise putrescine were shown to accumulate exogenous putrescine. The dihydrolase pathway which converts arginine in a two step reaction to ornithine has also been detected in *Trichomonas vaginalis* (Linstead and Cranshaw 1983). This together with ornithine decarboxylase probably constitutes the major biosynthetic pathway of polyamines in trichomonads. The synthesis of spermidine from putrescine and spermine requires decarboxylated S-adenosyl-L-methionine (SAM). The enzyme responsible for the decarboxylation of SAM, SAM decarboxylase was identified in *T. vaginalis* and *Tritrichomonas foetus*. A schematic representation of the metabolism of polyamines in *Trichomonas vaginalis* is shown in Fig 8.

Although the exact function of polyamines in trichomonads is not known, a possible link between polyamines and pathogenicity has been

suggested by Bremner *et al.* (1987a). It was demonstrated that DFMO prevented the cytotoxic effects of *T. vaginalis* towards myeloma cells in culture. When the inhibitor was administered orally to mice, the drug inhibited the development of subcutaneous abscesses resulting from *T. vaginalis* infection.

1.7.5. Purine and pyrimidine metabolism.

T. vaginalis and *Tritrichomonas foetus*, in common with the majority of parasitic protozoa, are unable to synthesise purines *de novo* but depend solely on salvage pathways to meet their requirements for purine nucleotides (Wang 1983, Wang *et al.* 1983a,b; Wang and Cheng 1984). *T. foetus* generates nucleotides by salvaging purine bases with phosphoribosyl transferases. Unusually, a single enzyme accounts for the activity towards hypoxanthine, guanine and xanthine (Wang *et al.* 1983). In contrast *Trichomonas vaginalis* converts adenosine and guanosine to their respective monophosphates through the action of kinases (Miller and Linstead 1983). *T. vaginalis* cannot inter-convert the two mononucleotides, whereas nucleotide interconversions do occur in *Tritrichomonas foetus* (Wang 1984).

Pyrimidines can be synthesised *de novo* by most parasitic protozoa, but not by trichomonads or *Giardia* species. Of the enzymes of the *de novo* pyrimidine synthesis pathway only low activities of carbamoyl phosphate synthase and aspartate transcarbamoylase have been detected in *Trichomonas vaginalis* (Hill *et al.* 1981). Trichomonads salvage pyrimidines and pyrimidine nucleosides from their environment and several enzymes in the salvage pathway have been identified (Jarroll *et al.* 1983). Thymidine phosphotransferase which generates thymidine monophosphate

from thymidine, is an unusual enzyme and has been suggested as a good target for chemotherapeutic attack as it is responsible for the supply of all the thymidine nucleotides for DNA synthesis. *T. vaginalis* salvages cytidine and uridine in a similar manner to thymidine, despite possessing very high and as yet unexplained activities of cytidine deaminase and uridine phosphorylase, whereas *Tritrichomonas foetus* uses these enzymes to convert both cytidine and uridine to uracil, which is salvaged by uracil phosphoribosyl transferase. The UTP finally produced can be converted to CTP to satisfy the cytidine nucleotide requirement. *T. foetus* contains ribonucleotide reductase. This enzyme is not present in *Trichomonas vaginalis*, which obtains the required deoxyribonucleotides directly from deoxyribonucleosides through the action of deoxyribonucleoside phosphotransferase, an enzyme very similar to and possibly the same as thymidine phosphotransferase (Wang and Cheng 1984).

1.7.6. Lipid Metabolism.

The lipid metabolism of trichomonads is poorly understood. It is thought that they are unable to carry out oxygen-dependent steps of lipid biosynthesis and degradation and are unable to convert or retroconvert long chain fatty acids or cholesterol (Lindmark 1983). Roitman *et al.* (1978) demonstrated only slight incorporation of labelled acetate into sterols and fatty acids and consequently trichomonads were considered to be almost entirely dependent on exogenous lipids. Studies of the growth of *Trichomonas vaginalis* in defined medium demonstrated this requirement for fatty acids (oleic, palmitic and stearic) and cholesterol *in vitro* (Linstead 1981). Bromke (1986) described a similar serum-free growth

medium for *T. vaginalis* that was supplemented with only oleic acid and cholesterol. *In vivo* *T. vaginalis* obtains lipids from the host via binding human low density lipoproteins to specific surface receptors. Lipid-lipid interactions between the lipoproteins and trichomonad membranes and the subsequent internalisation of the lipid have been demonstrated (Peterson and Alderete 1984a,b).

The major lipid of intact trichomonads are; i) neutral lipids (30% of the total), free cholesterol > cholesteryl esters > triacylglycerols > unesterified fatty acids > wax esters, ii) phospholipids (65% of total), phosphatidylethanolamine > phosphatidylinositol > phosphatidylserine > sphingolipid > lysophospholipids > polyphosphoinositides and iii) glycolipids (5% of total).

It has recently been reported that trichomonads can convert saturated fatty acids to fatty alcohols, perhaps by a reductase-dehydrogenase mechanism and that the alcohols act as precursors in the synthesis of glyceryl ether lipids (Holz 1985). This along with minor chain elongation and sterol modification assumed to account for the slight incorporation of labelled acetate described by Roitman et al. (1978) are the only known examples of lipid metabolism in trichomonads.

1.7.7. Protein and amino acid metabolism.

Trichomonads require an exogenous source of amino acids for growth (Linstead 1981). At least twelve are essential for the axenic culture of *Tritrichomonas foetus*, including arginine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine and valine (Gutteridge and Coombs 1979). The mechanism of uptake is unknown. Host proteins are thought to provide the parasite's amino acid

requirements *in vivo*. The predominant amino acid found in *Trichomonas vaginalis* is alanine, which along with glutamic acid, proline and leucine accounts for 72% of the total free amino acids found in whole cells (Rowe and Lowe 1986).

There is very limited information available on *de novo* synthesis and interconversion of amino acids in trichomonads, although a wide range of aminotransferase activities have recently been described (Lowe and Rowe 1986b). High activities of aspartate, aromatic amino acid, branched chain amino acid and ω -amino acid (lysine and ornithine) aminotransferases have been detected. In contrast, no transaminase activity with β -alanine γ -aminobutyrate, arginine, glutamine, glycine or serine was detected (Lowe and Rowe 1986b). The aspartate and aromatic amino acid aminotransferase activities have been purified and are believed to be due to a single enzyme. Studies involving gostatin (5-amino-2-carboxy-4-oxo 1,4,5,6-tetrahydropyridine-3-acetic acid), an irreversible inhibitor of this enzyme, have demonstrated that activity was not essential for the growth of *T. vaginalis* in complex medium, presumably because any requirement for the products of the aspartate transaminase reaction can be obtained directly from the medium. In order to investigate activity in the absence of exogenous amino acids, semi-defined medium depleted of amino acids was used; even under these conditions, however, gostatin had no effect on parasite growth although it was reported that some amino acids, notably aspartate, were found to be present in the medium. Since aspartate was unlikely to be produced other than from oxaloacetate by the action of the transaminase, it is probable that its presence is due to proteolysis of serum proteins in the medium or leakage from the cells. It was concluded that in the absence

of non-protein containing media the significance of the aminotransferase in the interconversion or metabolism of amino acids in trichomonads could not be established (Lowe and Rowe 1986a).

Amino acids are required by trichomonads as precursors of other important molecules, e.g. polyamines. Arginine is broken down to ornithine, ammonia and carbon dioxide by the three enzymes of the dihydrolase pathway (Linstead and Cranshaw 1983). The importance of ornithine as the direct precursor of the polyamine, putrescine has been described in section 1.7.4.

Cysteine and thiol compounds are thought to play an important role in countering oxygen toxicity and maintaining cell integrity (Gillin et al. 1984) and thus sulphur amino acid metabolism in trichomonads may differ significantly from that in mammalian cells. One example of this is the high level of homocysteine desulphurase activity detected in *T. vaginalis*. Homocysteine desulphurase catalyses the desulphuration of homocysteine to hydrogen sulphide, ammonia and α -ketobutyrate, a reaction thought to be of little significance in mammals (Thong and Coombs 1985a). In addition, trichomonads were shown to possess very high levels of L-serine sulphhydrylase activity which is quite distinct from the homocysteine desulphurase activity (Thong and Coombs 1985b). A detailed account of the metabolism of sulphur amino acids in trichomonads was provided by Thong (1986)

Despite the extent to which protein synthesis has been studied in mammals and bacteria, there have been very few reports on protein synthesis in trichomonads. Proteolysis has received a little more attention. Coombs (1982) compared the levels of proteinase activity in a range of flagellate protozoa and demonstrated that *T. vaginalis* contained

relatively high levels of proteinase activity. Subsequent investigation of the enzymes responsible for this activity by electrophoretic analysis on gels containing denatured haemoglobin demonstrated that multiple forms of proteinase could be detected in cell lysates of *T. vaginalis*. All of the enzymes were stimulated by dithiothreitol (DTT) and had inhibitor sensitivities characteristic of cysteine proteinases, however differences between the forms were observed with respect to pH optima and their relative sensitivities to inhibitors (Coombs and North 1983).

A low molecular weight cysteine proteinase has been purified and characterised from homogenates of *Tritrichomonas foetus*, which was capable of hydrolysing a range of low molecular weight and protein substrates. The proteinase was considered to be the major, and possibly the only endopeptidase of *T. foetus* (McLaughlin and Muller 1979).

1.8. Proteolytic enzymes.

The nomenclature used for proteolytic enzymes is not always clear and often misleading. In an attempt to clarify this situation, the International Union of Biochemistry recommended that the general term 'peptidase' should be used to describe any enzyme that hydrolyses peptide bonds, in preference to the less acceptable but widely used term 'protease'. Peptidases are subdivided into two classes, the endopeptidases, generally referred to as proteinases and the exopeptidases, including carboxypeptidases, aminopeptidases and dipeptidases (Barrett 1986).

1.8.1. Classification of endopeptidases.

Endopeptidases (proteinases) can be classified in a number of different ways. Often, they are classified on the basis of their ability to hydrolyse specific proteins eg elastase, collagenase etc. This is usually unsuitable since many proteinases are not highly specific and are capable of hydrolysing a range of different substrates, nor does it convey any information on the physical properties of the enzyme. Another widely adopted method of classifying proteinases is on the basis of their similarities to well characterised proteins such as trypsin, chymotrypsin and the mammalian cathepsins. This can often prove misleading, especially if only a restricted number of properties are compared. Classification based on the pH range over which the proteinases are active is equally unsuitable since many proteinases are active over a broad pH range. Furthermore the optimum pH of a proteinase often differs with different substrates and thus may be considered to be as much a property of the substrate as of the enzyme.

The most satisfactory classification scheme is that proposed by Hartley (1960) based on the catalytic mechanism of the proteinase. This forms the basis for the Enzyme Commission classification given in Table 3. There are four types of proteinase and these can be distinguished on the basis of their sensitivity to various inhibitors (Table 3). Some commonly used inhibitors are not specific to one type of proteinase. For example, the chloromethyl ketone derivatives N- α tosyl-L-lysine chloromethyl ketone (TLCK) and L-tosylamido-2-phenylethyl chloromethyl ketone (TPCK) and the inhibitors of microbial origin leupeptin and antipain all inhibit some serine proteinases as well as many cysteine proteinases.

Table 3. Classification of proteinases.

Type	Specific inhibitors: characteristic of enzyme type	Other inhibitors	Activators
Aspartic protein- ases (EC 3.4.23)	Pepstatin S-PI (acetyl pepstatin) Diazocetyl norleucine methyl ester Epoxy (p-nitrophenoxy) propane		
Metalloproteinases (EC 3.4.24)	Chelating agents EDTA o-phenanthroline 8-hydroxyquinoline α,β-dipyridyl Phosphoramidon (not acid metal- loproteinases)		
Serine proteinases (EC 3.4.21)	PMSF DIFP	TLCK TPCK Antipain Leupeptin Chymostatin	
Cysteine protein- ases (EC 3.4.22)	Iodoacetamide, iodoacetate Heavy metals N-Ethyl maleimide	p-Chloromercuribenzoate (also inhibits some serine proteinases) TLCK TPCK Antipain Leupeptin Chymostatin	Reducing agents Cysteine DTT EDTA

Table taken from North (1982).

In general, proteinases of the same class exhibit some similar properties. In particular, there is some overlap between the EC classification scheme and that based on pH dependence. The aspartic (carboxyl) proteinases are all active at acid pH values and in the absence of any supporting data many acid proteinases have been assumed to be of the aspartic type. Some acid proteinases have, however, been shown to be cysteine (thiol) proteinases which are usually more active at slightly acidic pH values. Metalloproteinases are active around neutrality and serine proteinases are most active at alkaline pH values. Indeed the terms neutral proteinase and alkaline proteinase are often used synonymously for these two types (North 1982).

1.8.2. Properties of proteinases.

1) Cysteine proteinases.

The best characterised cysteine proteinases are the mammalian cathepsins B, H, and L (Turk 1986) and the plant proteinase papain (Glazer and Smith 1971). Cysteine proteinases have also been detected in birds, fish, invertebrates and eukaryotic microorganisms, including protozoa and slime moulds. The frequency of cysteine proteinases in protozoa is particularly high (North 1982).

For the cysteine proteinases that have been sequenced to date, a considerable degree of homology has been demonstrated and it has been suggested that they may have evolved from a common ancestral gene (see North 1986). Mechanistic studies of cysteine proteinases have shown a cysteine-histidine interactive system within the catalytic site and it

seems probable that this could be a common feature of all enzymes in this group (Brocklehurst 1986).

Recently there has been a growth in interest in cysteine proteinases (see Turk 1986). This may in part be owing to the suggestion that these enzymes may have a role in tumour growth and metastasis (Sloane *et al.* 1986) and partly due to the recent isolation of endogenous cysteine proteinase inhibitors which are believed to form part of the inhibitory defence system to protect cells against the harmful effects of unspecific proteolysis by cysteine proteinases (Turk 1986). Cysteine proteinase inhibitors may be divided into two classes, low M_r inhibitors including stefins and cystatins and high M_r inhibitors represented by kininogens. Sequence comparisons among the various cysteine proteinase inhibitors have revealed the presence of the peptide Gln-Val-Val-Ala-Gly which is thought to form part of the reactive site of the inhibitors (Muller-Esterl *et al.* 1986).

Many cysteine proteinases, especially those of mammalian lysosomes, are acid proteinases with very similar properties to one another, but other activities have also been described. Ca^{2+} -dependent cysteine proteinases, e.g. calpain represent a subclass of cysteine proteinases. Although they have an absolute requirement for Ca^{2+} , the possibility that these are metalloproteinases has been eliminated as a cysteine residue has been identified at the active site and the amino acid sequence around the cysteine residue is homologous to those of typical cysteine proteinases (Kawashima *et al.* 1986). It has been suggested that these non-lysosomal enzymes have different roles to those of the cathepsins.

ii) Serine proteinases.

Serine proteinases have been detected in a wide range of organisms including eukaryotic microorganisms, although there are few reports of serine proteinases in protozoa (North 1982). The best characterised of the microbial eukaryote enzymes are those isolated from fungi. Serine proteinases have a similar catalytic mechanism to cysteine proteinases with a serine residue at the active site in place of cysteine. Almost all serine proteinases are inhibited by PMSF but many are also inhibited by some thiol reagents. This may reflect the close proximity of a cysteine residue to the active site. Binding of a bulky residue to the cysteine may indirectly interfere with substrate binding, as suggested for the serine carboxypeptidase of yeast, carboxypeptidase Y (Bai and Hayashi 1979). It is unlikely that the cysteine participates directly in the catalytic mechanism. Serine proteinases are generally active around neutral pH and have a broad specificity as demonstrated by their ability to cleave the oxidised insulin B chain at a large number of different sites (Lindberg et al. 1981).

iii) Aspartic proteinases

Aspartic proteinases are normally acid proteinases with pH optima between pH 3 and 5. They are not inhibited by serine proteinase inhibitors, chelating agents or thiol compounds. Inhibitors of aspartic proteinases, e.g. pepstatin, have been used to isolate active site peptides and to directly demonstrate the involvement of one or two aspartic acid residues. Recent sequence data for a number of aspartic proteinases has revealed two conserved aspartic acid residues (see Takahashi 1987). Acid proteinases have been detected in a range of eukaryotic micro-organisms

including protozoa. Aspartic proteinases isolated from fungi have been shown to have properties similar to those of pepsin and rennin, proteinases of the digestive tract of mammals, and have attracted particular interest owing to their industrial applications. Two such processes include the hydrolysis of soybean protein to make soy sauce and milk coagulation in the preparation of cheese (Aunstrup 1980). Most of the fungal proteinases have broad specificity but preferentially hydrolyse peptide bonds between two bulky amino acids: for example most of the fungal proteinases cleave the B chain of insulin at the Tyr(16)-Leu(17) and Phe(24)-Phe(25) bonds (North 1982).

iv) Metalloproteinases

Metalloproteinases are endopeptidases that contain an essential metal ion, usually zinc. They have been reported in both prokaryotic and eukaryotic microorganisms. Generally metalloproteinases have optimum activities at neutral or alkaline pH, but are stable over a broad pH range. The stability is increased considerably by the addition of Ca^{2+} to the reaction mixture, but there is no requirement for Ca^{2+} for activity. Chelating agents, e.g. EDTA, inhibit metalloproteinases by removing the metal atom. The best characterised metalloproteinases preferentially hydrolyse peptides with hydrophobic side chains such as phenylalanine and leucine and often have very weak esterase activity (Aunstrup 1980).

v) Others.

Although most of the proteinases isolated and characterised to date fall into one of the above classes, there are exceptions. Most of these

may be due to incomplete characterisation of the enzymes, but there are examples of proteinases that clearly do not fit into the above categories (Titani *et al.* 1987).

1.8.3. Assessment of proteolytic activity.

A range of substrates have been used to detect proteinase activity including low molecular weight chromogenic or fluorogenic substrates, high molecular weight protein substrates or chromogenic or radioactively labelled derivatives. No assay system is of universal value because individual proteolytic enzymes require their own specific and optimal assay conditions. This situation becomes even more complicated in the case of determining proteolytic activity in a mixture of unknown proteinases present in total cell extracts. Electrophoretic techniques have been used with some success to separate and identify proteolytic species from complex mixtures such as cell lysates. The enzymes are separated on a polyacrylamide gel, which is then incubated under conditions that allow digestion of the substrate, which may have been copolymerised in the gel or added after electrophoresis. However, this type of technique will only detect proteinases active on the particular substrate under the particular set of conditions used.

Although the *in vitro* properties of a proteinase may provide some indication of its *in vivo* role, the use of non-physiological substrates can only provide limited information. Only in rare cases is the natural protein substrate(s) of an individual proteinase available or even known. A knowledge of the localisation of the enzyme, and alterations of proteinase levels during physiological responses or developmental changes may provide further information on *in vivo* roles.

A more direct approach is to manipulate proteinase activity *in vivo* using proteinase inhibitors. There are, however, few inhibitors specific for individual proteolytic enzymes and the possibility that other cellular processes not involving proteinase activity may be affected can not be excluded. A more precise means of manipulating proteinase activity *in vivo* is through the isolation of appropriate mutants, or naturally occurring strains lacking particular proteolytic enzymes. As yet this type of study has been confined mainly to investigations carried out on yeast mutants (Wolf 1986).

Studies on protozoa have been mainly confined to assessments of the roles of proteinases based on comparisons of activity between related species, for example between pathogenic and non-pathogenic species. Such comparisons have often been made, however, by measuring proteinase levels found under culture conditions. These might not bear any relationship to those experienced during infection and so should be interpreted with great caution.

1.8.4. Endopeptidase (proteinase) activity in protozoa.

The possibility that proteinases may play a role in the pathogenesis of parasitic protozoa and other aspects of the host-parasite interaction has stimulated a significant amount of research into the proteolytic systems of these parasites. Proteolytic activity has been detected in many species and some of the enzymes responsible have been purified and characterised. The roles suggested for some of these enzymes are discussed in section 1.8.6.

1) Flagellates.

Proteinase activity in *Trypanosoma cruzi*, the causative agent of American trypanosomiasis, Chagas' disease, was first reported by Goncalves et al. 1958, since when several enzymes have been reported and partially characterised (see Cazzulo 1984). Itow and Camargo (1977) detected four proteolytic activities from the epimastigotes of the Y strain, which differed from one another with respect to substrate specificity, pH optima, inhibitor sensitivity, electrophoretic mobility and whether they were particulate or soluble. Some of the activities may be released into the extracellular medium (Jankevicius and Jankevicius 1981). Avila et al. (1979) detected five different activities, which they named exopeptidases I, II and III and cathepsins A and D. Only one of these enzymes, cathepsin D had activity towards a protein substrate, haemoglobin, and it was shown to be insensitive to thiol compounds. Toruella et al. (1981) confirmed the presence of a proteinase able to hydrolyse azocasein and α -L-benzoyl-D-L-arginine-p-nitroanilide (BAPA) in six stocks of *T. cruzi*.

Three proteinases have been purified from *T. cruzi*. Bongertz and Hungerer (1978) purified a high molecular weight proteinase from the amastigotes of the D₁ strain that hydrolysed BAPA. It was strongly inhibited by sulphhydryl reagents and activated by 2-mercaptoethanol. Rangel et al. (1981), purified a proteinase of molecular weight 60 kDa from epimastigotes of the Y strain which hydrolysed casein at pH 7.0 and haemoglobin at pH 3.0. This enzyme was also reported to have the characteristics of a cysteine proteinase. A third cysteine proteinase of a similar molecular weight to that reported by Rangel et al. (1981) was described by Bontempi et al. (1984). It was shown to be active at acid

pH towards several protein substrates but did not hydrolyse BAPA nor any of the *p*-nitroanilide derivatives tested.

Proteolytic activity has been detected in a number of species of African trypanosomes. A proteinase isolated from *T. brucei rhodesiense* trypomastigotes was originally described as an aspartic proteinase (Venkatesan *et al.* 1977). However, it has since been shown that the major proteinase of bloodstream forms of *T. brucei* is a cysteine proteinase, stimulated by thiol compounds, inhibited by *p*-chloromercuric benzoate and unaffected by pepstatin (Steiger *et al.* 1979). North *et al.* (1983) investigated the proteolytic enzymes of four species of trypanosomes. Four types of proteolytic activity were detected in the bloodstream forms of the four *Trypanosoma* species; i) an activity towards hide powder azure, ii) an activity towards azocasein, iii) an activity towards Bz-Pro-Phe-Arg-Man in the presence of DTT (type 1) and iv) an activity towards several *p*-nitroanilide derivatives in the absence of DTT (type 2). Studies of the pH optima, DTT requirements and inhibitor sensitivities suggested that the activity towards hide powder azure and type 1 activities could be due to the same enzymes, probably cysteine proteinases. AZCase had some characteristics of a cysteine proteinase activity but was not identical to the HPAase and type 2 activities and it was suggested that it could be due to a serine proteinase. Proteolytic enzymes were also separated by electrophoresis on gels containing denatured haemoglobin. Cell lysates of *T. brucei*, *T. evansi* and *T. equiperdum* were shown to contain at least three proteolytic species, however no proteolytic activity was detected in *T. vivax* using this technique.

Cysteine proteinases were also detected in a range of African trypanosomatids by SDS-PAGE using gels containing fibrinogen or collagen (Lonsdale-Eccles and Npimbaza 1986). The proteinases were found to have pH optima between 5 and 6, required DTT or 2-mercaptoethanol for full expression and were inhibited by cysteine proteinase inhibitors and trypanocidal drugs. *T. evansi*, *T. b. brucei* and *T. b. gambiense* had similar activities of molecular weight 28 kDa. In addition, higher molecular weight proteinases (up to 105 kDa) were also detected. *T. congolense* was found to have a low molecular weight proteinase (31 kDa) in addition to several higher molecular weight proteinases (Lonsdale-Eccles and Npimbaza 1986).

Proteinases have been reported to be present in much smaller amounts in the procyclic forms than in the metacyclic or mammalian bloodstream forms of African trypanosomes (North et al. 1983; Lonsdale-Eccles and Npimbaza 1986).

The proteolytic activities in *Leishmania* species have been relatively little studied. Proteinases have been reported to be present in the cultured promastigotes of several species (Camargo et al. 1978; Steiger et al. 1979; Fong and Chang 1981; Simon and Makkoda 1983), however the activities of amastigotes have been studied in detail only for *Leishmania mexicana mexicana* (Pupkis and Coombs 1984). The proteinases of *L. m. mexicana* amastigotes and promastigotes have been analysed by electrophoresis on polyacrylamide gels containing denatured haemoglobin (North and Coombs 1981). Eleven bands of activity were detected indicating that multiple proteinases were present in the cells. As with trypanosomes there were significant quantitative, and furthermore qualitative, differences between the proteinases of the two major

developmental forms. Most of the proteinases showed characteristics of cysteine proteinases. Subsequent purification of proteinases from the promastigotes and amastigotes of *L. m. mexicana* revealed that the major proteinase activity in amastigotes was indeed due to a cysteine proteinase, molecular weight 31 kDa, whereas a second minor activity was due to an enzyme with a molecular weight of 67 kDa that showed some characteristics of a metalloproteinase. A soluble proteinase purified from the promastigotes was shown to be similar to the amastigote minor activity. It was suggested that these results indicate that a highly active substrate-specific soluble proteinase with characteristics of a cysteine proteinase is produced upon transformation of the *L. m. mexicana* promastigotes to amastigotes (Pupkis and Coombs 1984).

It has recently been reported that a highly conserved protein, present on the surface of promastigotes of *L. donovani*, *L. major*, *L. tropica*, *L. m. mexicana* and *L. braziliensis* (Lepay et al. 1983; Bouvier et al. 1985; Etges et al. 1985) has proteinase activity in *L. major* (Etges et al. 1986). The enzyme has a molecular weight of between 60 and 70 kDa but has not yet been fully characterised.

Proteolytic activity has also been detected in other trypanosomatids and phytoflagellates and the proteolytic system of *Euglena gracilis* has been studied in some detail. Cysteine, serine and aspartic proteinases have all been described (see North 1982). There have been several reports of intracellular serine proteinases (Langner et al. 1979). In addition, a proteinase has been isolated from the culture medium and purified; it is severely inhibited by diisopropylfluorophosphate and to some degree by phenanthroline, EDTA and p-chloromercuric-benzoate (Wakano et al. 1979). More recently soluble and particulate acid proteinases have

been demonstrated in different *E. gracilis* cell types. Cysteine proteinase activity was present in both the soluble and particulate fractions and aspartic proteinases in the particulate fractions. The enzymes responsible have not been purified (Krauspe et al. 1986).

ii) Amoebae.

The organism that has been most frequently studied is *Entamoeba histolytica* and there have been a number of reports of cysteine proteinases in this organism. Jarumilinta and Maegraith (1961) showed that both the trophozoites and extracts prepared from them were able to hydrolyse a number of proteins and synthetic substrates. The enzyme responsible was suggested to be trypsin-like on the basis of its specificity. McLaughlin and Faubert (1977) partially purified two proteinases. One was shown to be a cysteine proteinase, the other was insensitive to cysteine proteinase inhibitors and it is not known which class it belongs to. A cytotoxic cysteine proteinase with a molecular weight of 16 kDa was isolated more recently from trophozoites (Lushbaugh et al. 1985), the purified enzyme had similar properties to the cysteine proteinase described by McLaughlin and Faubert (1977). Scholze and Verries (1984) have also isolated cysteine proteinases from *E. histolytica* that are active towards native enzyme substrates. Cysteine proteinase activity has also been demonstrated in *E. invadens* (Avila et al. 1985). There have been very few reports of proteinases in other amoebae. Proteinase activity has been reported in various species of *Acanthamoeba* (Auriault and Desmazeaud 1979). Two proteinases are released by *Hartmannella culbertsoni* during encystment, one of which has

been purified and characterised. It was not affected by thiol reagents but was inhibited by PMSF and is believed to be a serine proteinase.

iii) Sporozoa.

The species most studied are those of the genus *Plasmodium*, the malarial parasites. Successful analysis of their proteinases, however, has been hindered by the difficulty of obtaining samples of parasites that are free of host cell material, particularly erythrocyte membranes which themselves contain both acid and neutral proteinases (North 1982).

The asexual erythrocytic stages of malaria parasites contain proteinases which are responsible for the digestion of haemoglobin, however they are also known to produce several other stage-specific proteolytic enzymes which are less well characterised. An endopeptidase that digests haemoglobin at acid pH (Chan and Lee 1974, Levy et al. 1974) has been partially characterised (Gyang et al. 1982; Sherman and Tanigoshi 1983). An alkaline endopeptidase active at pH 7 and 8 has been isolated from soluble fractions of *P. knowlesi* (Cook et al. 1969) and *P. berghei* (Schrevel et al. 1984). Intracellular enzymes with endoarylamidase and aminopeptidase activities have also been described (Slomianny et al. 1983) and partially characterised (Vander Jagt et al. 1984). There have been a number of reports on the proteinases of *P. falciparum*. Gyang et al. (1982) described three proteolytic activities in *P. falciparum*. Two of these, an acid proteinase that degrades haemoglobin and an aminopeptidase were shown to be inhibited by anti-malarial compounds. The proteinase has been purified (Vander Jagt et al. 1986). It has a molecular weight of less than 10 kDa, and is strongly inhibited by pepstatin but only partially by bestatin, antipain and

phosphoramidon. It was suggested that it was one of the proteinases responsible for the degradation of haemoglobin.

iv) Ciliates.

Fok and Paeste (1982) described the presence of sixteen hydrolase activities in *Paramecium caudatum* and *P. tetraurella*, including equivalents of cathepsins B, C, D and E. 'Cathepsin B' was shown to have an acidic pH optimum and was localised in the lysosomal fraction. They also demonstrated changes in the levels of 'cathepsin C' during growth of the organism. The only other reports of proteinases in *Paramecium* concern a proteinase which affects the structure of the cell surface immobilization antigen in *P. aurelia* which has the characteristics of a cysteine proteinase (Steers and Davis 1977).

Tetrahymena proteinases have been studied in more detail. Lawrie (1937) first detected proteinase activity towards casein, gelatin and α -glutinin. Dickie and Liener (1962) purified three proteinases from *Tetrahymena*, one intracellular and two extracellular proteinases. Later studies, however, revealed multiple forms of both intracellular (Levy et al. 1976) and extracellular proteinases (Blum 1976). Although no detailed characterisations were carried out, Blum (1976) reported that although the activity was inhibited by leupeptin, antipain and chymostatin but not totally suggesting that there may be more than one class of proteinase present. Banno et al. (1982) described four forms of both intracellular and extracellular proteinases active towards azocasein and Bz-Arg-Man active at neutral pH. These also had the properties of cysteine proteinases. Multiple forms of intracellular proteinases in *Tetrahymena pyriformis* were described by North and Walker (1984). Acid and neutral

proteinases were detected using a variety of different assay conditions. The neutral activities could be resolved into at least three forms by ion-exchange chromatography, each form had a different substrate specificity. The acid proteinase activity was similarly separated into different fractions. The intracellular activity of the acid proteinases responded differently from that of the neutral activities to culture age or resuspension of the cells in fresh medium and starvation buffer. Furthermore, analysis on sucrose density gradients showed that although the neutral activities had a similar subcellular distribution to one another, this differed from that of the acid proteinase activity. This suggests that the physiological roles of the acid and neutral proteinases may differ. A lysosomal proteinase has been reported which has the property of splitting 80S ribosomes. The cells also contain a cytoplasmic proteinase inhibitor which inhibits the ribosomal splitting enzyme (Klemperer and Pilley 1985). The enzyme and inhibitor were subsequently purified and characterised and shown to have cathepsin B and cystatin-like properties, respectively (Murrice 1986).

1.8.5. Exopeptidase activity in protozoa.

Despite the large amount of information now available on endopeptidases in protozoa relatively little attention has been given to the exopeptidases, and although activity has been demonstrated in a number of species, very few of the enzymes responsible have been purified and characterized in detail.

1) Flagellates.

Six peptidases which correspond closely to those present in human erythrocytes have been identified in the blood stream forms of *Trypanosoma brucei* and can be distinguished from one another on the basis of their specificities to a range of dipeptide and tripeptide substrates (Letch and Gibson 1981). No variation in electrophoretic mobility was found for the six peptidases among the four stocks studied although polymorphism had been reported previously (Gibson et al. 1980). Stocks did, however, differ in the presence or absence of particular peptidase bands.

No correlation has been found between peptidases of *Leishmania* sp. and those of human erythrocytes (Letch and Gibson 1981), although both *Trypanosoma brucei* and *Leishmania mexicana mexicana* peptidases have been reported to vary between different developmental stages of the parasite (see North 1982).

Palmer (1974) demonstrated the presence of arylamidases in *Crithidia fasciculata* which hydrolyse the β -naphthylamides of leucine, arginine, lysine and glutamic acid. More recently Oe et al. (1984) reported that a folate-hydrolysing enzyme previously purified from *C. fasciculata* hydrolysed various 2-L-dipeptides indicating a new type of carboxypeptidase in this species.

At least two aminopeptidases active on glycyl-L-phenylalanine- β -naphthylamide are present in *Tritrichomonas foetus* (McLaughlin and Muller 1979).

Six major types of aminopeptidases and an acid carboxypeptidase have been detected in *Euglena gracilis* many of which have been purified and characterised (Richter et al. 1972; Senkpiel et al. 1973, 1974).

Further multiplicity of the peptidases was revealed on purification and more recently the molecular and physiological characteristics of the apparently complex aminopeptidase system of *E. gracilis* have been described (Barth *et al.* 1980a,b).

ii) Amoebae.

There have been very few reports of exopeptidases in amoebae. Jarumilinta and Naegraith (1961) described peptidase activity in *Entamoeba histolytica*. More recently Jonckheere and Dierickx (1982) reported finding only low levels of leucine aminopeptidase activity in the pathogen *Naegleria fowleri* in comparison to that found in other *Naegleria* species. The authors suggested that determination of the specific activity of this enzyme would allow for a quick identification of the pathogenic species.

iii) Sporozoa.

Aminopeptidases have been identified in *Plasmodium yoelii nigeriensis* and *P. chabaudi* (Charet *et al.* 1980b), *Babesia hylomysci* (Assi and Charet 1981), *Eimeria nieschulzi* (Charet *et al.* 1980b) and *E. tenella* (Wang and Stotish 1978). All of the enzymes were found to have similar properties and notably were all inhibited by antimalarial drugs including chloroquine, quinacrine, primaquine and quinine. Charet *et al.* (1980b) however, concluded that the action of antimalarial drugs on aminopeptidase was probably of limited interest since inhibition was obtained with high concentrations and was relatively unspecific. On the other hand the remarkable similarity between the aminopeptidases of the *Eimeria* genus, *Plasmodium* and also *Babesia*, considering their different

nutritional requirements, suggests that this enzyme plays a role in the parasites' own basic metabolism rather than in host protein degradation.

The latter has been supported by the findings of Gyang *et al.* (1982) who purified three proteolytic enzymes from *P. falciparum*, one of which was a neutral aminopeptidase with similar properties to the enzymes described previously. It was suggested from its pH profile that this aminopeptidase may be cytosolic and that unlike the haemoglobin-splitting peptidase did not act in an acid environment such as the lysosome.

Vander Jagt *et al.* (1984) have also described the purification of an aminopeptidase from *P. falciparum* which had similar inhibitor specificities, pH optimum, sensitivity to metal ions and inhibition by antimalarials to the previous enzyme (Gyang *et al.* 1982), and the authors concluded it to be cytosolic (again this was mainly based on the pH dependence of the enzyme). They claimed, however, it to be different from the aminopeptidase activity purified by Gyang *et al.* (1982).

iv) Ciliates.

Peptidase activity has been detected in *Tetrahymena*, the most recent report being that of Zdanowski and Rasmussen (1979) which described peptidases in the cytoplasm and on the outer cell surface of *Tetrahymena thermophila*.

Abou Akkada and Howard (1962) described peptidase activity detected at neutral pH with glycyl-L-leucine as substrate in the rumen protozoan *Entodinium ecaudatum*.

1.8.6. Role of proteinases in protozoa.

Traditionally proteinases have been regarded as primarily degradative enzymes responsible for the breakdown of proteins into small peptides and amino acids to meet nutritional requirements and participate in cellular protein turnover. More recently it has been shown that some proteolytic enzymes have the ability to carry out selective modification of proteins by limited cleavage and that they have a role in a whole range of cellular processes. Although these roles have been investigated in most detail in mammalian and bacterial systems, there is an increasing amount of information available on the roles of proteinases in protozoa.

1) Post-translational processing.

Primary translation products are often larger than the final product and proteolysis must be involved in the subsequent processing (Johnson and Brown 1974). One recently reported example of this in protozoa is in the synthesis of trichomonad ferredoxins (Weiss and Muller 1985). Other examples have been described for *Tetrahymena*. An enzyme in the postmicrosomal supernatant which could hydrolyse *N*-benzoyl-L-tyrosine ethyl ester is believed to be responsible for the proteolytic cleavage of pellicular proteins (Collins and Willhelm 1978). The inhibitor TPCK increased the level of the precursor protein. Proteolytic processing has also been implicated in the processing of micronuclear histones in *Tetrahymena* (Allis et al. 1980; Allis and Wiggins 1984; Allis et al. 1984) and in the processing of crystalline secretion products of *Paramecium* (Adoutte et al. 1984)

ii) Protein turnover.

The turnover of cellular protein which was first described in animals by Schoenheimer (1942), allows cells to remove abnormal proteins and to adapt their complement of protein more rapidly to changing physiological needs. One protozoan in which a proteolytic response to physiological changes has been reported is *Tetrahymena*. *Tetrahymena thermophila* is usually grown in a medium containing proteose peptone and yeast extract as organic nutrients. However, when the ciliate is transferred to an inorganic medium the cells respond by rapidly increasing their rate of protein degradation. *Tetrahymena* responds in much the same way as has been described in mammalian cell types, by a stimulation of autophagy. Autophagy is a process whereby proteins and other cytoplasmic components are sequestered by membranes and subsequently degraded by lysosomal enzymes (Jonassen and Grinde 1986).

iii) Activation and inactivation of enzymes.

Many proteolytic events are more specific than the general changes in protein content described above and result in the selective activation and inactivation of individual proteins. For example, when statically grown, stationary phase cultures of *Tetrahymena pyriformis* are shaken, the specific activity of several peroxisomal enzymes decreases (Levy and McConkey 1977). During the same period, the level of intracellular proteinase activity increases two- to three-fold. Actinomycin D and cycloheximide prevent both the enzyme inactivation and the increase in proteinase activity. Furthermore, it was observed that a purified preparation of the proteinase was able to inactivate several commercially available enzymes, including ones equivalent to those inactivated *in vivo*.

However, the increased level of proteinase activity is not necessarily responsible for the *in vivo* inactivation, since Supryniewicz and Allewell (1979) have noted that under other conditions, parallel decreases in peroxisomal enzyme activity and proteinase activity occur.

iv) Nutrition.

An obvious role for proteolytic enzymes in protozoa which utilize proteins as a nutritional source is in the digestion of food. This may involve extracellular enzymes breaking down proteins outside the cell or intracellular lysosomal enzymes that degrade proteins taken up by the organism by phagocytosis. Both extra- and intra-cellular proteinases have been implicated in protozoan nutrition. The extracellular proteinase level of *Euglena gracilis* has been shown to be increased by the addition of peptone to the medium and was higher in a bleached mutant, suggesting that in this case the extracellular enzyme may have a role in heterotrophic growth (Nakano et al. 1979). Not all extracellular enzymes have a role in nutrition. Normal strains of *Tetrahymena* can utilize egg-albumin as an exogenous source of amino acids. Phagocytosis-deficient mutants, however, are unable to utilize the protein. This suggests that the protein is not broken down by extracellular proteinases but is taken up by phagocytosis and degraded intracellularly (Rasmussen and Orias 1977). An increase in the level of intracellular proteinase activity in *T. pyriformis* has been noted after ingestion of yeast (Ricketts and Rappitt 1974)

The role of proteinase in the breakdown of more complex substrates has been studied in most detail in pathogenic species. The most well documented is the proteolytic digestion of haemoglobin in endocytic

vacuoles in *Plasmodium* (Sherman 1977). Studies have shown that dibrominated derivatives of haemoglobin, in which the β chains are covalently linked, not only resist digestion by the parasite proteinases but also prevent intraerythrocytic parasite development (Geary *et al.* 1983).

v) Pathogenicity.

There have been a number of suggestions that the proteinases of parasitic protozoa might have a role in the host-parasite relationship and in particular in the pathogenicity of the parasites.

Proteinase activity may be involved in host cell penetration by *Trypanosoma cruzi* (Bongertz and Hungerer 1978). Support for this idea came from the finding that antibodies against a purified cysteine proteinase bind to the cell surface of amastigotes (Rangel *et al.* 1981), although proteinase activity has also been found on the surface of other developmental forms. Alternatively, it was suggested that proteolytic modification of the parasites own surface membrane may be involved in the generation of molecules required for adhesion of the parasite to the host cell, prior to penetration (Piras *et al.* 1983). In contrast, a surface proteinase has been detected on the cell surface of promastigotes of *Leishmania major* which was suggested to have a role in the insect rather than in the mammalian host (Etges *et al.* 1986).

Qualitative and quantitative differences between the intracellular proteinases of the two developmental forms of *L. m. mexicana* have been demonstrated (North and Coombs 1981; Coombs 1982; Pupkis and Coombs 1984). This stage specificity of the enzymes has led to the suggestion that they may have a role in the survival of the amastigote in the host

macrophage. This could be achieved through the release of amino acids which on further metabolism would yield ammonia and amines. These could elevate the lysosomal pH and thus indirectly reduce the activity of potentially harmful lysosomal hydrolases or alternatively could directly inhibit hydrolase activity. The involvement of proteinases in parasite survival has been further indicated by the finding that inhibitors of amastigote proteinases have potent antileishmanial activity (Coombs et al. 1982; Coombs and Baxter 1984).

Another role of proteinases in pathogenicity has been reported for *Entamoeba histolytica*. The idea that the tissue lesions associated with invasive amoebiasis might be caused by hydrolytic enzymes including proteinases has been widely discussed. Two mechanisms have been proposed for the production of the cytopathogenic effects *in vitro*. These include contact cytolysis (Jarumilinta and Kradolfer 1964; Ravdin et al. 1980) and action of a soluble toxin at some distance from the amoebae (Bos 1979; Lushbaugh et al. 1979). Several workers have described cytotoxins in cell free extracts prepared from axenically cultured trophozoites (Mattern et al. 1980; Bos et al. 1980; McGowan et al. 1982). Lushbaugh et al. (1981) have provided direct evidence in support of a relationship between this cytotoxin, whose concentration correlates with strain virulence, and proteinase activity. Cytotoxin activity can be inhibited by α -1 antiprotease and α -2 macroglobulin. Leupeptin and antipain have also been shown to inhibit cytopathogenic effects (McGowan et al. 1982; Lushbaugh et al. 1984). A purified preparation of the neutral cysteine proteinase was completely inhibited by human and rabbit sera and McLaughlin and Faubert (1977) suggested that the inhibition was likely to be due to the α -2 macroglobulin fraction. A similar proteinase

was also purified by Lushbaugh et al. (1985) which was proposed to promote the metastatic and invasive behaviour of *E. histolytica*.

The role of proteinases in supplying the erythrocytic stages of *Plasmodium* with amino acids is well documented. Mahoney and Eaton (1981) reported that a chloroquine resistant strain of *P. berghei* contains higher levels of proteinase activity than a normal chloroquine-sensitive strain. Chloroquine is a widely used antimalarial agent and the appearance of resistant strains is of considerable concern. During infection of erythrocytes a malarial pigment, hemozoin, forms which may be responsible for binding and trapping the drug within the cells. The pigment possibly forms from the incomplete breakdown of haemoglobin. Mahoney and Eaton (1981) suggested that the higher proteinase activity of the resistant strain prevents malarial pigment formation and, consequently, chloroquine accumulation. However, it has also been reported that the hemozoin does not contain protein and probably consists entirely of hemozoin released by autooxidation of haemoglobin (Sherman 1977). The levels of proteolysis in resistant and sensitive strains have since been investigated (Charet et al. 1982; Wood et al. 1984) but as yet the relationship between proteolysis, haemoglobin breakdown, pigment formation and chloroquine sensitivity remains unclear.

Proteinases may have a role in the invasion of host cells by merozoites. It has been shown that *in vitro* invasion of monkey cells by merozoites of *P. knowlesi* can be inhibited by proteinase inhibitors, particularly chymostatin and leupeptin (Banyal et al. 1981). Merozoite proteinases that may be responsible have been detected. Similar results were obtained for *P. falciparum*. Leupeptin, TLCK and pepstatin were shown to be deleterious to both parasite development and parasite

invasion whereas aprotinin, α -1-antitrypsin and soybean trypsin inhibitor had no effect. In another study TPCK and PMSF inhibited merozoite invasion but not parasite development (Dejkriengkraikhul and Vilariat 1983). A serine proteinase purified from *P. lophurae* was demonstrated to be active on the inner surface of the red cell membrane. It was suggested that proteinase-induced modifications to the erythrocyte cytoskeleton could lead to merozoite release.

1.9. Aims of the project.

Over recent years it has become increasingly apparent that proteolytic enzymes play important roles in the life cycle of many species of protozoa. In particular, proteinases in several parasitic species have been implicated in a variety of aspects of the host-parasite relationship and pathogenicity. This has led to the suggestion that proteolytic enzymes may provide a potential target for chemotherapeutic attack. In order to investigate this possibility it is first necessary to characterise the proteolytic systems in detail and therefore one of the main aims of this project was to do this for two pathogenic species of trichomonads, *Trichomonas vaginalis* and *Tritrichomonas foetus*.

Comparison of the levels of proteinase activity in a range of flagellate protozoa revealed that *Trichomonas vaginalis* possessed particularly high levels of activity (Coombs 1982). This activity was demonstrated to be due to a group of high activity cysteine proteinases (Coombs and North 1983). In contrast, the proteolytic activity in *Tritrichomonas foetus* was reported to be due to a single low molecular weight cysteine proteinases (McLaughlin and Muller 1979). To

investigate further the differences between these two species a comparative study of the proteolytic systems, in terms of the levels of activity present and the numbers, properties and localisation of the enzymes responsible was carried out. Two intestinal trichomonads, *Pentatrichomonas hominis* and *Trichomitus batrachorum* were also included in this study in order to compare the characteristics of pathogenic and non-pathogenic species. Any link between proteolytic enzymes and pathogenicity could be further investigated by comparing the proteinases in a range of isolates of *Trichomonas vaginalis* that have previously been shown to possess varying degrees of virulence using a number of *in vitro* models. It was anticipated that methodology developed for analysing intracellular proteinases would be appropriate for detecting extracellular activities. It was also envisaged that the development of selective and sensitive assays for trichomonad proteinases, for example using peptide nitroanilide derivatives, would be a useful step in the development of specific inhibitors that could be used in identifying the roles of these enzymes and that might have potential antitrichomonal properties.

Another aim of this project was to purify individual proteolytic enzymes from *T. vaginalis*, both in order to characterise the activity in detail and also to raise anti-proteinase antiserum. The production of anti-proteinase antibodies would provide a useful tool for further investigation of the proteolytic systems of trichomonads. For example, antibodies could be used in subsequent purifications, for investigating the relationship between the multiple forms and intra- and extracellular proteinases and in studies on the localisation and role of the enzyme in *in vivo* models.

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METHODS

2.1. Organisms, cultivation and harvesting.

2.1.1. Trichomonads.

Trichomonas vaginalis, clone G3, has been cultivated *in vitro* for several years (Coombs 1976). This line was used for all experiments except where otherwise indicated. A number of other lines of *T. vaginalis* were also used. Lines 39, 45, 46, 55, 61, and 64 are recent isolates, that have been maintained as stabulates since isolation from patients attending the Genito-Urinary Clinic, Royal Infirmary, Glasgow, Scotland and were obtained from Dr I.B. Tait. Two lines, 6950♂, originally obtained from Dr. V.S. Latter (Wellcome Research Laboratories, Beckenham, Kent, England) and 39 *in vivo* (derived from line 39) were maintained intravaginally in mice, being passaged approximately every 2 weeks over a twelve month period prior to use in this study (Bremner *et al.* 1987b). Line 39 *in vitro*, was maintained axenically by serial passage every 3-4 days over the same twelve month period. A number of metronidazole resistant strains were also used. Line IR78 was originally obtained from Dr J.G. Meingassner (Sandoz Forschungsinstitut, Vienna, Austria). Lines 2755 (obtained from Dr. S. Al-Egally, Huddersfield Royal Infirmary, Huddersfield, England), 45733 (obtained from Dr B.L. Radford, Public Health Laboratory, Exeter, England) and F1297 (obtained from Dr. F.M. Tobin, St Mary's General Hospital, Portsmouth, England) are recent metronidazole resistant isolates that have been maintained as stabulates since isolation.

Tritrichomonas foetus (clone F2) was derived from the Pfizer strain originally obtained from Dr. D.J. Linstead (Wellcome Research Laboratories, Beckenham, Kent, England). *Trichomitus batrachorum* (clone

B2) was derived from an isolate obtained from the intestine of the leopard frog, *Rana pipiens* (Coombs 1976). *Pentatrachomonas hominis* (ATCC 30098) was obtained from Dr. A. Yule (London School of Hygiene and Tropical Medicine, University of London, London, England). All trichomonads were maintained prior to use in this study by Ms. A.F. Bremner (Dept. of Zoology, Glasgow University, Glasgow, Scotland).

2.1.2. Other species.

Leishmania mexicana mexicana (MHYC/BZ/62/M379), *L. donovani* (MHOM/ET/67/L82), *L. major* (MHOM/SA/83/RKK), *L. tarentolae* (LV 414), *Crithidia fasciculata* (ATCC 11745), *Herpetomonas muscarum muscarum* (ATCC 30260), *H. m. ingenoplastis* (30269), *Tetrahymena pyriformis* (CCAP/1630/1f), *Trypanosoma brucei* (BATRO 1125) *T. rhodesiense* (BATRO 95) and *Plasmodium chaubaudi* were obtained as frozen pellets from Dr. G.H. Coombs (Dept of Zoology, University of Glasgow, Glasgow, Scotland).

2.1.3. Cultivation of trichomonads in vitro.

All trichomonads were routinely cultivated axenically in modified Diamond's medium (MDM) (Table 4) supplemented with 10% (v/v) heat-inactivated horse serum, benzyl penicillin (1 000 units ml⁻¹) and streptomycin sulphate (1 mg ml⁻¹). Improved growth of *Pentatrachomonas hominis* was achieved using MDM supplemented with foetal calf serum (instead of horse serum) and a supplement of 10 mg galactose ml⁻¹ (Thong 1986). All cultures were checked routinely for contamination by microscopic observation. Contaminated cultures were discarded. *Trichomitus batrachorum* was grown at 25°C and other trichomonads at 37°C. Cultures of *T. batrachorum*, *Trichomonas vaginalis* and

Table 4. Modified Diamond's medium (Diamond 1957).

Ingredients	g
trypticase	20
yeast extract	10
maltose	5
ascorbic acid	1
KCl	1
KHCO ₃	1
KH ₂ PO ₄	1
K ₂ HPO ₄	0.5
FeSO ₄	0.1

The ingredients were dissolved in 900 ml distilled water and the pH adjusted to 6.3-6.4. The medium was dispensed into glass bottles and autoclaved for 15 mins at 15 lb in⁻². The sterilized medium, without any further additions, is stable at room temperature for several months. Sterile heat-inactivated horse serum (10% v/v), benzylpenicillin (1000 units ml⁻¹) and streptomycin sulphate (1 mg ml⁻¹) were added to the sterile medium under aseptic conditions prior to use.

Tritrichomonas foetus were initiated at a starting density of approximately 10^4 organisms ml^{-1} . *T. foetus* and *Trichomonas vaginalis* were subpassaged every 1-2 days and *Trichomonitus batrachorum* every 4-5 days. Cultures of *Pentatrichomonas hominis* were initiated with a starting density of about 10^5 organisms ml^{-1} and were subpassaged every 5 days.

2.1.4. Harvesting.

Trichomonads were routinely harvested at late log-phase of growth (approximately 10^6 organisms ml^{-1}) by centrifugation at 3 000 rpm for 15 min at room temperature in a MSE bench centrifuge. The harvested cells were washed twice with 0.25 M sucrose and used immediately or stored at -20°C until required. No loss of proteinase activity was apparent on storage.

Recent isolates of *Trichomonas vaginalis* and other species of protozoa were supplied as frozen pellets, stored at -70°C , by Ms A.F. Brenner, Mr. D.J. Mallinson and Dr. G.H. Coombs (Dept. of Zoology, University of Glasgow, Glasgow, Scotland).

Cell free lysates of trichomonads and other protozoa in 0.25 M sucrose were produced using 0.25% (v/v) Triton X-100.

2.1.5. Homogenisation of trichomonads for subcellular fractionation studies.

Freshly harvested pellets of *Trichomonas vaginalis* and *Tritrichomonas foetus* resuspended in 0.25 M sucrose were disrupted by 30-40 strokes with a Potter tissue homogeniser fitted with a serrated Teflon plunger, type A (A.H. Thomas Co., Philadelphia, USA) operating at

2 500 rpm. The resulting homogenate was centrifuged at 2 500 rpm for 5 min in a Sorval RC 5B refrigerated centrifuge. The pellet was resuspended in 0.25 M sucrose and the homogenisation repeated, following which it was combined with the original supernatant to give the homogenate for fractionation (described below). This procedure resulted in lysis of more than 95% of the parasites as judged by microscopic observation.

2.1.6. Subcellular fractionation.

Homogenates of *Trichomonas vaginalis* and *Tritrichomonas foetus* were fractionated into four fractions (nuclear, large particle, small particle/microsomal and non-sedimentable fractions) by differential centrifugation using the method of Steinbuchel and Müller (1986b). All low speed centrifugations were carried out in a Sorvall RC 5B refrigerated centrifuge at 4°C.

The nuclear fraction was sedimented at 2 500 rpm for 4 min, resuspended and washed once in 0.25 M sucrose. The wash was discarded. The supernatant was centrifuged at 7 750 rpm (*Trichomonas vaginalis*) and 5 500 rpm (*Tritrichomonas foetus*) for ten min to sediment the large particle fraction. The small-particle fraction was sedimented at 30 000 rpm for 60 min at 4°C in an MSE 65 ultracentrifuge; the resulting supernatant formed the non-sedimentable fraction.

The particulate fractions were further investigated by density gradient centrifugation on Percoll gradients. Samples (2 ml) of the particulate fractions in 0.25 M sucrose were layered on to 12 ml of Percoll solution, 20% or 50% (v/v) in 0.25 M sucrose in 0.1 M MES buffer

(Wood and Kaplan 1985). The gradients were centrifuged, with the brake off, at 31 000 rpm for 20 min at 4°C in an MSE 65 Ultracentrifuge.

The gradients were calibrated using density marker beads (Pharmacia). 50 µl of each marker bead were combined and resuspended in 2 ml of 0.25 M sucrose and layered onto the gradients and treated identically to tubes containing trichomonad samples. After centrifugation the gradients were fractionated by pumping sucrose (50% w/v) into the bottom of the tube and collecting the fractions from the top of the gradient using an LKB Redirac fraction collector.

Since Percoll may interfere with certain assays and electrophoretic procedures, the Percoll was removed from the fractions by centrifugation at 50 000 rpm for 60 min at 4°C in an MSE 65 ultracentrifuge. The Percoll formed a solid pellet at the base of the centrifuge tube and the supernatant was decanted. The fractions so produced were used immediately or stored at -20°C until required.

2.1.7. Materials.

Trypticase, a product of Beckton Dickinson and Co. was supplied by A.J. Beveridge (Edinburgh, Scotland); heat inactivated horse serum by Gibco Biocult (Paisley, Scotland) and yeast extract by Difco Laboratories (West Molesey, Surrey, England). All other chemicals were analar grade and obtained from either Sigma Chemical Co. Ltd., (Poole, Dorset, England) or BDH, (Glasgow, Scotland).

2.2. Peptidase assays.

2.2.1. Endopeptidase (proteinase) assays.

Proteinase activity was assayed by previously published methods using hide powder azure (North and Whyte 1984) and azocasein (Coombs 1982). Hide powder azure was ground to a fine powder under liquid nitrogen and a suspension in distilled water (10mg ml^{-1}) produced by sonication (8x15 sec). For the standard assay procedure, 0.5 ml of the substrate suspension was incubated with 0.5 ml buffer (0.1 M acetate/acetic acid pH 5.5), 0.1 ml sample and 10 μl 0.1 M DTT for 30 min at 37°C. The reaction was stopped by the addition of 0.2 ml 50% (w/v) trichloroacetic acid and the tubes were stood at 4°C for 30 min. The precipitate formed was removed by centrifugation at 13 400 g for 5 min in an MSE Microcentaur microcentrifuge. The absorbance of the supernatant was determined at 595 nm.

The standard assay procedure used to determine the activity towards azocasein was as follows: 50 μl sample was incubated with 100 μl of 100 mg ml^{-1} azocasein solution, 0.64 ml buffer (0.1 M sodium acetate acetic acid and 10 μl 0.1 M DTT at 37°C for 30 min. The reaction was stopped by the addition of 750 μl 5% trichloroacetic acid. The tubes were stood at 4°C for 30 min and the precipitate formed removed by centrifugation at 13 400 g for 5 min in an MSE Microcentaur microcentrifuge. The absorbance of the supernatant was determined at 366 nm. Activity is given in units of μg protein hydrolysed min^{-1} . An increase of 1 absorbance unit was caused by the hydrolysis of 3.4 mg hide powder azure and 0.4 mg azocasein under the reaction conditions stated.

Assays with 4-nitroanilide derivatives of peptides were based on the method of North et al. (1983); 50 μ l samples of enzyme were incubated with 1.0 ml buffer, 10 μ l 0.1M DTT (when required) and 50 μ l 1 mM substrate. The release of nitroaniline was measured spectrophotometrically at 405 nm. Activity is given in units of nmol nitroaniline released min^{-1} calculated using 9500 as the molar extinction coefficient of *p*-nitroaniline.

2.2.2. Carboxypeptidase assays.

A number of methods were used to determine the most suitable procedure for the measurement of carboxypeptidase activity in trichomonad lysates. All methods tested were as described by Doi et al. (1981). The method selected was a modification of the conventional ninhydrin method. Ninhydrin reagent was prepared by dissolving 200 mg of ninhydrin and 20 mg SnCl_2 in 10 ml of methyl cellosolve followed by the addition of 10 ml acetate citrate buffer (0.5 M acetic acid, 0.1 M citric acid adjusted to pH 4.0 with NaOH). The reaction mixture contained 0.2 ml enzyme sample and 0.4 ml of freshly prepared ninhydrin reagent which was heated in a tube in a boiling water bath. After 5 min the tube was transferred to an ice-cold water bath and 1 ml of a solution containing acetone/0.1M Na_2PO_4 /water 4/2.4/3.6 (v/v/v) was added. The absorbance was determined at 570 nm.

2.2.3. Amino-peptidase assays.

Amino-peptidase activity in trichomonad cell lysates was determined using the following method. 0.1 ml sample was incubated with 0.16 ml substrate and 0.05 ml 0.1 M sodium phosphate buffer pH 7.2. The assay

mixture was incubated at 37°C for 60 min, then the reaction was stopped by the addition of 0.5 ml 1 M sodium carbonate. The tube was centrifuged at 890 g for 5 min and the *p*-nitroaniline in the supernatant determined spectrophotometrically at 420 nm. Substrates used were S-Benzyl-L-cysteine-4'-nitroanilide, L-leucine *p*-nitroanilide, L-alanine *p*-nitroanilide, L-proline *p*-nitroanilide and glycine *p*-nitroanilide.

2.2.4. Materials

All substrates were obtained from Sigma Chemical Co. Ltd. (Poole, Dorset, England). Other chemicals were analar grade and obtained from either Sigma or BDH (Glasgow, Scotland).

2.3. Electrophoretic analysis of proteolytic activity.

2.3.1. Haemoglobin gels.

Proteinases were separated and visualized on polyacrylamide gels containing denatured haemoglobin as described by North and Coombs (1981). Approximately 100 µg of cell protein in 50 µl was loaded onto each gel. Proteinase bands were developed at pH 4.0 in 0.1 M sodium acetate/acetic acid buffer or at pH 6.0 in 0.1 M sodium phosphate buffer. 1 mM DTT was added to the incubation buffer when required. The gels were incubated at 37°C with several changes of buffer. They were stained and destained as described by North and Coombs (1981). Densitometric scans of the gels were made at 550 nm using a Gelman DCD-16 scanner.

To test the effect of proteinase inhibitors on the activities detected on the haemoglobin gels, cell lysates were preincubated with each inhibitor for 1 h at room temperature. Inhibitors used were

leupeptin, antipain, pepstatin and chymostatin at $100 \mu\text{g ml}^{-1}$, 1,10 phenanthroline, PMSF, TLCK and TPCK at 1 mM . All inhibitors were made up as solutions or suspensions in water. Following electrophoresis, inhibitors were included in the incubation buffer at the same concentrations as those used for the pre-electrophoresis incubation except for leupeptin, antipain, pepstatin and chymostatin which were added at a concentration of $20 \mu\text{g ml}^{-1}$.

2.3.2. Gelatin gels

1). Intracellular activities.

Samples were prepared by 1:1 dilution of cell lysates with sample buffer (0.125 M Tris HCl pH 6.8, 4% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 20% (v/v) glycerol) and subjected to polyacrylamide gel electrophoresis using the SDS discontinuous buffer (SDS-PAGE) system as described by Hames (1981). Gelatin was copolymerised into separating gels at a final concentration of 0.2% (w/v). The acrylamide concentration of the separating gels was normally 7.5% (w/v). Electrophoresis was carried out over night at a constant current of 6mA per gel.

After electrophoresis the gels were immersed for 1 h in 1 litre of 2.5% (v/v) Triton X-100 to remove the SDS and restore proteolytic activity. The proteinase bands were usually developed by immersing the gels in incubation buffer (0.1 M sodium acetate/acetic acid buffer pH 5.5 containing 1 mM DTT) for 4 h at 37°C ; this standard procedure was used except where otherwise indicated. Other buffers used were 0.1 M glycine/HCl (pH 2.0-3.0), 0.1 M sodium acetate/acetic acid (pH 4.0-6.0) and sodium phosphate (pH 7.0-8.0). The bands were visualised by staining in

0.05% (w/v) PAGE blue 83, 25% (v/v) isopropanol, 10% (v/v) acetic acid for at least 3 h before destaining in 10% (v/v) acetic acid. Densitometric scans of the gels were made as previously described. Molecular weights of individual proteinases were determined from their mobility compared to those of protein standards (High molecular weight standard mixture SDS-6H, Sigma Chemical Co. Ltd.).

ii). Extracellular activities.

Samples of medium were collected after removing the cells by centrifugation (15 min, 3 000g) and filtering at low pressure through a Millipore nitrocellulose filter (pore size, 0.45 μm). The absence of cells was confirmed by microscopic observation. Proteinase activity was detected by the same procedure as for intracellular proteinases described above.

iii). Inhibitors.

To determine the effects of inhibitors on the development of the proteinase bands, 1 ml samples containing approximately 1 mg of protein were loaded onto a continuous stacking gel of 1.5 cm depth. After electrophoresis and treatment with Triton X-100, each gel was cut into five 3 cm width strips and immersed in incubation buffer containing one of the following inhibitors; leupeptin, antipain or pepstatin at 20 $\mu\text{g ml}^{-1}$, TLCK, TPCK, PMSF, 1,10 phenanthroline, iodoacetic acid, EDTA or mercuric chloride at 1 mM, except where otherwise stated. All the inhibitors were dissolved in water except pepstatin and TPCK which were dissolved in 5% (v/v) dimethylsulphoxide (DMSO) and PMSF which was dissolved in ethanol.

2.3.3. Materials.

Leupeptin, antipain, chymostatin and pepstatin were supplied by the Protein Research Foundation (Osaka, Japan) and PMSF by BDH (Glasgow Scotland). All other inhibitors were supplied by Sigma Chemical Co. Ltd. (Poole, Dorset, England). All other reagents were analar grade and supplied by BDH or Sigma.

2.4. Other enzyme assays.

All hydrolase assays were carried out using chromogenic substrates at 37°C. Released nitrophenyl phosphate was determined by measuring the absorbance using an LKB Ultrospec 4050 spectrophotometer.

2.4.1. β -N-acetylglucosaminidase.

Activity was assayed using the method of Loomis (1969). The reaction mixture contained 0.25 ml 0.1 M *p*-nitrophenyl- β -D-glucosamine, 0.1 ml 0.1 M sodium acetate/acetic acid buffer, pH 5.0 and 0.15 ml enzyme sample. After 15 min incubation the reaction was stopped by the addition of 1.5 ml 1 M sodium carbonate and the mixture diluted with 1 ml distilled water. The absorbance of *p*-nitrophenol released was determined at 420 nm.

2.4.2. β -glucosidase.

Activity was assayed using the method described by Coston and Loomis (1969). The reaction mixture contained 0.25 ml *p*-nitrophenyl- β -D-glucopyranoside, 0.1 ml 0.2 M sodium acetate/acetic acid buffer, pH 5.0 and 0.15 ml enzyme sample. The assay mixture was incubated for 60 min

and the reaction stopped, diluted and the absorbance determined as for β -N-acetylglucosaminidase.

2.4.3. α -mannosidase.

Activity was assayed using the method of Loomis (1970). The reaction mixture contained 0.25 ml 0.1 M *p*-nitrophenyl- α -D-mannopyranoside, 0.1 ml 0.025 M sodium acetate/acetic acid buffer pH 5.0 and 0.15 ml enzyme sample. The assay mixture was incubated for 60 min and the reaction stopped, diluted and the absorbance determined as for β -N-acetylglucosaminidase.

2.4.4. Acid phosphatase.

Acid phosphatase activity was determined using 4-nitrophenyl disodium orthophosphate by the method of Bergmeyer *et al.* (1974). The reaction mixture contained 0.1 ml enzyme sample, 1.37 ml 0.1 M glycine/HCl buffer, 0.13 ml 2% (v/v) Triton X-100, 0.4 ml distilled water and 0.5 ml 0.01 M substrate. After 30 min incubation the reaction was stopped by the addition of 2.5 ml 0.4 M Tris/PO₄ buffer pH 8.5 and the absorbance measured at 420 nm

2.4.5. Lactate dehydrogenase.

Lactate dehydrogenase was assayed as an intracellular marker enzyme using a colorometric method, based on that described by Cabaud and Wroblewski (1958). The assay mixture contained 1 ml 0.75 mM sodium pyruvate pH 7.5, 1.28 mM NADH and 0.1 ml enzyme sample. The mixture was incubated at 37°C for 30 min before addition of 1 ml of the colour reagent, 2,4-dinitrophenyl hydrazine, approximately 200 mg ml⁻¹ in 1 M

HCl. The assay mixture was left for 20 min at room temperature and then 10.0 ml 0.4 M NaOH was added. After at least five min but not more than 30 min the absorbance at 525 nm was determined. Lactate dehydrogenase activity is given in units of pyruvate reduced $\text{min}^{-1} (\text{mg protein})^{-1}$

2.4.6. Malate dehydrogenase (decarboxylating).

Malate dehydrogenase (decarboxylating) activity was determined by following the reduction of NADP⁺ at 340 nm as described by Muller and Steinbuechel (1986b). The assay mixture contained 53 mM Tris/HCl buffer pH 7.3, 3.3 mM L(-)-malate, 0.67% (v/v) Triton X-100 and 0.33 mM NADP in a final volume of 3.0 ml. 0.1 ml enzyme sample was added to the assay mixture and the change in absorbance followed in a Varian Series 634 spectrophotometer connected to a Bryans 28 000 chart recorder.

2.4.7. Materials.

All chemicals were purchased from either Sigma Chemical Co. Ltd. (Poole, Dorset, England) or BDH (Glasgow, Scotland).

2.5. Protein determination.

Protein was routinely determined using the method of Sedmak and Grossberg (1977) using bovine serum albumin as standard. In samples containing Percoll, protein was determined using the Lowry method. Protein content in the range 5-50 μg was determined from standard curves constructed for each fresh batch of reagents.

2.6. Protein purification

Cell lysates of *Trichomonas vaginalis* were centrifuged at 5 000 rpm for 15 min in a Sorval RC-5B refrigerated centrifuge and the resulting supernatant used as the starting material for the purification of intracellular proteinases. All purification procedures were carried out at 4°C.

2.6.1. Gel filtration.

2 ml samples of the *T. vaginalis* supernatant were loaded on to a column of Sephadex G 75 superfine (height 96 cm, diameter 1.5 cm) and eluted at a flow rate of 17.5 ml h⁻¹ with 0.1 M sodium acetate/acetic acid buffer pH 5.5. 5 ml fractions were collected using an LKB 2112 Redirac fraction collector. The protein concentration in the fractions was estimated from the absorbance at 280 nm, measured using an LKB ultrospec 4050 spectrophotometer. Proteinase activity was determined throughout the purification as described in section 2.2.1.

Fractions containing proteinase activity were pooled and concentrated under nitrogen using an Amicon Ultrafiltration Cell fitted with a PM 10 membrane.

10% glycerol was added to the concentrated high-*M_r* activity and 1.5 ml samples were loaded on to a column of Sephacryl S300 (height 48 cm, 1.5 cm diameter). Protein was eluted from the column at a flow rate of 42 ml h⁻¹ and fractions (2 ml) collected and analysed as described above.

2.6.2. Ion-exchange chromatography.

A column of DEAE-cellulose (height 22 cm, diameter 2.5 cm) was equilibrated with starting buffer, 10 mM Tris/HCl pH 7.2. The low-*M_r* activity from the preceding gel filtration step was dialysed against starting buffer (3 x 250 ml) before loading samples of 5 ml on to the column. Unbound protein was eluted at a flow rate of approximately 25 ml h⁻¹ with starting buffer. Bound protein was eluted using a gradient of 0-300 mM NaCl (500 ml volume). 5 ml fractions were collected as described above. Any remaining bound protein was removed using 1 M NaCl and the column equilibrated with starting buffer before reuse.

2.6.3. Organomercurial Sepharose affinity chromatography.

Cyanogen bromide-activated Sepharose 4B was coupled to 4-aminophenylmercuric acetate as described by Barrett (1973). 4 g of CNBr-activated Sepharose 4B were resuspended in 8 ml of 10% (v/v) DMSO and 1 mg of 4-aminophenylmercuric acetate in 0.8 ml DMSO was introduced slowly. The mixture was stirred at 4°C overnight, transferred to a chromatographic column and washed with 20% (v/v) DMSO until the E₂₆₀ of the effluent fell below 0.10. The column was washed with starting buffer, 50 mM sodium acetate/acetic acid buffer pH 5.5 containing 20 mM NaCl, 1 mM EDTA. The sample (2.0 ml) was dialysed against the same buffer (3x250 ml) prior to loading on the column. Unadsorbed protein was eluted in starting buffer at a flow rate of 20 ml h⁻¹ (cm²)⁻¹. The adsorbed proteins were eluted with DTT gradients of 0-10 mM (proteinase D) and 0-20 mM (High-*M_r* proteinase) in a total volume of 100 ml. After use the column was regenerated using 0.05 M sodium acetate/acetic acid buffer pH

4.8 containing 10 mM mercuric chloride and 20 mM EDTA and then washed with starting buffer before reuse.

2.6.4. Materials.

Sephadex G75 superfine and Sephacryl S300 were obtained from Pharmacia Fine Chemicals AB (Milton Keynes, England.), DE 52 was obtained from Whatman Chemical Separation Ltd. (Maidstone, Kent, England.) and CN-Br activated Sepharose 4B was obtained from Sigma Chemical Co. Ltd. (Poole, Dorset, England). All other chemicals were purchased from either BDH (Glasgow, Scotland) or Sigma.

2.7. Characterisation of proteinase activity.

2.7.1. Determination of molecular weight.

The molecular weights of the proteinase activities were determined using gel filtration by comparison of their elution volumes with those of proteins of known molecular weights. Calibration proteins used were cytochrome c (*M*. 12 500), chymotrypsinogen A (*M*. 25 000), egg albumin (*M*. 45 000), bovine serum albumin (*M*. 158 000), catalase (*M*. 240 000) and ferritin (*M*. 450 000). 5 mg of each protein was dissolved in 0.5 ml running buffer, loaded on to the column and eluted under identical conditions to those used for elution of the proteinase activities

The molecular weights of the proteinases were also determined using the gelatin-PAGE technique as described in section 2.3.2.

2.7.2. Determination of isoelectric point.

i). Chromatofocusing.

Chromatofocusing is a specialised form of ion-exchange chromatography first described by Lampson and Tytell (1965) where a pH gradient is produced on an ion-exchange column using the buffering capacity of the charged groups of the exchanger.

If an elution buffer at one pH is applied to an ion-exchange column of another pH, the elution buffer will titrate the charged groups of the column to generate a pH gradient. Any proteins applied and bound to such a column will elute according to their isoelectric points. Proteins of similar isoelectric points are also focused by this technique and therefore this method has very good resolution of mixtures of protein.

The quality of the pH gradient determines the resolution and the use of specially designed amphoteric Polybuffer elution buffers produces smooth and even pH gradients.

1 ml samples of the purified proteins were applied to a column of Polybuffer PBE 94 exchanger (10 cm length, 1 cm diameter) equilibrated with 0.025 M imidazole-HCl buffer pH 7.4. The proteins were eluted with approximately 100 ml Polybuffer 74 adjusted to pH 4.0 with HCl. 1 ml fractions were collected and the pH value of fractions containing proteinase activity was determined.

ii). Isoelectric focusing.

Samples were analysed on polyacrylamide gels by isoelectric focusing in the pH range 3.5-9.5 using an LKB 2117 Multiphor system as described

by Winter *et al.* (1977). After focusing, proteinase activity was determined in the gel.

The activity towards hide powder azure was determined by slicing the gel into 1 cm sections. Each section was placed in a test tube containing the standard hide powder azure reaction mixture (see section 2.2.1). After 3 h incubation, the reaction was stopped and the precipitated protein and gel removed by centrifugation. The absorbance of the supernatant was determined at 595 nm.

The activity towards azocasein was determined by incubating the gel in 100 ml of azocasein (10 mg ml^{-1}) in 0.1 M acetate buffer pH 5.5 containing 1 mM DTT for 2 h at 37°C. After incubation the gel was fixed and stained as described by Winter *et al.* (1977). Proteinase activity was represented by clear areas on the gel.

2.7.3. Materials.

Combithek calibration proteins for gel filtration were obtained from Boehringer Mannheim (Lewes, E. Sussex, England.). Polybuffer 74 and Polybuffer Exchanger PBE 94 were obtained from Pharmacia (Milton Keynes, London.). Ampholytes were obtained from either Bio-Rad Laboratories Ltd. (Watford, Herts, England.) or LKB Instruments Ltd. (S. Croydon, Surrey, England). All other chemicals were purchased from either Sigma Chemical Co. Ltd. (Poole, Dorset, England) or BDH (Glasgow, Scotland).

2.8. Purification of extracellular proteinase activity.

Extracellular proteinase activity was purified from medium (100 ml) in which *Trichomonas vaginalis* had been grown to late log-phase. Cells

were removed by centrifugation at 3 000 rpm for 15 min at 4°C in a Sorval RC-5B refrigerated centrifuge. The medium was then filtered at low pressure through a Millipore nitrocellulose filter (pore size 0.45 µm) to ensure complete removal of cells. The absence of cells was confirmed by microscopic observation.

The medium was concentrated approximately 10-fold in an Amicon ultrafiltration cell fitted with a PM30 membrane. ~~Mercaptoethanol and~~

SDS were added to the concentrated medium to give final concentrations of 5% (v/v) and 2% (w/v) respectively, which was then refiltered through a PM 30 membrane. The filtrate, containing the extracellular proteinase was filtered through a PM 10 membrane and washed with starting buffer for organomercurial Sepharose affinity chromatography. The proteinase was bound to the affinity column and eluted with 10mM DTT as described in section 2.6.3.

2.9. Anti-proteinase antiserum.

2.9.1. Production of antiserum.

0.5 ml containing 1 mg of purified extracellular proteinase was mixed with 0.5 ml Freund's complete adjuvant and injected subcutaneously into three sites of a single New Zealand White female rabbit. Three weeks later the rabbit was given a booster injection of 0.5 ml purified proteinase mixed with 0.5 ml Freund's incomplete adjuvant. After one week, the rabbit was bled from one ear and 30 ml of blood obtained. The antiserum was decanted after the blood had coagulated in a glass tube stored at 4°C for 24 h. The serum was stored at -70°C until required.

2.9.2. Characterisation of antiserum.

i). Western blotting.

Trichomonad proteins were separated by SDS-PAGE using the discontinuous buffer system as described by Hanes (1981). After electrophoresis, the gel was immersed in transfer buffer, 1.92 M glycine, 0.25 M Tris/methanol/water 1/2/7 (v/v/v) and blotted onto nitrocellulose paper at 200 mamp for 2 h and 150 mamp overnight. After blotting the gel was incubated in phosphate buffered saline containing 2 g l⁻¹ gelatin and 0.1% (v/v) Triton X-100 (PBS gelatin) for 4 h. The nitrocellulose was incubated with shaking for 1 h with serum diluted 1: 1000 in fresh PBS gelatin. The gel was given 3x5 min washes in PBS gelatin and then goat antirabbit antibody diluted 1: 500 in the same buffer was added. After incubation with the antibody for 1 h, the nitrocellulose was washed for 3x5 min in PBS containing 200 mM NaCl followed by one wash in PBS for 5 min. 50 mg 4-chloronaphthol was dissolved in 17 ml cold methanol and 10 ml 1 M Tris-HCl pH 7.4 added. The solution made up to 100 ml with water and poured onto the paper. After 20-30 sec, 50 µl H₂O₂ were added. Colour development was stopped with 5 ml 10% SDS.

ii). Immunoprecipitation.

200 µl samples of either trichomonad cell lysates or medium in which the parasites had been grown were incubated with 10 µl non-immune serum, 10 µl immune serum or 50 µl immune serum for 2 h at room temperature with constant shaking. 50 µl Pansorbin was added to each sample and the mixture incubated for a further 2 h at room temperature prior to centrifugation in an MSE Microcentaur microcentrifuge at high

speed for 5 min. The pellet was washed twice in PBS and then resuspended in PAGE sample buffer. After shaking for 30 min at room temperature the mixture was centrifuged as described above and the supernatant loaded onto a 7.5% acrylamide gel containing 0.2% copolymerised gelatin. Electrophoresis was carried out as described in section 2.3.2. After electrophoresis the gel was incubated in 0.1 M sodium acetate acetic/acid buffer pH 5.5 containing 1 mM DTT for 8 h. The gel was then stained and destained according to the standard procedure (section 2.3.2.).

2.9.3. Materials.

Pansorbin was purchased from Calbiochem, Behring Diagnostics, La Jolla, California, USA. All other chemicals were analar grade and obtained from either Sigma Chemical Co. Ltd. (Poole, Dorset, England) or BDH (Glasgow, Scotland).

3.1. Proteolytic activity in trichomonad cell lysates.

In initial experiments enzyme activities were determined in whole cell lysates. Endopeptidase (proteinase) activity was assayed in trichomonad lysates using two chromogenic proteins, hide powder azure and azocasein, and a range of peptide nitroanilide derivatives that have been used previously to detect proteolytic enzymes in a range of other organisms (North et al. 1983; North and Walker 1984; North and Whyte 1984). Exopeptidase (carboxypeptidase and aminopeptidase) activities were determined using blocked amino acid derivatives.

3.1.1 Detection of activity using protein substrates.

Proteolytic activity could be detected in cell lysates prepared from all four species of trichomonad when either hide powder azure or azocasein was used as the substrate. The activity varied between species but in all cases it was significantly increased by the addition of 1 mM dithiothreitol (DTT) to the assay mixture. The effect of DTT concentration on the activities towards azocasein and hide powder azure is shown in Fig. 9. Activity towards both substrates increased linearly over time of incubation for at least 3 h and was proportional to the volume of lysate added up to at least 200 μ l. Three of the species, *Trichomonas vaginalis*, *Tritrichomonas foetus* and *Trichomitus batrachorum* had similar levels of activity towards azocasein (AZCase) whilst *Fentatrichomonas hominis* had a significantly lower level of activity (Table 5). Only *Trichomonas vaginalis* and *Trichomitus batrachorum* had similar levels of proteinase activity towards hide powder azure (HPAase)

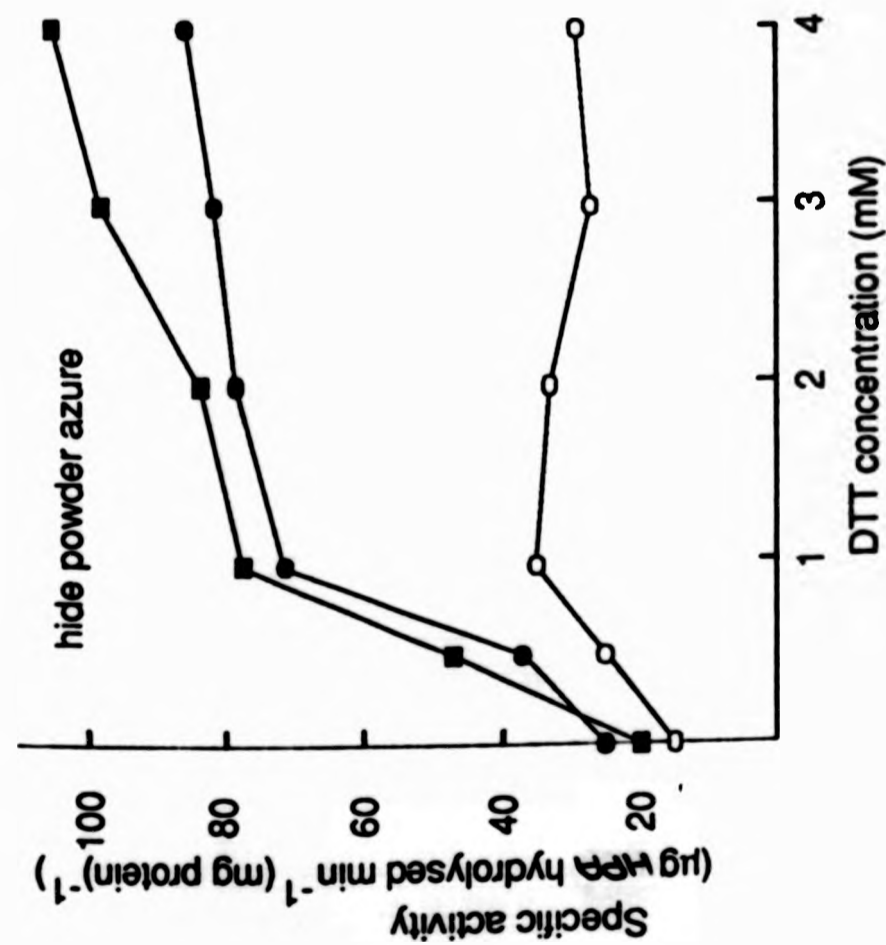
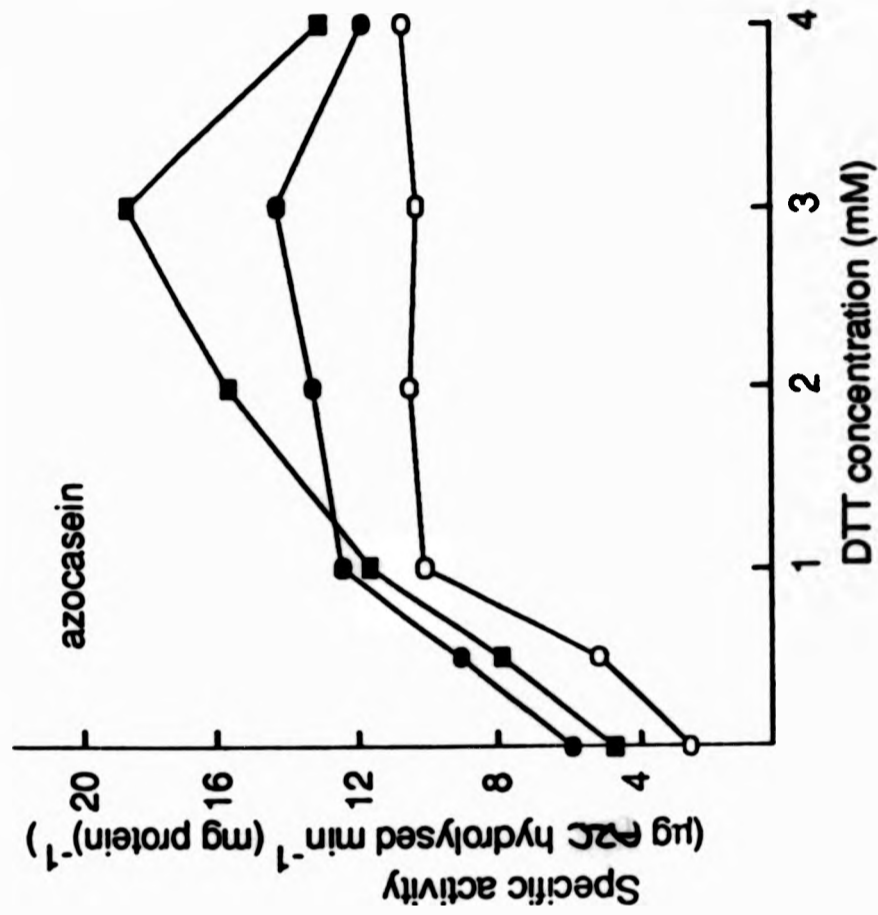


Fig. 9. Effect of DTT concentration on proteinase activity. Assays were carried out on lysates of *Trichomonas batrachorum* (●), *Trichomonas vaginalis* (■) and *Trichomonas foetus* (○). The results shown in this and other figures are from one experiment which is representative of at least three separate experiments.

Table 5. Proteinase activity in trichomonad cell lysates.

Species	Specific Activity	
	HPAase	AZCase
<u>Trichomonas vaginalis</u>	76.3 \pm 4.3	12.8 \pm 1.2
<u>Trichomitus batrachorum</u>	72.4 \pm 2.9	11.2 \pm 0.9
<u>Tritrichomonas foetus</u>	35.4 \pm 5.3	12.7 \pm 1.9
<u>Pentatrichomonas hominis</u>	17.0 \pm 2.2	3.3 \pm 0.3

Results are given in units of μ Ag substrate hydrolysed min^{-1} $(\text{mg protein})^{-1}$ and are the mean \pm standard deviation from at least three independent determinations.

whilst both *Tritrichomonas foetus* and *P. hominis* had significantly lower HPAase activities.

In all cases, proteolytic activity was apparent over a broad pH range. The activities shown in Fig. 10 were obtained using three buffers, but other buffers were also used, namely glycine-HCl (pH 2.0-3.5), sodium acetate/acetic acid (pH 4.0-5.0), sodium phosphate (pH 6.0-8.0), Bicine (pH 7.5-8.5) and Caps (pH 9.5-10.0) and no differences in the pH dependence of the activities was observed using these alternative buffers. All the activities were maximal in the range pH 4.5-6.5, but within this range the optimal pH value differed with both the substrate used and the species.

There are four different classes of proteinase which can be distinguished from one another on the basis of their sensitivity to specific inhibitors (see section 1.8.1.). Therefore, to determine the type of proteinase responsible for the activities towards hide powder azure and azocasein the effects of a range of proteinase inhibitors on the activities were examined (Table 6). None of the activities was greatly affected by the aspartic proteinase inhibitor, pepstatin, nor by the serine proteinase inhibitor PMSF (Barrett 1977) but all the activities were inhibited to some extent by TLCK, TPCK, iodoacetic acid, leupeptin, chymostatin and antipain, each of which has been shown to inactivate a number of cysteine proteinases (Barrett 1977). Overall the HPAase activities were less sensitive to these inhibitors than the AZCase activities, particularly with leupeptin and antipain. Phenanthroline, an inhibitor of metalloproteinases which nevertheless inhibits cysteine proteinases in other protozoa (North 1982) was found to be effective against HPAase but not AZCase.

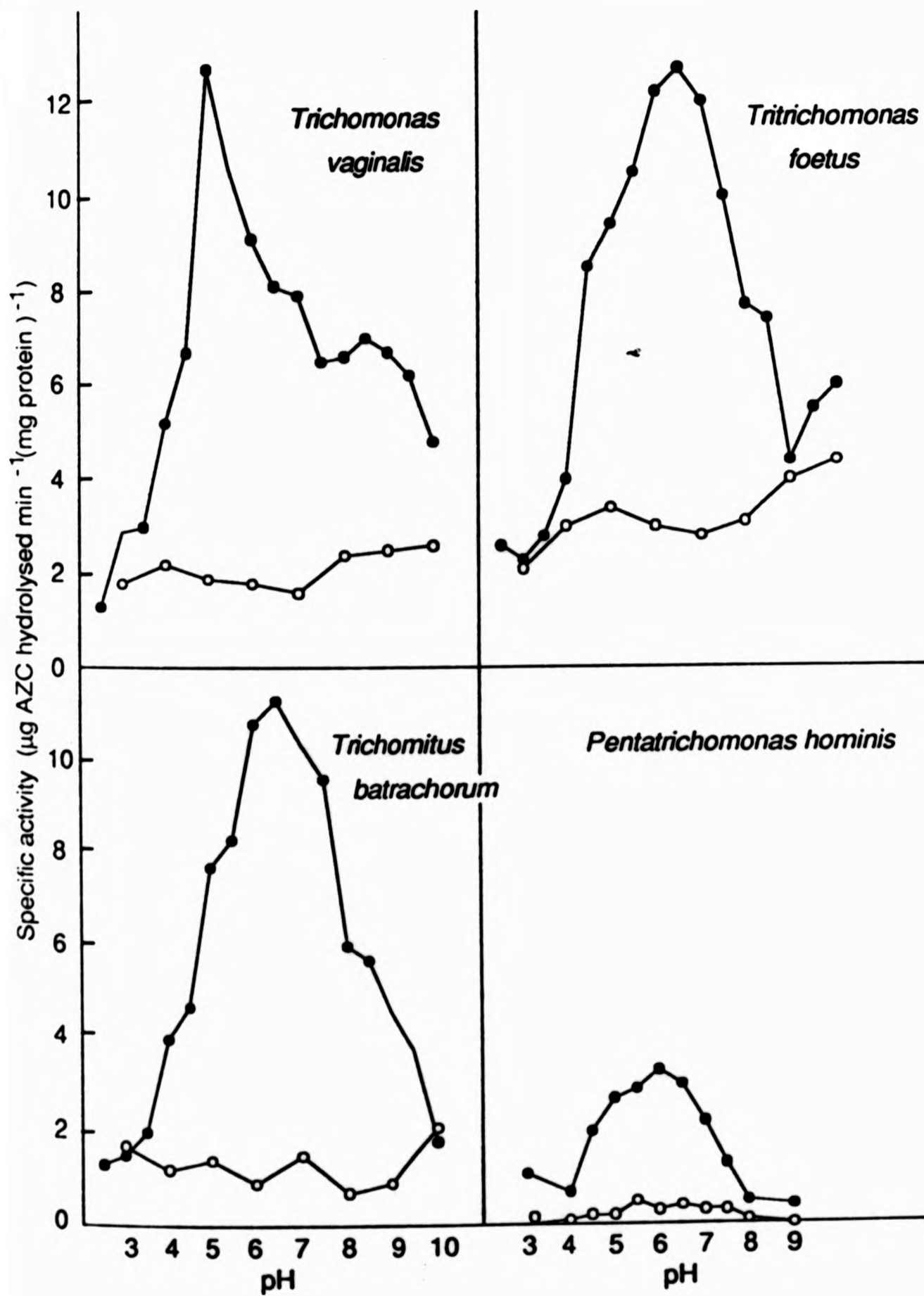


Fig. 10. pH dependence of proteinase activity.

(a) Activity towards azocasein was determined in the presence (●) and absence (○) of 1 mM DTT.

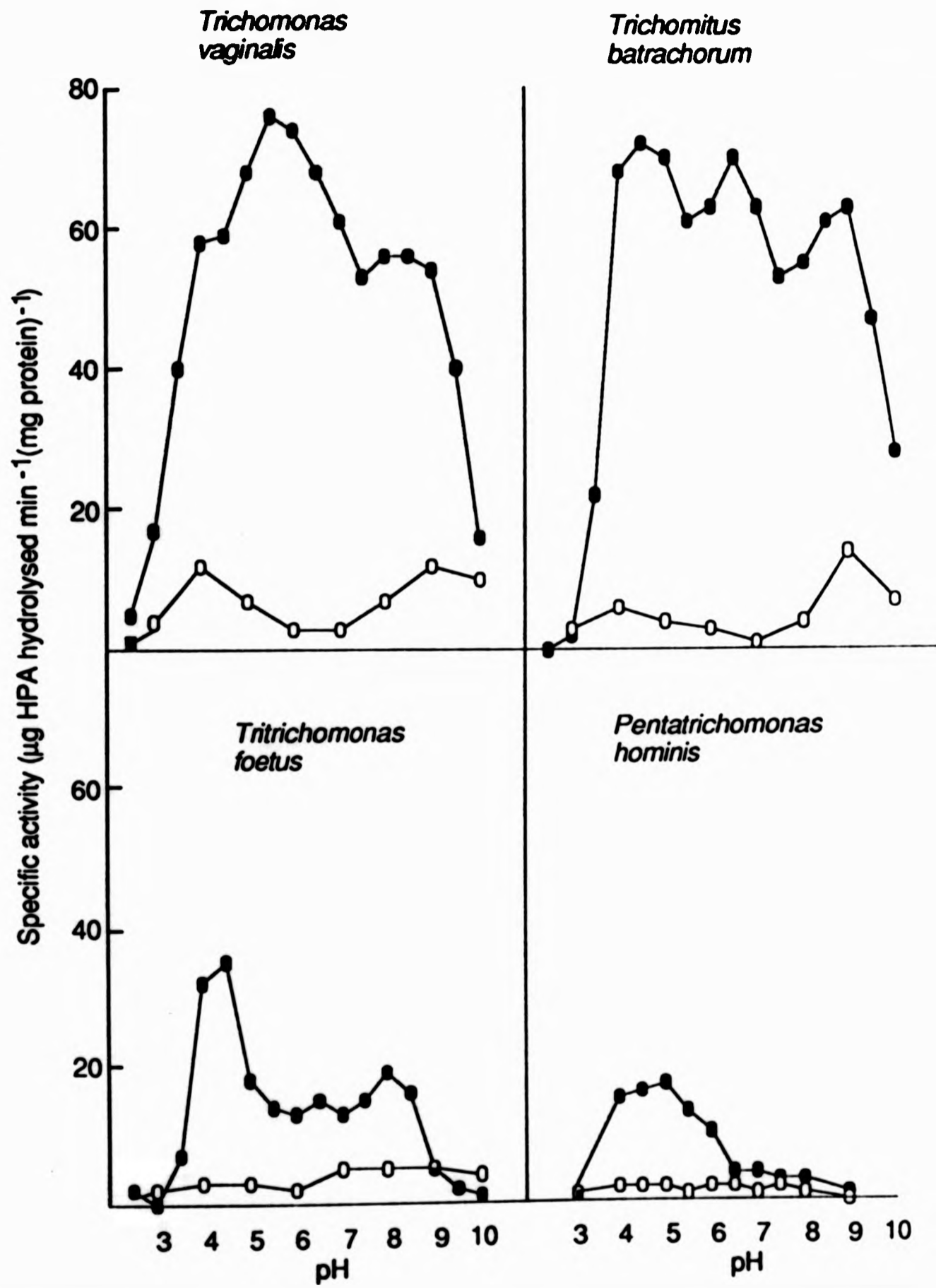


Fig. 10. pH dependence of proteinase activity.

(b) Activity towards hide powder azure was determined in the presence (●) and absence (○) of 1 mM DTT

Table 6. Effect of inhibitors on proteinase activity.

Inhibitor	Trichomonas vaginalis				Activity Remaining (%)				Trichomonas foetus		Pentatrachomonas hominis	
	HPAase pH 5.5	AZCase pH 5.0	HPAase pH 4.5	AZCase pH 6.5	Trichomitus batrachorum	HPAase pH 4.5	AZCase pH 6.5	HPAase pH 4.5	AZCase pH 6.5	HPAase pH 5.0	AZCase pH 6.0	
Pepstatin	91 + 10	102 + 5	98 + 8	100 + 3	83 + 3	83 + 6	101 + 4	94 + 3	97 + 5	94 + 3	97 + 5	
PMSF	97 + 8	102 + 3	102 + 6	98 + 5	95 + 5	95 + 6	97 + 14	104 + 7	99 + 1	104 + 7	99 + 1	
TLCK	26 + 7	21 + 5	19 + 7	25 + 2	43 + 2	43 + 9	36 + 2	34 + 7	26 + 4	34 + 7	26 + 4	
TPCK	42 + 8	82 + 8	80 + 11	90 + 1	73 + 1	73 + 6	83 + 3	53 + 11	38 + 6	53 + 11	38 + 6	
Iodoacetic acid	73 + 12	63 + 5	53 + 3	69 + 16	86 + 15	86 + 15	61 + 8	54 + 2	63 + 11	54 + 2	63 + 11	
Leupeptin	41 + 10	12 + 3	54 + 9	38 + 8	69 + 6	69 + 6	23 + 2	41 + 7	32 + 5	41 + 7	32 + 5	
Chymostatin	60 + 15	27 + 4	63 + 2	80 + 6	74 + 9	74 + 9	33 + 3	70 + 14	38 + 7	70 + 14	38 + 7	
Antipain	48 + 7	15 + 1	57 + 2	80 + 5	59 + 5	59 + 5	26 + 2	39 + 3	40 + 5	39 + 3	40 + 5	
Phenanthroline	58 + 32	100 + 7	46 + 32	97 + 12	42 + 12	42 + 34	88 + 13	59 + 14	96 + 9	59 + 14	96 + 9	

Activities are given as a percentage of that in control samples (values of controls are given in table 5). The results are the mean + standard deviation from at least three independent determinations.

3.1.2. Detection of activity using peptide nitroanilide substrates.

A range of peptide nitroanilides was used to detect proteolytic enzyme activity in an attempt to find a selective and sensitive assay. It was found that the rate of substrate hydrolysis was dependent upon the specific amino acid residues present (Table 7). Bz-Pro-Phe-Arg-Nan, Tos-Gly-Pro-Arg-Nan and Bz-Phe-Val-Arg-Nan were the substrates hydrolysed most rapidly, as had been found previously in other protozoa (North et al. 1983; North and Walker 1984). All of the peptide nitroanilides used originally had arginine in the P₁ position (as defined by Schechter and Burger 1967) since they have previously been shown to be good substrates for cysteine proteinases. The results also suggest some specificity at the P₂ and perhaps the P₃ positions. Two of these three substrates have bulky amino acids at the P₂ position: the two substrates with glycine at the P₂ position and Bz-Arg-Nan with no amino acid at the P₂ position were poorly hydrolysed. Preliminary results with three other substrates demonstrated that *Trichomonas vaginalis* lysates hydrolysed cBz-Arg-Arg-Nan and cBz-Tyr-Lys-Arg-Nan relatively rapidly whilst cBz-Gly-Pro-Cit-Nan, where the arginine in the P₁ position was replaced by citrulline, was hydrolysed very slowly.

At pH 7.0 the hydrolysis of Bz-Pro-Phe-Arg-Nan, Tos-Gly-Pro-Arg-Nan and Bz-Phe-Val-Arg-Nan was markedly enhanced by the addition of 1mM DTT. There was no stimulatory effect of DTT with the other substrates tested at pH 7.0, nor did the reducing agent enhance the hydrolysis at pH 5.0 of any of the nitroanilide derivatives and indeed in some cases it was inhibitory (Table 7).

For further investigation assays were carried out with two substrates; Bz-Pro-Phe-Arg-Nan, which was hydrolysed most rapidly by

Table 7. Activity of trichomonad cell lysates towards peptide p-nitroanilides.

Substrate	pH	Specific Activity (nmol min ⁻¹ (mg protein) ⁻¹)							
		Trichomonas vaginalis		Trichomitus batrachorum		Tritrichomonas foetus		Pentatrichomonas hominis	
		+	-	+	-	+	-	+	-
Bz-Pro-Phe-Arg-Nan	5	18 + 3	15 + 7	16 + 3	16 + 5	12 + 6	13 + 2	4 + 1	-
Tos-Gly-Pro-Arg-Nan	5	12 + 2	11 + 4	15 + 5	14 + 4	9 + 8	11 + 6	1 + 2	3 + 2
Bz-Phe-Val-Arg-Nan	5	10 + 1	11 + 3	11 + 5	12 + 4	10 + 3	8 + 4	2 + 1	1 + 1
Bz-Val-Gly-Arg-Nan	5	8 + 2	6 + 2	10 + 2	9 + 2	6 + 4	5 + 2	ND	ND
Bz-Arg-Nan	5	6 + 4	6 + 1	5 + 3	6 + 3	4 + 2	7 + 1	ND	ND
Bz-Ile-Glu-Gly-Arg-Nan	5	3 + 2	4 + 3	2 + 1	1 + 2	1 + 3	2 + 1	ND	ND
Bz-Pro-Phe-Arg-Nan	7	26 + 8	4 + 2	18 + 5	1 + 1	16 + 4	3 + 2	6 + 2	2 + 1
Tos-Gly-Pro-Arg-Nan	7	18 + 9	6 + 3	12 + 4	3 + 2	10 + 3	2 + 2	1 + 2	1 + 1
Bz-Phe-Val-Arg-Nan	7	14 + 3	2 + 1	9 + 2	1 + 1	9 + 3	1 + 1	3 + 2	ND
Bz-Val-Gly-Arg-Nan	7	3 + 2	3 + 2	1 + 3	1 + 2	7 + 4	4 + 3	ND	ND
Bz-Arg-Nan	7	1 + 2	2 + 3	ND*	ND	3 + 1	2 + 1	ND	ND
Bz-Ile-Glu-Gly-Arg-Nan	7	1 + 1	2 + 1	ND	ND	2 + 1	3 + 2	ND	ND

Results are the mean + standard deviation from at least three independent determinations. Assays were carried out in the presence (+) and absence (-) of 1 mM DTT.
 * No detectable activity (0.1 nmol min⁻¹ (mg protein)⁻¹).

trichomonad lysates and Bz-Arg-Nan, which has been widely used to detect activity in other protozoa. Activities were determined in both the presence and absence of DTT. The pH dependence of these activities is shown in Fig. 11. The effects of proteinase inhibitors on the hydrolysis of Bz-Pro-Phe-Arg-Nan was investigated at both pH 5.0 and 7.0. None of the activities was inhibited by PMSF but all were significantly affected by cysteine proteinase inhibitors (Table 8). In general, the latter were more inhibitory against the activities at pH 7.0 than at 5.0, although this was less apparent for *Tritrichomonas foetus* and *P. hominis* than the other two species. Pepstatin, generally considered to be an inhibitor of aspartic proteinases (Barrett 1977), significantly decreased the activity at pH 5.0 towards Bz-Pro-Phe-Arg-Nan in *Trichomonas vaginalis* and *Trichomitus batrachorum*, but not in *Tritrichomonas foetus*. At pH 5.0 the activities towards Bz-Arg-Nan and Bz-Pro-Phe-Arg-Nan showed similar sensitivities to the inhibitors tested (Table 8).

3.1.3. Carboxypeptidase activity.

A number of previously published methods were used to determine the optimum conditions for the assay of carboxypeptidase activity (see section 2.2.2.). Activity was detected in cell lysates of *Trichomonas vaginalis*, *Tritrichomonas foetus* and *Trichomitus batrachorum* using three substrates (Table 9). All three species had similar specificities for the three substrates used, although the level of activity varied between species. *Trichomonas vaginalis* had the highest activity towards all three of the substrates. The pH optima for the activities towards N-cBz-Gly-Leu are shown in Fig. 12. Carboxypeptidase activity in lysates of *P. hominis* was not determined.

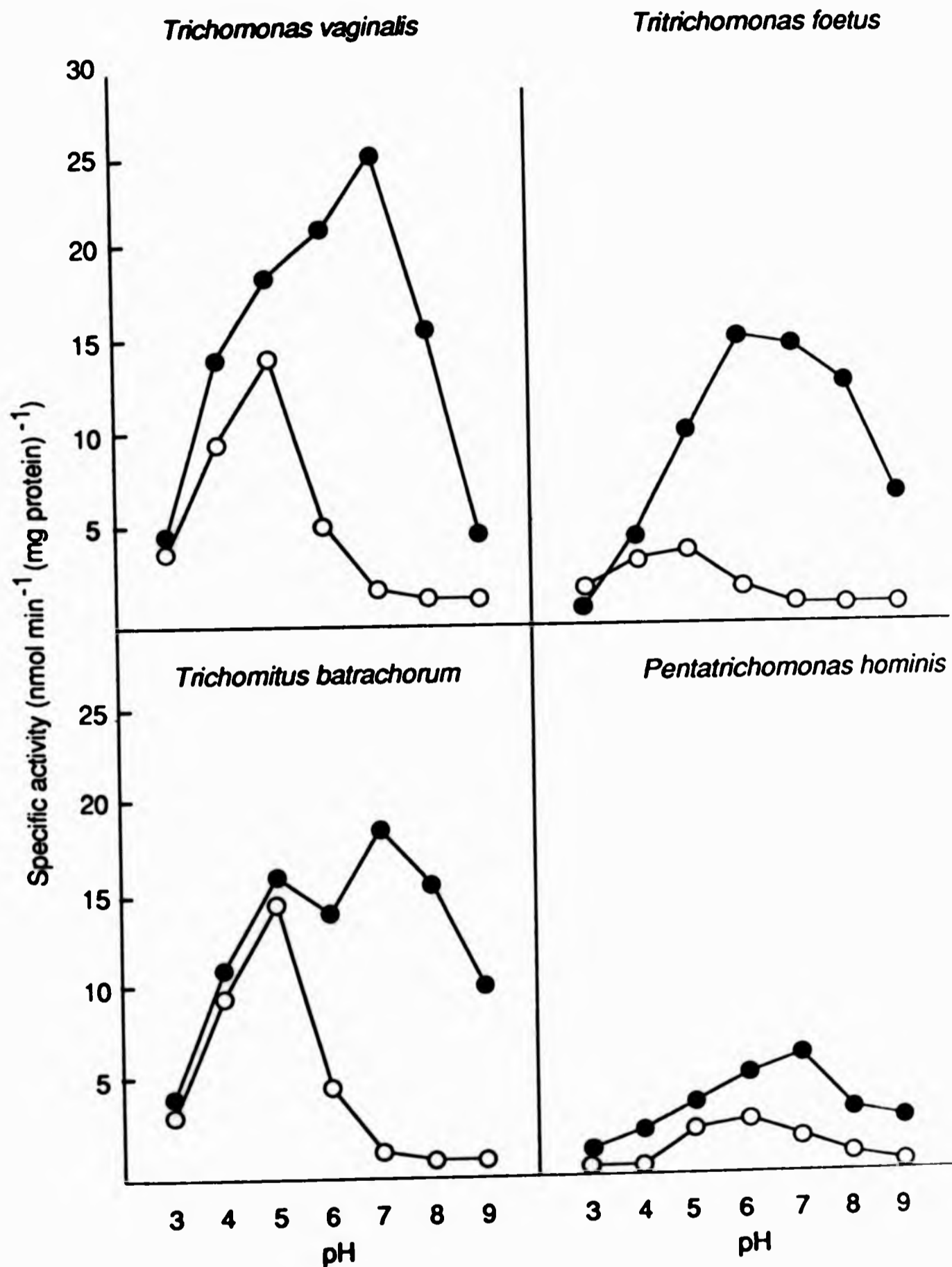


Fig 11. pH dependence of peptide nitroanilide hydrolysing activity.
(a) Activity towards Bz-Pro-Phe-Arg-Nan was determined in the presence (●) and absence (○) of 1 mM DTT

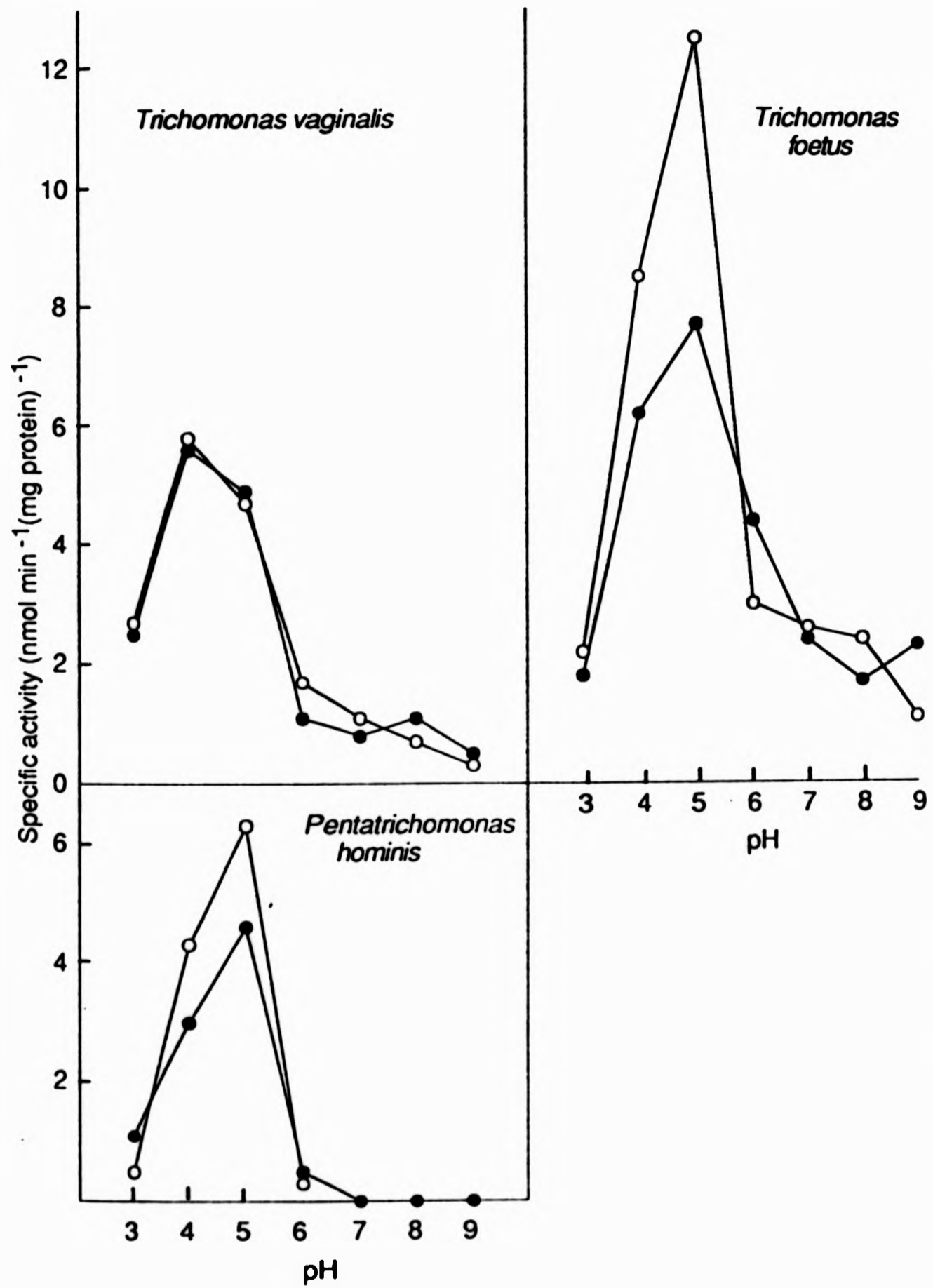


Fig. 11. pH dependence of peptide nitroanilide hydrolysing activity.
(b) Activity towards Bz-Arg-Man was determined in the presence (●) and absence (○) of 1 mM DTT.

Table 8a. Effect of inhibitors on the hydrolysis of Bz-Pro-Phe-Arg-Nan and Bz-Arg-Nan by Trichomonas vaginalis and Trichomonas foetus.

	Activity remaining (%)					
	<u>Trichomonas vaginalis</u>			<u>Trichomonas foetus</u>		
	Bz-Phe-Pro-Arg-Nan pH 5.0	Bz-Phe-Pro-Arg-Nan pH 7.0	Bz-Arg-Nan pH 5.0	Bz-Pro-Phe-Arg-Nan pH 5.0	Bz-Pro-Phe-Arg-Nan pH 7.0	Bz-Arg-Nan pH 5.0
Pepstatin	56 + 17	95 + 7	98 + 11	96 + 1	100 + 2	92 + 17
PMSF	93 + 2	98 + 4	100 + 15	106 + 6	98 + 5	101 + 7
TLCK	52 + 14	21 + 7	16 + 21	20 + 12	18 + 4	11 + 25
TPCK	49 + 12	34 + 18	26 + 19	24 + 15	9 + 1	38 + 12
Iodoacetic acid	62 + 15	37 + 11	42 + 32	25 + 14	13 + 2	17 + 31
Leupeptin	54 + 9	12 + 6	18 + 27	14 + 10	15 + 3	18 + 18
Chymostatin	43 + 12	17 + 12	32 + 16	16 + 9	19 + 5	31 + 22
Antipain	51 + 16	15 + 8	11 + 9	14 + 6	8 + 4	22 + 12
Phenanthroline	60 + 2	61 + 31	63 + 48	51 + 14	70 + 41	71 + 16

Activities are given as a percentage of that in control samples (values given in Table 7). The results are the mean + standard deviation from at least three independent determinations.

Table 8b. Effect of inhibitors on the hydrolysis of Bz-Pro-Phe-Arg-Nan and Bz-Arg-Nan by Trichomitus batrachorum and Pentatrachomonas hominis.

	Activity Remaining (%)				
	<u>Trichomitus batrachorum</u>		<u>Pentatrachomonas hominis</u>		
	Bz-Pro-Phe-Arg-Nan pH 5.0	Bz-Arg-Nan pH 7.0	Bz-Arg-Nan pH 5.0	Bz-Pro-Phe-Arg-Nan pH 5.0	Bz-Pro-Phe-Arg-Nan pH 7.0
Pepstatin	51 + 5	66 + 19	100 + 3	101 + 4	99 + 3
PMSF	99 + 2	99 + 4	96 + 6	98 + 9	95 + 5
TLCK	21 + 8	17 + 2	21 + 31	26 + 3	18 + 4
TPCK	34 + 4	31 + 9	16 + 28	22 + 11	20 + 4
Iodoacetic acid	29 + 6	15 + 11	31 + 19	22 + 7	12 + 3
Leupeptin	33 + 3	14 + 3	32 + 17	15 + 6	17 + 2
Chymostatin	32 + 14	24 + 5	46 + 17	24 + 12	21 + 6
Antipain	44 + 14	29 + 2	30 + 26	31 + 7	15 + 4
Phenanthroline	68 + 5	86 + 21	41 + 31	65 + 8	72 + 31

Activities are given as a percentage of that in control samples (values given in Table 7). The results are the mean + standard deviation from at least three independent determinations.

Table 9. Carboxypeptidase activity in trichomonad cell lysates.

	<u>Trichomonas</u> <u>vaginalis</u>	Specific Activity <u>Trichomonas</u> <u>foetus</u>	<u>Trichomitus</u> <u>batrachorum</u>
Substrate			
N-Cbz-Gly-Leu	0.211 \pm 0.038	0.133 \pm 0.066	0.115 \pm 0.019
N-Cbz-Glu-Tyr	0.075 \pm 0.041	0.054 \pm 0.023	0.031 \pm 0.028
N-Cbz-Leu-Tyr	0.074 \pm 0.012	0.074 \pm 0.014	0.038 \pm 0.008

Results are given in units of change in absorbance (measured at 570 nm) h^{-1} (mg protein) $^{-1}$ \pm standard deviation from at least three independent determinations.

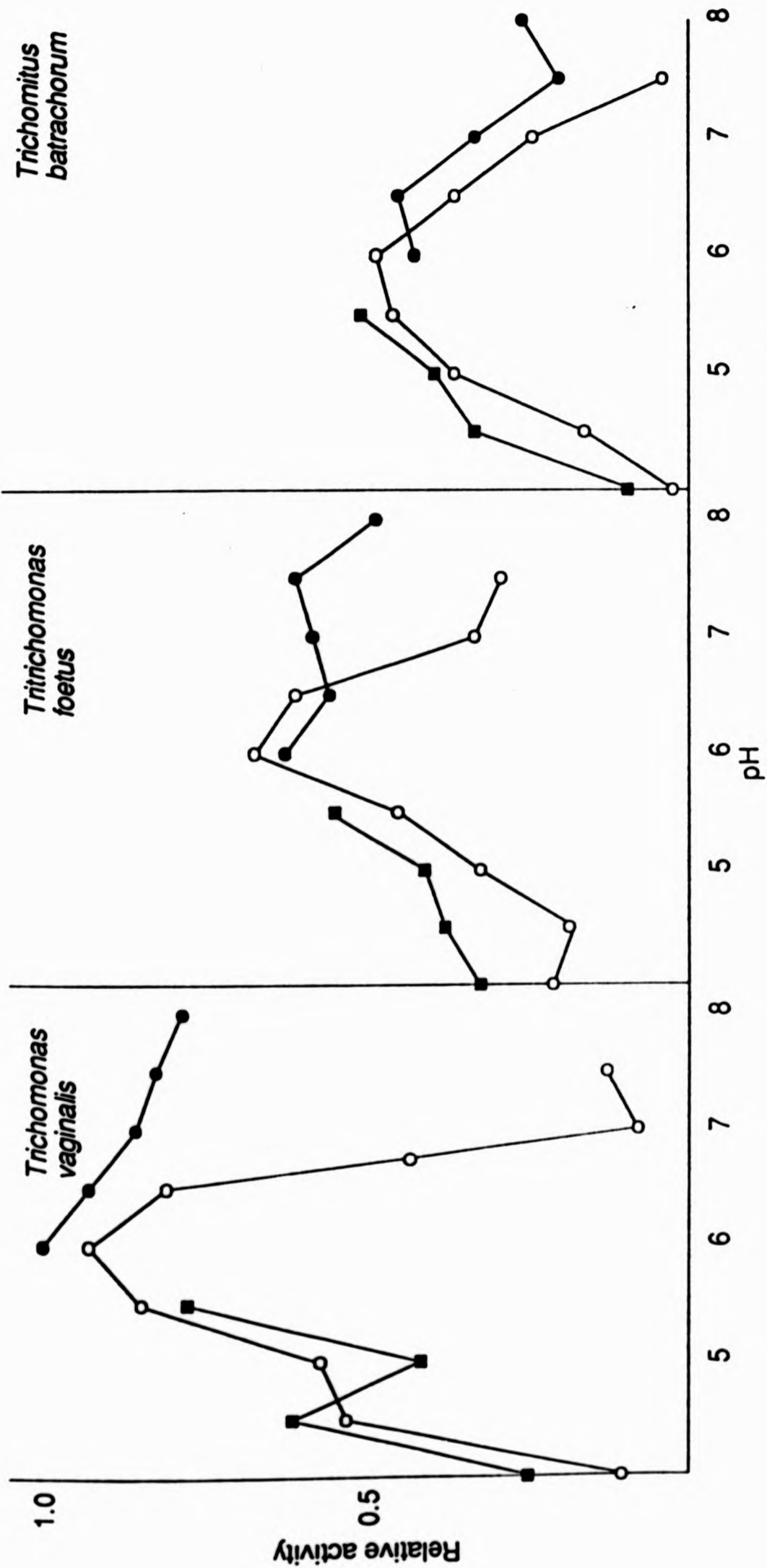


Fig 12. pH dependence of carboxypeptidase activity. Buffers used were citrate phosphate (O), sodium acetate/acetic acid (■) and sodium phosphate (●).

3.1.4. Aminopeptidase activity.

Aminopeptidase activities were detected towards five substrates in the lysates of all four trichomonad species (Table 10). The highest activity was detected towards L-alanine *p*-nitroanilide in *T. vaginalis* lysates. In general, the activities detected towards all the substrates were higher in *T. vaginalis* and *Tritrichomonas foetus* than in *Trichomitus batrachorum* and *F. hominis*.

3.1.5. Summary.

Proteinase activities were detected in the lysates of each of the four species of trichomonad using the protein substrates hide powder azure and azocasein. Both activities appeared to be due to cysteine proteinases since they were DTT-dependent and inhibited by cysteine proteinase inhibitors. The activities, however, differed from one another with respect to their pH optimum and relative sensitivity to inhibitors. Although these differences could result from the properties of the substrates it is likely that the activities are due at least in part to different enzymes. This is supported by the finding that the ratio of HPAase to AZCase is not the same for all four species.

At least two types of enzyme in trichomonad lysates appear to be capable of hydrolysing Bz-Pro-Phe-Arg-Nan. The first, a DTT-dependent activity, may be similar to those responsible for the HPAase and AZCase activities. The second, a DTT-independent activity was similar to the activity towards Bz-Arg-Nan with respect to sensitivity to proteinase inhibitors and pH optima.

Table 10. Aminopeptidase activity in trichomonad cell lysates.

Substrate	Specific Activity			
	<u>Trichomonas vaginalis</u>	<u>Tritrichomonas foetus</u>	<u>Trichomitrus batrachorum</u>	<u>Pentatrichomonas hominis</u>
S-Benzyl-L-Cys-Nan	11.97 + 3.3	20.71 + 1.7	5.03 + 1.3	11.93 + 2.0
L-Ala-Nan	78.24 + 30.0	24.55 + 4.4	5.20 + 0.8	11.94 + 3.1
L-Pro-Nan	15.08 + 2.9	12.60 + 1.9	7.36 + 2.5	4.29 + 1.1
Gly-Nan	13.92 + 2.3	16.91 + 3.4	5.64 + 1.8	7.50 + 2.1
L-Leu-Nan	27.66 + 3.4	19.14 + 2.1	8.10 + 0.7	12.22 + 1.8

Results are given in units of $\text{nmol min}^{-1} (\text{mg protein})^{-1}$ + standard deviation from at least three independent determinations.

A number of carboxypeptidase and aminopeptidase activities were also detected in the lysates of all four species of trichomonad but the enzymes responsible have not been investigated further.

3.2. Detection and separation of proteolytic activity in trichomonads using electrophoretic techniques.

Since the results of the investigations of trichomonad proteinases using conventional assays indicated that more than one activity was present in each species, electrophoretic techniques were used to analyse these enzymes in more detail. The advantage of these techniques is that the activities can be detected and separated simultaneously. Initially the investigation involved a continuation of the earlier work by Coombs and North (1983) using haemoglobin gels but subsequently an improved method involving SDS-PAGE of gels containing copolymerised gelatin was adopted.

3.2.1. Haemoglobin-disc gel electrophoresis.

The trichomonad proteinases were analysed using electrophoresis in polyacrylamide gels containing denatured haemoglobin. After electrophoresis and incubation in appropriate buffer, proteinase activities were detected as clear bands on the gels. The densitometric scans of gels for the four trichomonad species are shown in Fig. 13.

The results obtained for *Trichomonas vaginalis* confirmed those reported previously (Coombs and North 1983). At least seven bands of proteolytic activity could be detected following electrophoresis of *T. vaginalis* lysates and the proteinases could be distinguished from one

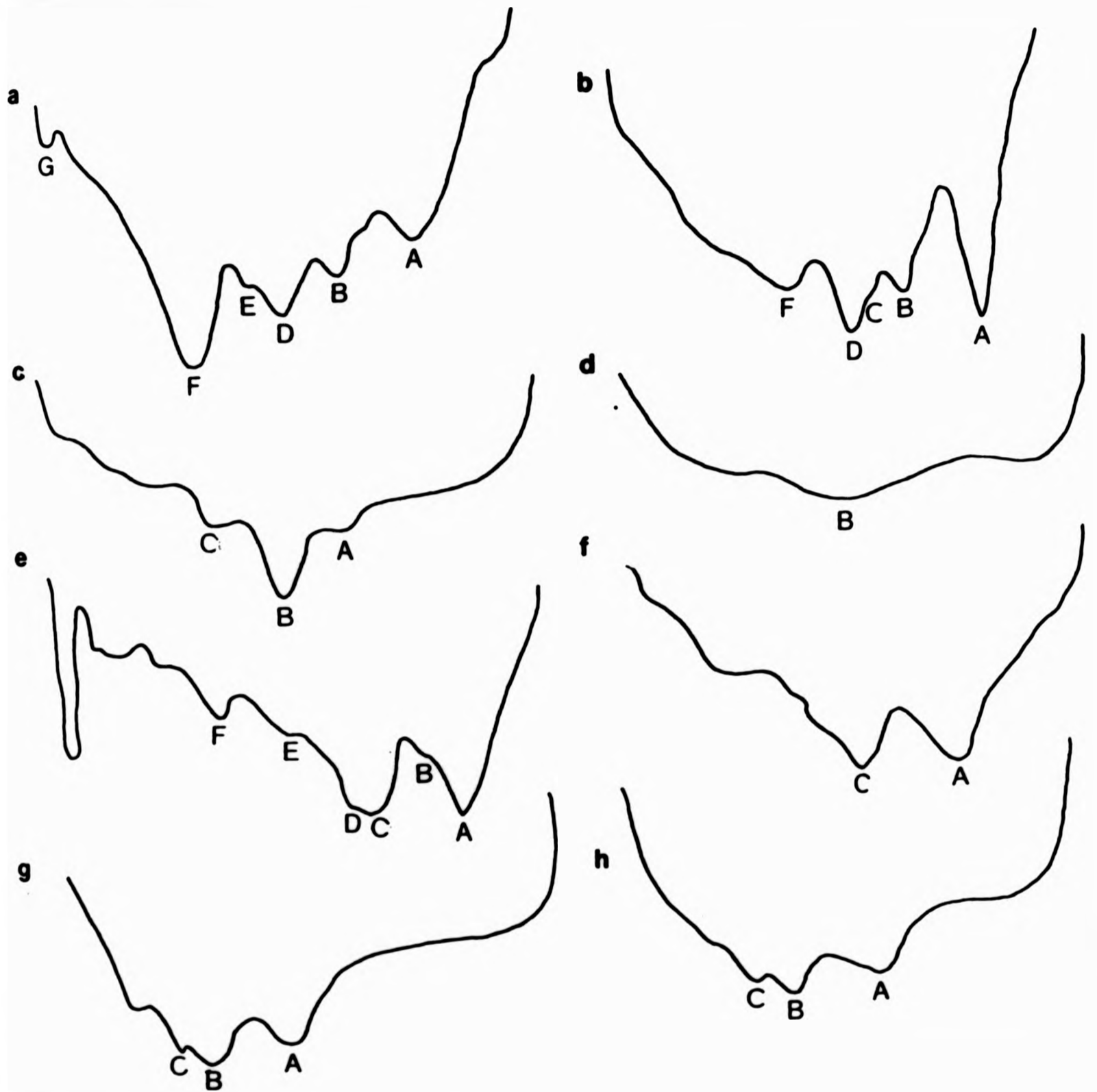


Fig. 13. Representative densitometric scans of proteinase band patterns on haemoglobin gels. Samples which were run from the cathode (left) to the anode (right), were of *Trichomonas vaginalis* (a and b), *Trichomitus batrachorum* (c and d), *Tritrichomonas foetus* (e and f) and *Pentatrichomonas hominis* (g and h). After electrophoresis gels were incubated at pH 4.0 in the presence (a, c, e and g) or absence (b, d, f and h) of 1 mM DTT.

another by their relative activities, pH dependence and their sensitivities to proteinase inhibitors. A similarly complex pattern of proteolytic activity was demonstrated after electrophoresis of *Iritrichomonas foetus* lysates, with six proteinase bands detected on the gels. At pH 4 bands A and C appeared most rapidly (within 2 h), band D appeared more slowly (4-8 h) and bands E and F were usually visible after 12 h incubation. Band B was not always visible by eye but appeared as a distinct shoulder on the densitometric scan. All the bands appeared optimally when the gels were incubated at pH 4.0, and only bands A and C became visible when the gels were incubated at pH 6.0. Similarly, only bands A and C became visible when 1 mM DTT was omitted from the pH 4.0 incubation buffer.

With *Trichomitus batrachorum* and *F. hominis* the band patterns on the gels were much simpler. On incubation of *T. batrachorum* at pH 4.0 only one band (B) was visible in the absence of DTT, while in the presence of DTT, bands A and C were also seen but only after a long incubation (up to 18 h): band B was usually visible within 1 h but appeared much more slowly in the absence of the reducing agent. With *F. hominis* three relatively low activity bands were detected at pH 4.0 in the presence of 1 mM DTT. In the absence of DTT all three bands were still visible but reduced. At pH 6.0 only bands A and B were visible but band A could be distinguished as a shoulder on the densitometric scan. In the absence of DTT none of the bands were apparent.

With both *Trichomitus batrachorum* and *Iritrichomonas foetus* the appearance of all of the bands was inhibited by all the cysteine proteinase inhibitors tested, namely TLCK, TPCK, iodoacetic acid, leupeptin, chymostatin and antipain. Pepstatin and PMSF had no effect on

the appearance of any of the bands. This was similar to the result obtained previously with *Trichomonas vaginalis* (Coombs and North 1983). The effects of proteinase inhibitors on the activities of *P. hominis* were not investigated.

3.2.2. Gelatin-PAGE.

There are a number of limitations associated with analysing the proteinases in cell lysates using the haemoglobin gel system. In particular, it provides little information about the physical properties of the enzymes detected and it is relatively insensitive. In addition, there are a number of inherent practical disadvantages. For example, it is inconvenient for handling large numbers of samples, and it is difficult to make direct comparisons between the proteinases detected on individual gels. The use of polyacrylamide slab gels which contain copolymerised gelatin as the substrate provides an improved and highly sensitive electrophoretic method for separating and detecting the trichomonad proteinases. The use of denaturing conditions makes it possible to obtain molecular weight data directly from the gels and allows a better comparison to be made between the activities present in different samples.

The sensitivity of the method for detecting proteinases was tested using both suspensions of live cells and also cell lysates. Samples containing between 10^2 and 10^6 live cells of *Trichomonas vaginalis* were mixed directly with an equal volume of sample buffer and subjected to SDS-PAGE. Proteolytic activity could be detected in all samples that contained 10^4 or more cells. When cell lysates containing varying amounts of protein were analysed using this method, proteinase activity

could be detected in samples containing as little as 1 μg protein. The conventional assays using azocasein and hide powder azure as substrates were unable to detect proteinase activity in trichomonad lysates containing less than 10 μg protein and an individual assay could not distinguish between different forms of proteinase.

There were differences between the four species of trichomonad with respect to (i) the number of proteinase activities detected (ii) the molecular weights of the enzymes responsible (see Table 11) and (iii) the specific activities of the proteinases. At least eleven bands of proteolytic activity were detected in lysates of *T. vaginalis* which ranged in molecular weight from less than 20 kDa to 110 kDa. Eight bands of activity were detected in *Tritrichomonas foetus* and *Trichomitus batrachorum*. The highest activity bands of *Tritrichomonas foetus* had low molecular weights (less than 32 kDa) whereas those of *Trichomitus batrachorum* were of higher molecular weight (32 kDa to 45 kDa). Only four bands of activity were detected in *P. hominis*. A range of different polyacrylamide concentrations were used to achieve optimal separation of the trichomonad proteinases and Fig. 14 shows the band patterns obtained using a 7.5% (w/v) polyacrylamide gel. The apparent molecular weights of the individual proteinases were determined from their mobility on a number of gels containing different concentrations of polyacrylamide (Table 11).

The gel shown in Fig. 14 was incubated for a sufficient length of time (24 h) to allow all bands to appear. In other experiments the gels were incubated for 4 h, which was sufficient only for the major (those with apparent molecular weights of 28, 38, 42, 48, 68, 96 and 110 kDa (*Trichomonas vaginalis*); <20, 25, 32 and 34 kDa (*Tritrichomonas foetus*);

Table 11. Apparent molecular weights (in kDa) of trichomonad proteinases detected by gelatin-PAGE.

<u>Pentatrichomonas</u> <u>hominis</u>	<u>Trichomitus</u> <u>batrachorum</u>	<u>Tritrichomonas</u> <u>foetus</u>	<u>Trichomonas</u> <u>vaginalis</u>
32	32	20	20
45	34	25	28
52	40	32	34
60	43	34	38
	45	64	42
	48	70	48
	60	86	54
	84	120	68
			86
			96
			110

The data were derived from at least six gelatin-PAGE analyses using gels of different polyacrylamide concentration in the range 5% to 15%.

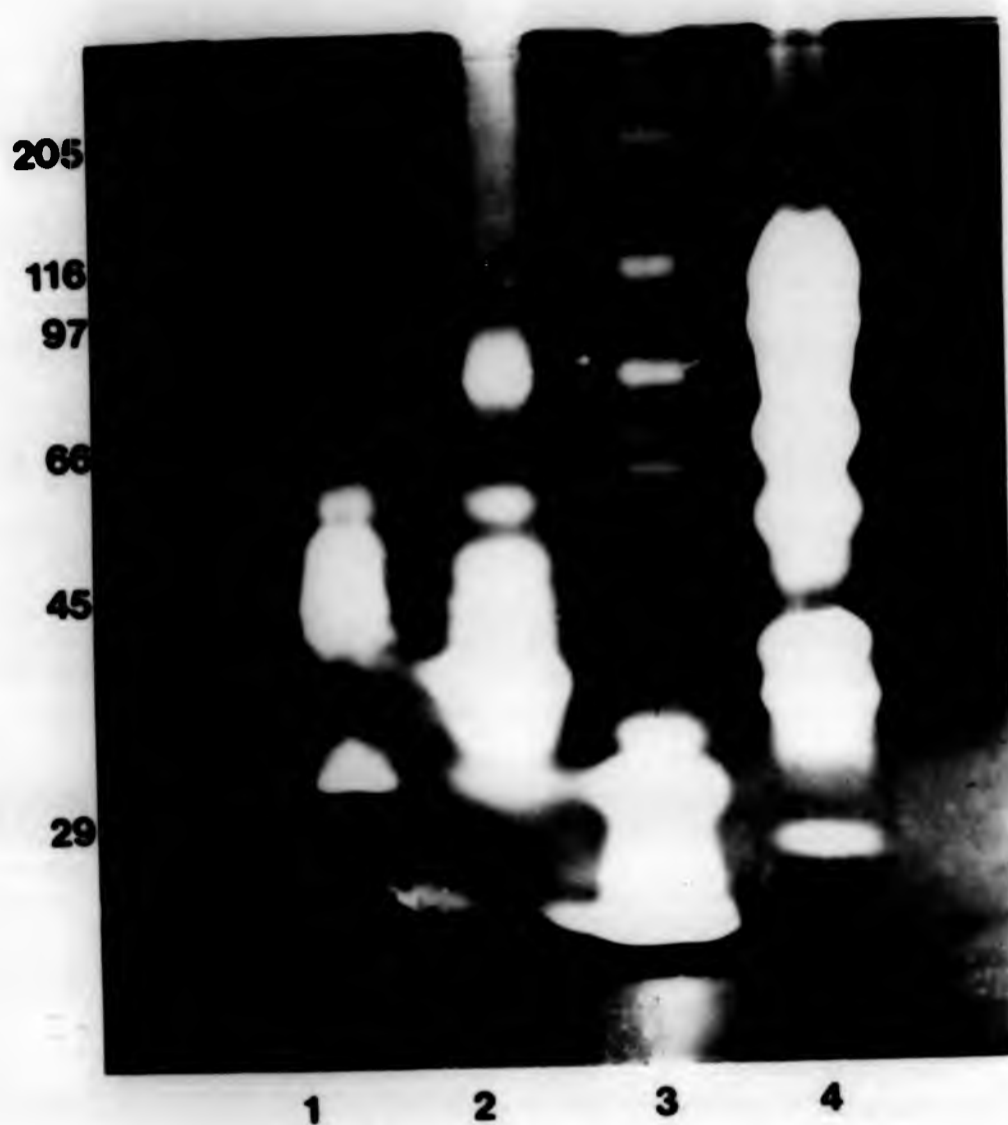


Fig. 14. Proteinase activities in trichomonad species analysed by gelatin-PAGE. Samples of cell lysate (containing the amount of protein indicated) were subjected to electrophoretic analysis as described in section 2.3.2. except that the gel was incubated at pH 5.5 for 24 h. Samples were run from the cathode (top) to anode (bottom). Lane 1, *Pentatrichomonas hominis* (25 μ g); lane 2, *Trichomitus batrachorum* (25 μ g); lane 3, *Tritrichomonas foetus* (10 μ g); lane 4, *Trichomonas vaginalis* (10 μ g). The standard proteins (left), in ascending molecular weight, were carbonic anhydrase, egg albumin, bovine albumin, phosphorylase b, β -galactosidase, myosin. The molecular weights are given in kDa.

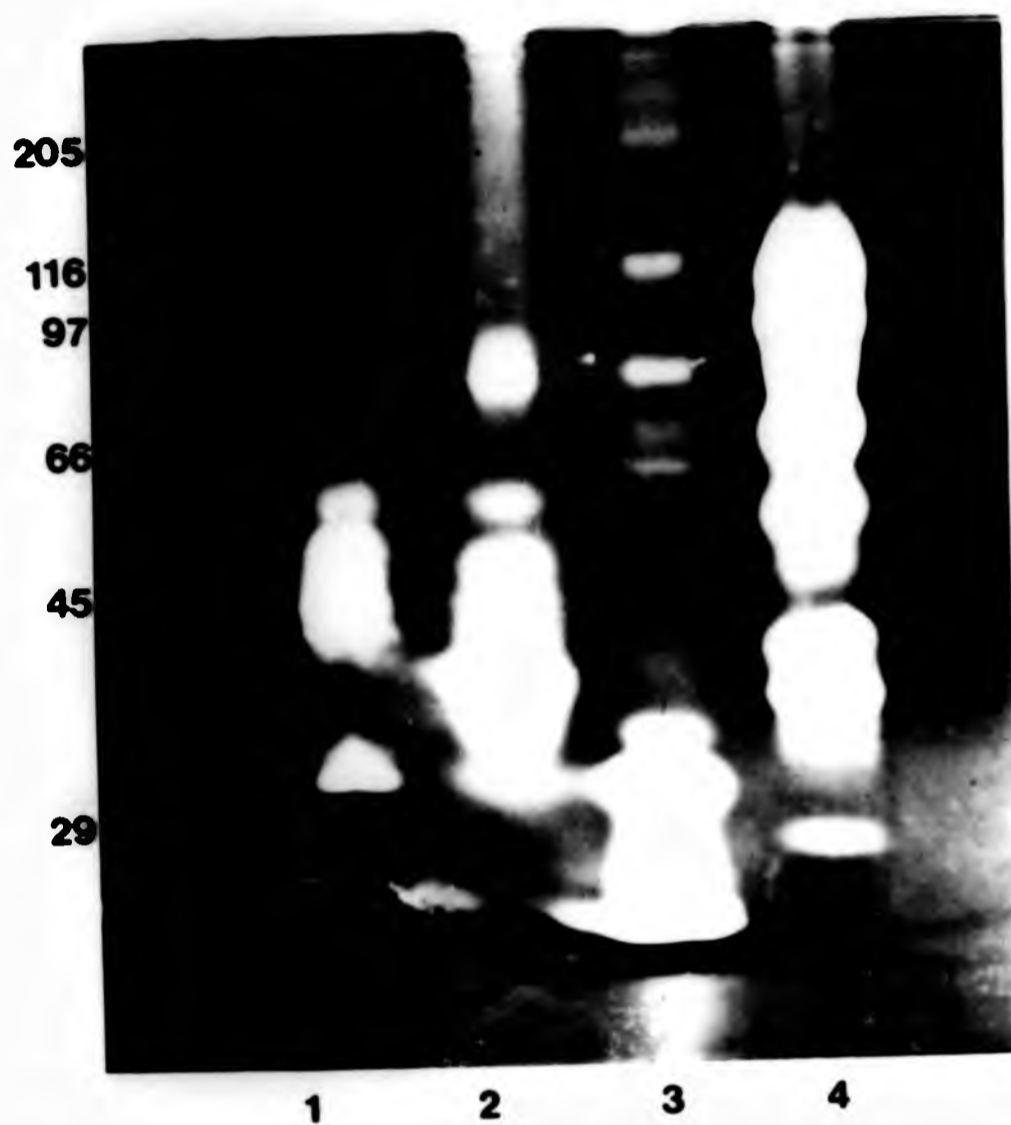


Fig. 14. Proteinase activities in trichomonad species analysed by gelatin-PAGE. Samples of cell lysate (containing the amount of protein indicated) were subjected to electrophoretic analysis as described in section 2.3.2. except that the gel was incubated at pH 5.5 for 24 h. Samples were run from the cathode (top) to anode (bottom). Lane 1, *Pentatrichomonas hominis* (25 μ g); lane 2, *Trichomitus batrachorum* (25 μ g); lane 3, *Tritrichomonas foetus* (10 μ g); lane 4, *Trichomonas vaginalis* (10 μ g). The standard proteins (left), in ascending molecular weight, were carbonic anhydrase, egg albumin, bovine albumin, phosphorylase b, β -galactosidase, myosin. The molecular weights are given in kDa.

34, 40, 43, 45, 48 and 84 kDa (*Tritrichomonas foetus*); 32, 45 and 52 kDa (*P. hominis*) bands to appear. The major activities of *Trichomonas vaginalis* and *Tritrichomonas foetus* appeared more rapidly (within 1 h) than those of the two intestinal parasites *Trichomitus batrachorum* and *P. hominis* suggesting that the enzymes in the former two species were at higher activity. All the activities were considerably enhanced by the addition of DTT to the incubation buffer, thus 1 mM DTT was included in the standard procedure. The properties of the proteinases of *Trichomonas vaginalis* and *Tritrichomonas foetus* were examined in more detail.

It was considered possible that the presence of multiple proteinase bands on the gels could be due to self-degradation of the enzymes after preparation of the lysates. To test this possibility lysates were incubated in sample buffer for 1 h at 37°C prior to electrophoresis. However, there was no change in the relative activities of any of the bands indicating that all were stable under these conditions and that no proteinase was likely to have been converted from one form to another during PAGE analysis.

To investigate the pH dependence of the proteinase activity the gels were incubated in different buffers in the pH range 2.0-8.0. The effect of pH was determined by visual observation of stained gels and confirmed by analysing densitometric scans (Fig. 15). The major proteinase activities of *Trichomonas vaginalis* and *Tritrichomonas foetus* were optimal in the pH range 5.0-6.0. Two of the minor proteinases of *Trichomonas vaginalis*, however, were found to have optimal activity outside this range: the 54 kDa-proteinase, although visible after incubation at pH 5.5, was optimally active at pH 8.0 and a low molecular

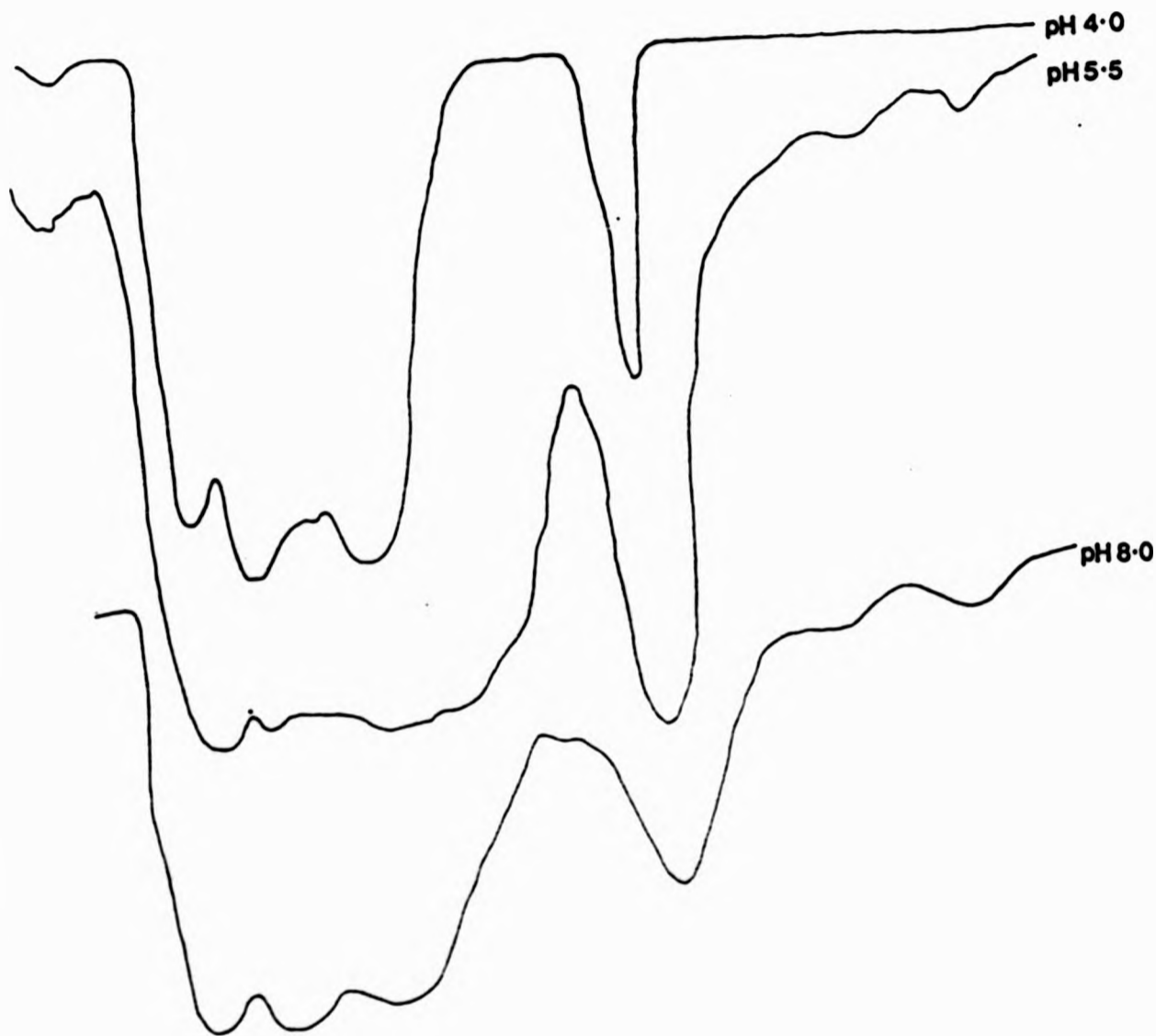


Fig. 15. Effect of pH on proteinase band patterns produced by gelatin-PAGE analysis of *Trichomonas vaginalis* lysates. Buffers used were sodium acetate/acetic acid (pH 4.0 and 5.5); sodium phosphate (pH 8.0). Densitometric scans of patterns produced after samples were run from the cathode (left) to the anode (right) are shown.

weight (<20 kDa)-proteinase was detectable only when the gels were incubated at pH 4.0.

Development of the proteinase bands observed for *T. vaginalis* and *Tritrichomonas foetus* was inhibited to varying degrees by agents which inactivate cysteine proteinases (Table 12). All bands of each species were similarly affected. In contrast, band appearance was not inhibited by pepstatin ($20 \mu\text{g ml}^{-1}$), PMSF (1 mM) nor EDTA (1 mM). Since antipain and leupeptin both had inhibitory effects they were used in further experiments to test whether self-degradation might be responsible for the presence of certain of the proteinases observed after PAGE. When either of the inhibitors was included at $100 \mu\text{g ml}^{-1}$ in the 0.25 M sucrose used for lysate preparation, all of the proteinase bands of *Trichomonas vaginalis* and *Tritrichomonas foetus* normally observed were still present, with no apparent change in the relative activities. Significantly the electrophoretic pattern of parasite proteins (stained with coomassie blue) was much clearer and sharper when the lysates were prepared in this way. Since the proteinase activities were unaffected they are apparently not formed by autolysis which confirms the results of the preincubation experiments (see previous page).

3.2.3. Summary.

Multiple forms of proteinases, active at acid pH, DTT-dependent and inhibited by cysteine proteinase inhibitors were detected by electrophoresis of trichomonad lysates on gels containing denatured haemoglobin or copolymerised gelatin. The number and relative activities of the enzymes detected differed between species. Using both techniques it was demonstrated that *T. vaginalis* possessed the greatest number of

Table 12. Effect of cysteine proteinase inhibitors on band appearance due to proteinases of Trichomonas vaginalis and Tritrichomonas foetus.

Inhibitor	Concentration	<u>Trichomonas vaginalis</u>	<u>Tritrichomonas foetus</u>
Antipain	5 μ g ml ⁻¹	+	+
	20 μ g ml ⁻¹	+	+
	100 μ g ml ⁻¹	+	-
Leupeptin	5 μ g ml ⁻¹	+	+
	20 μ g ml ⁻¹	+	+
	100 μ g ml ⁻¹	-	-
TLCK	1 mM	-	+
TPCK	1 mM	++	++
	5 mM	+	+
Mercuric chloride	1 mM	-	-
Iodoacetic acid	0.1 mM	+	+
	1 mM	-	-
	5 mM	-	-

++ as control
 + bands reduced but still visible
 - no bands visible

activities and *T. vaginalis* and *Tritrichomonas foetus* possessed forms of higher specific activity than *Trichomitus batrachorum* and *P. hominis*.

In all four species more bands were detected on the gelatin gels than on the haemoglobin gels. Since the gelatin gels were run in the presence of SDS whereas the haemoglobin gels were run under native conditions direct comparisons of the individual activities detected on the two types of gel were not possible. The gelatin-PAGE method provides a method for distinguishing between the four species on the basis of their characteristic proteinase band patterns.

3.3. Proteolytic activity in isolates of *Trichomonas vaginalis*.

Trichomonas vaginalis, clone G3, has been maintained in axenic culture for many years and so it was of interest to determine whether its level of proteolytic activity was typical of all *T. vaginalis* isolates, including those recently obtained from patients (see section 2.1.1.).

3.3.1. Activity towards protein substrates.

Cysteine proteinase activities were detected in lysates of the *T. vaginalis* clone G3, towards hide powder azure, optimal at pH 5.5 and towards azocasein, optimal at pH 5.0. The data in Table 13 show that the level of activity detected in G3 towards hide powder azure was similar to that of most of the other isolates tested. Nevertheless, there was some variation in activity between isolates, notably 6950 σ^A possessed less than half the HPAase activity of any of the other isolates. The AZCase activities showed more variation, with only two of the isolates, 39 *in vivo* and 39 *in vitro*, possessing a comparable level of activity to that

Table 13. Proteinase activity in a range of isolates of Trichomonas vaginalis.

Isolate	Specific Activity	
	HPAase	AZCase
G3	49.4 ± 4.5	10.2 ± 1.5
39 <u>in vitro</u>	62.8 ± 16.9	10.4 ± 1.1
39 <u>in vitro</u>	61.6 ± 15.3	7.8 ± 0.9
39	43.2 ± 10.5	4.1 ± 1.1
64	50.8 ± 12.0	5.6 ± 2.2
1297	50.6 ± 5.3	5.1 ± 2.3
2755	47.6 ± 2.5	5.6 ± 1.1
45	49.6 ± 5.1	5.0 ± 1.7
45733	47.4 ± 7.5	3.2 ± 2.4
61	49.4 ± 4.0	4.9 ± 2.7
55	36.8 ± 5.3	4.9 ± 1.7
46	34.2 ± 4.2	5.3 ± 1.6
IR 78	38.6 ± 6.6	3.9 ± 3.7
6950♂	16.2 ± 5.5	3.7 ± 1.8

Results are given in units of g substrate hydrolysed min⁻¹ (mg protein)⁻¹ and are the mean ± standard deviation from four independent determinations.

of G3. All of the others had significantly lower AZCase activity. The variation in the ratio of HPAase: AZCase activity confirm the suggestion that there may be differences in the proteinases detected by each of the two substrates. Further analysis of the enzymes responsible for the activities detected was carried out using the two electrophoretic methods.

3.3.2. Activity detected on haemoglobin gels.

All of the major bands detected in G3 could be detected in each of the isolates analysed by electrophoresis on haemoglobin-containing gels. Differences, however, were apparent in the relative activities of the bands as represented by the size of the troughs on the densitometric scans (Fig 16). For example, isolate 39 *in vivo* appeared to have a higher activity band F than band D whereas in all other isolates the reverse was true. Some of the minor bands present in G3 were not detected or severely reduced in some of the isolates. Band G was not detectable in isolates 2755 and 39 *in vivo* and band E was also absent from 2755. There was no evidence for bands in the isolates that were not also present in G3. All of the other isolates tested (not shown in Fig 16) had the same band pattern as G3.

3.3.3. Activity detected on gelatin gels.

Eight of the isolates were further investigated by SDS-PAGE on gelatin gels. All of the isolates had similar levels of proteinase activity to one another but all had significantly lower levels of activity than G3 (Fig. 17). There were also a number of qualitative differences between the isolates. The 54 kDa-proteinase detected in G3, was not detectable in isolates 45733 and 64. The latter two, however, had a

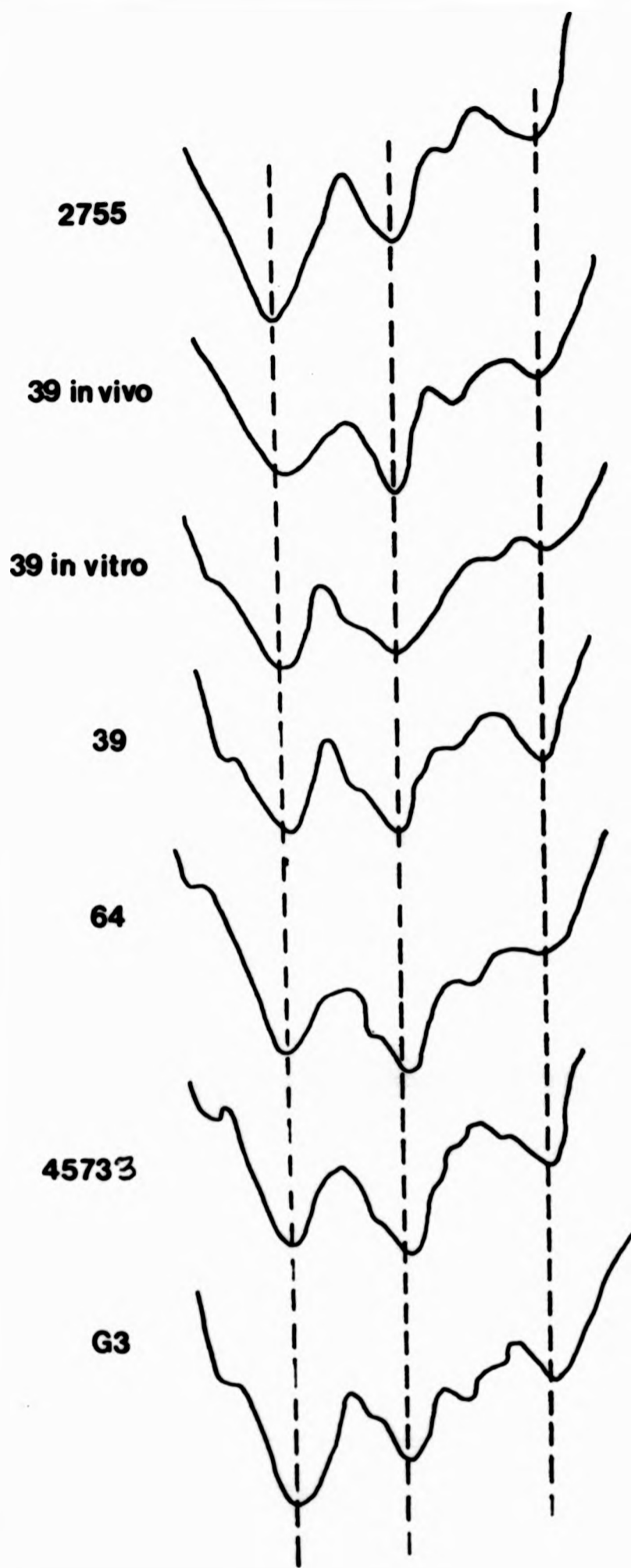


Fig. 16. Densitometric scans of proteinase band patterns produced by isolates of *Trichomonas vaginalis* on haemoglobin gels. Samples containing 50 μ g of protein were run from the cathode (left) to the anode (right).

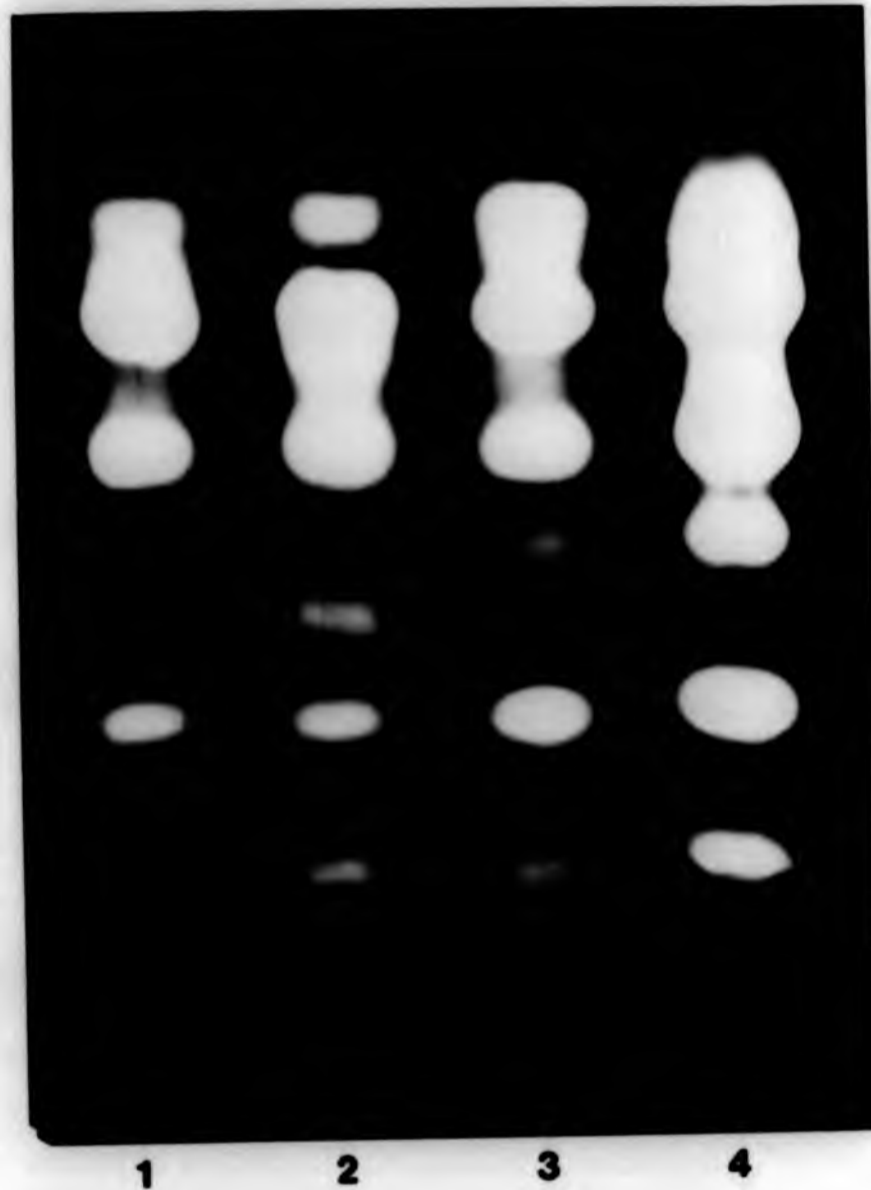


Fig. 17. Proteinase activities in isolates of *Trichomonas vaginalis*. Samples of cell lysate (containing 25 μ g protein) were subjected to gelatin-PAGE analysis as described in section 2.3.2. Lane 1, 45733; lane 2, 64; lane 3, 39; lane 4, G3.

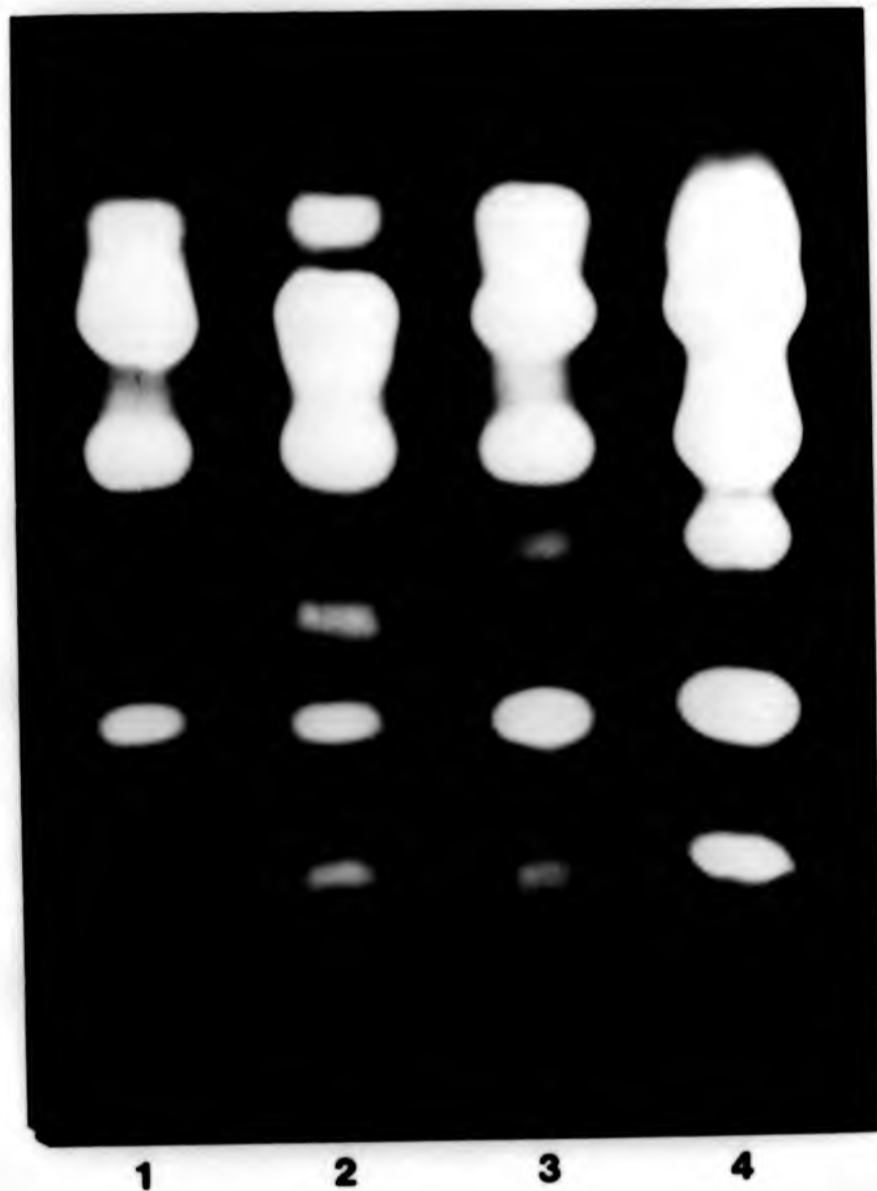


Fig. 17. Proteinase activities in isolates of *Trichomonas vaginalis*. Samples of cell lysate (containing 25 μ g protein) were subjected to gelatin-PAGE analysis as described in section 2.3.2. Lane 1, 45733; lane 2, 64; lane 3, 39; lane 4, G3.

proteinase with an apparent molecular weight of 45 kDa which was absent from G3. Isolates 45733 and 64 could be distinguished by differences in the high molecular weight activities. All of the other isolates examined by this method, 69500, 2755, 39 *in vivo* and 39 *in vitro*, had the same proteinase band pattern as 39. This isolate had a barely detectable 54 kDa-activity. The higher molecular weight proteinases more closely resembled those of G3, albeit with a lower specific activity.

3.3.4. Summary.

Using hide powder azure and azocasein, differences were demonstrated in the levels of proteinase activity present in the isolates. Some isolates had more than 3-fold higher activity than others. No correlation was apparent between the levels of proteinase detected and the way the isolates were maintained prior to this study or with resistance or sensitivity to metronidazole (see section 2.1.1.).

Differences in the activities of individual enzymes ~~were~~ observed by electrophoresis of the isolate lysates on gels containing denatured haemoglobin. Comparison of the isolates with G3 showed that several bands were absent or reduced in the band patterns produced by some of the isolates but no new bands were identified.

The results obtained using electrophoretic analysis of isolates on gelatin gels indicated both quantitative and qualitative differences between the isolates. Clone G3 possessed significantly higher levels of activity than any of the other isolates. A number of qualitative differences were also apparent in three of the isolates investigated. Bands present in G3 were not detectable or reduced in the other isolates and bands not present or not detectable due to low levels of activity in

G3 were present or of increased activity in the other isolates. This has shown that the gelatin-PAGE technique is capable of distinguishing between isolates.

3.4. Distribution of intracellular proteolytic activity in *Trichomonas vaginalis* and *Tritrichomonas foetus*

In order to establish the intracellular localisation of the proteinase activities detected in the cell lysates of *Trichomonas vaginalis* and *Tritrichomonas foetus*, the parasites were fractionated using a combination of differential and density gradient centrifugation. In addition to analysis of the proteinase activities a number of other hydrolase activities were investigated.

3.4.1. Activity in fractions separated by differential centrifugation.

Homogenates of *T. vaginalis* and *Tritrichomonas foetus* were fractionated by differential centrifugation into four components: the nuclear fraction, large particle fraction, small particle and microsomal fraction and the non-sedimentable supernatant (see section 2.1.6.). Most of the proteinase activity (towards hide powder azure and azocasein) was concentrated in the two particulate fractions (Fig 18). With *Trichomonas vaginalis* there were significant levels of activity in both of the fractions, which were combined together for further analysis. In contrast, most of the *Tritrichomonas foetus* activity was concentrated in the large particle fraction. Similar distributions were obtained for β -N-acetylglucosaminidase, acid phosphatase, α -mannosidase and β -glucosidase. Again, most of the hydrolase activities were concentrated in the

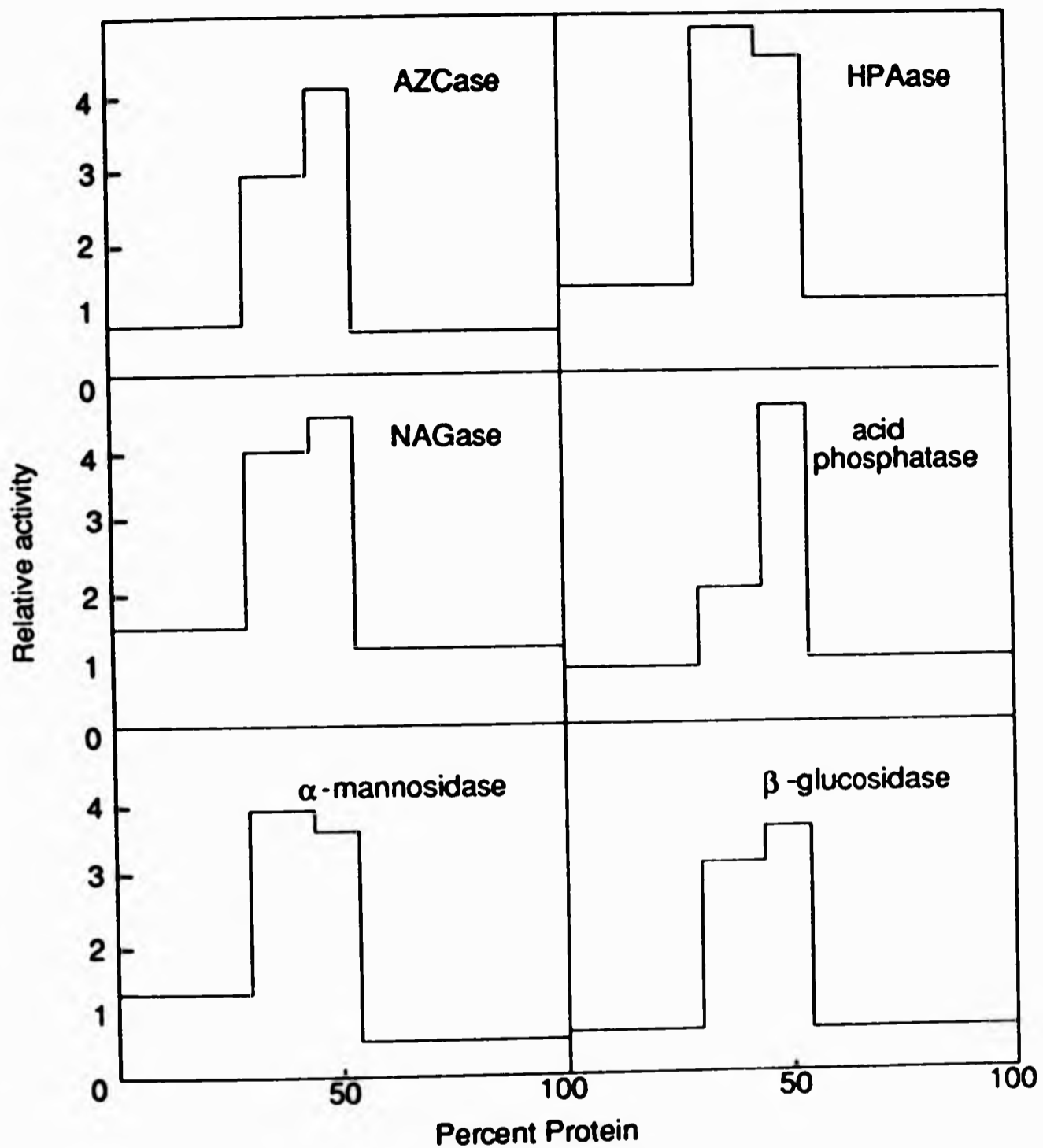


Fig. 18. Distribution of hydrolases after differential centrifugation.
(a) *Trichomonas vaginalis*. Relative activity was plotted against cumulative percentage protein recovered in each fraction. In the graph the direction from left to right corresponds to increasing centrifugal field. Percentage recoveries were: AZCase, 81.0; HPAase, 115.4; β -N-acetylglucosaminidase (NAGase), 91.4; acid phosphatase, 108.8; α -mannosidase, 85.0; β -glucosidase, 75.5.

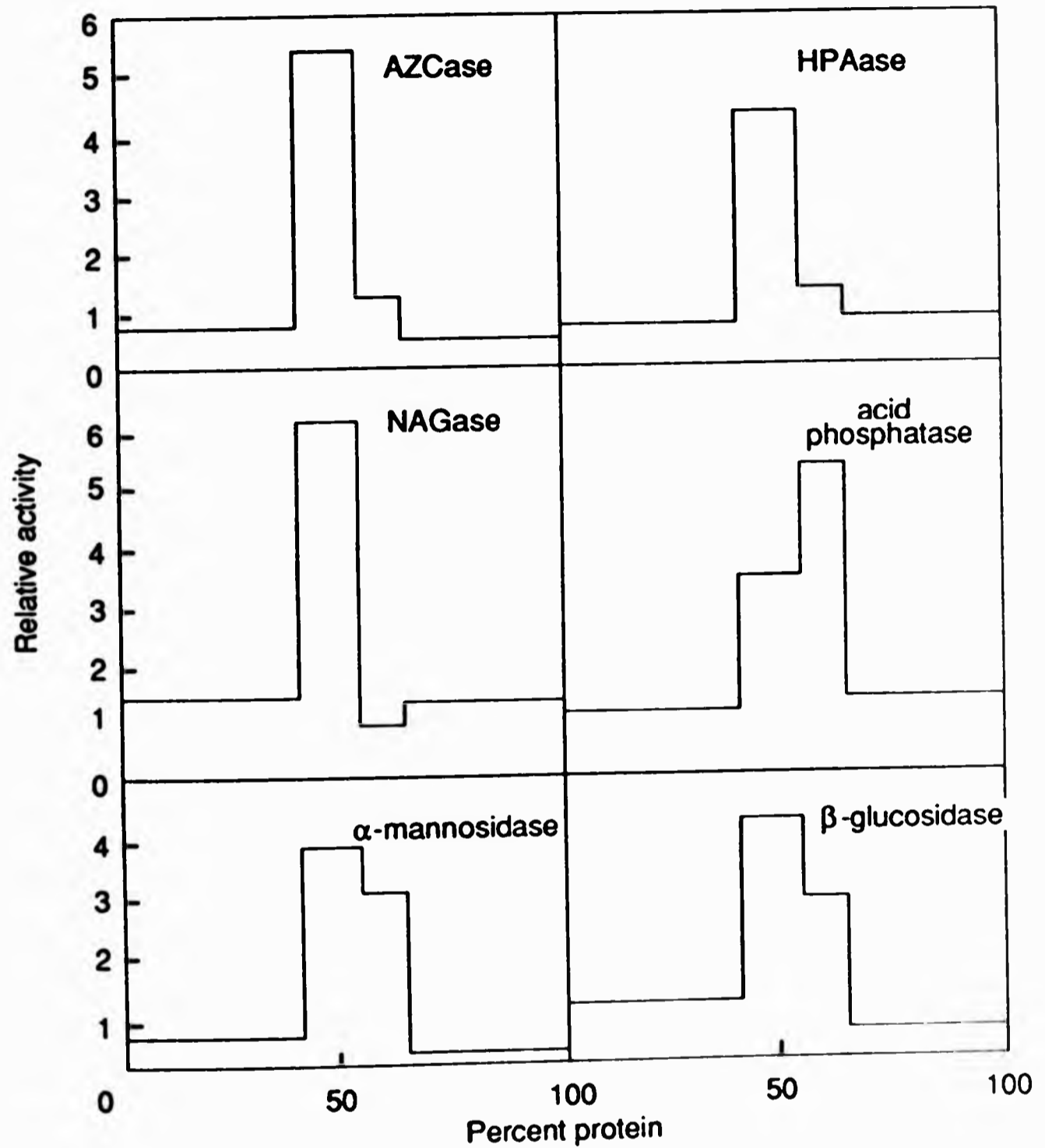


Fig. 18. Distribution of hydrolases after differential centrifugation.

(b) *Tritrichomonas foetus*. Relative activity was plotted against cumulative percentage protein recovered in each fraction. In the graph the direction from left to right corresponds to increasing centrifugal field. Percentage recoveries were: AZCase, 88.2; HPAase, 71.8; β-N-acetylglucosaminidase (NAGase), 86.8; acid phosphatase, 117.9; α-mannosidase, 86.3; β-glucosidase, 101.2.

particulate fractions. As with the proteinase activity significant levels of activity were present in both the large and small particle fractions of *Trichomonas vaginalis* whereas the *Tritrichomonas foetus* activity was higher in the large particle fraction with the exception of acid phosphatase which was higher in the small particle fraction (Fig. 18). The results are consistent with the proteinase activity being contained along with the other hydrolase activities in lysosome-like particles. The finding that, in *Trichomonas vaginalis* especially, the activity is present in both large and small particles suggests that there is a heterogeneous population of lysosomes in trichomonads.

3.4.2. Activity in fractions separated by density gradient centrifugation.

Analysis of the particulate fractions of *Trichomonas vaginalis* using a gradient containing 50% Percoll in 0.25 M sucrose gave two visible bands. One had an approximate equilibrium density of 1.040 g Percoll ml⁻¹ which on fractionation of the gradient was shown to correspond to the fraction containing the highest levels of hydrolytic activity. The second, fainter band had a higher equilibrium density of approximately 1.065 g ml⁻¹. The fractions corresponding to this band contained only relatively low levels of hydrolase activity. They were, however, shown to possess malate dehydrogenase (decarboxylating) activity which indicates the presence of hydrogenosomes (Fig. 19). A similar analysis of the large and small particle fractions of *Tritrichomonas foetus* did not produce any visible bands on the gradients. Further investigation, however, demonstrated that most of the hydrolytic activity was concentrated in fractions that had an equilibrium density of between

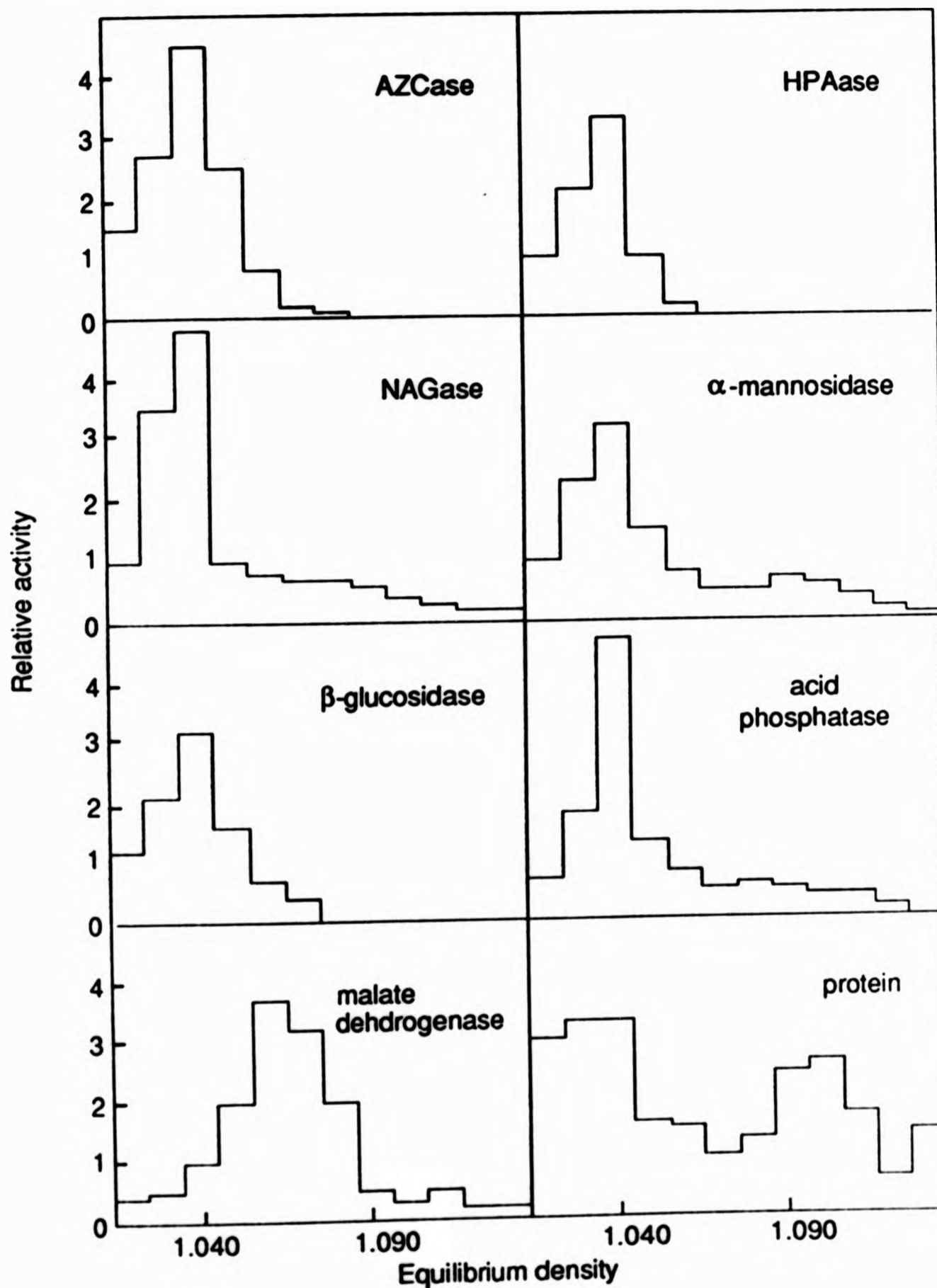


Fig. 19. Distribution of hydrolases after density gradient centrifugation on 50% Percoll gradients.

(a) *Trichomonas vaginalis*. Relative activity is plotted against Percoll density (g ml⁻¹).

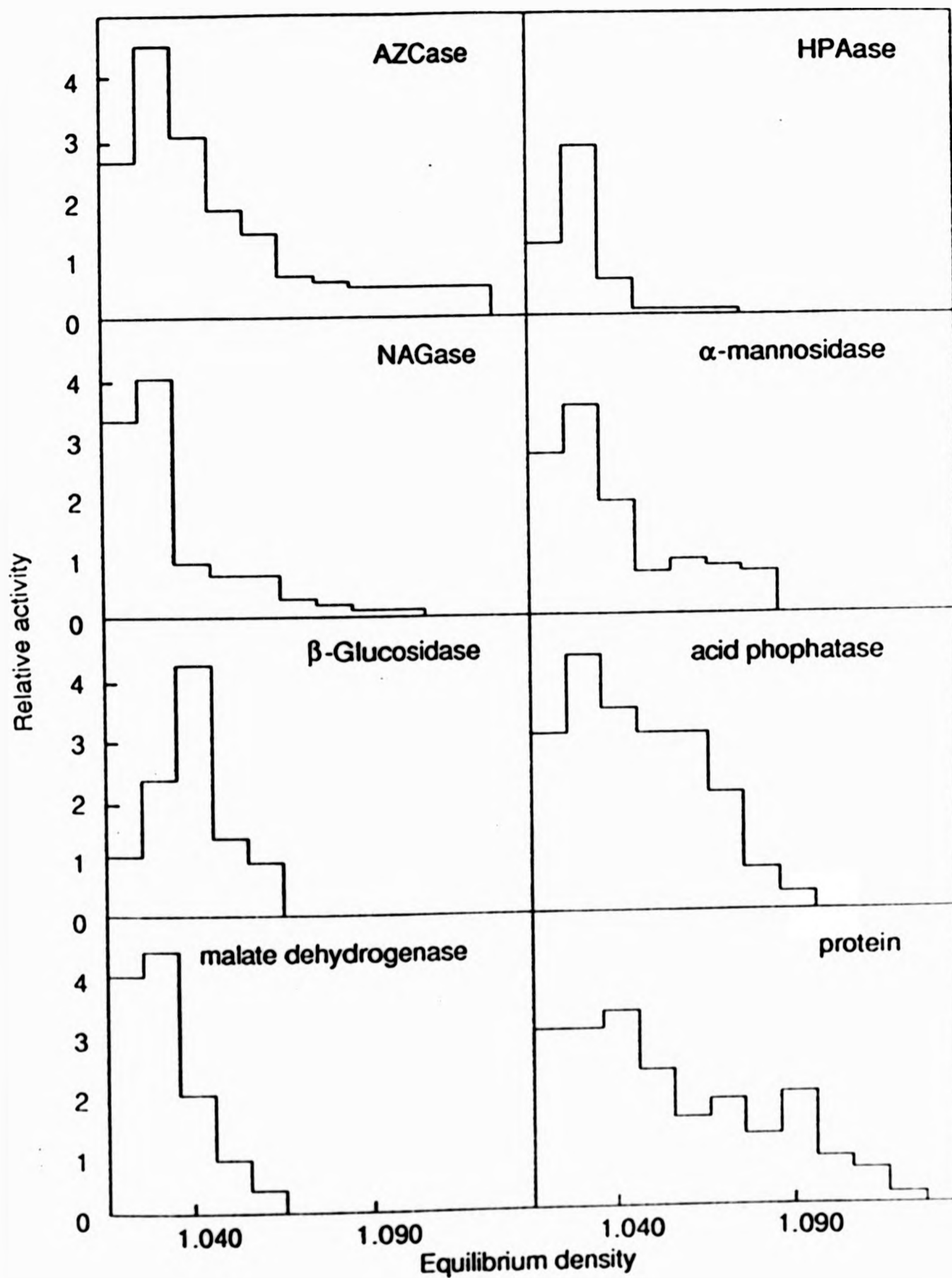


Fig. 19. Distribution of enzymes after density gradient centrifugation on 50% Percoll gradients.

(b) *Tritrichomonas foetus*. Relative activity is plotted against Percoll density (g ml^{-1}).

1.040 and 1.050 g ml⁻¹. Relatively low levels of malate dehydrogenase (decarboxylating) were also detected in these fractions, indicating the presence of a mixed particle population of similar densities (Fig 19). These results show that in *Trichomonas vaginalis* hydrogenosomes have a higher density than lysosomes although in *Tritrichomonas foetus* the lysosomes appear to have a higher density than the ~~hydrogenosomes~~.

An improved separation of the hydrolase-containing fractions was achieved using gradients containing 20% Percoll. Visual observations of the *Trichomonas vaginalis* gradient again showed two bands, one had an equilibrium density of 1.035 g ml⁻¹, the other had a density of 1.050 g ml⁻¹. Analysis of the fractions showed that these two bands corresponded to two peaks in the hydrolase activity (Fig. 20). Although all of the hydrolase activities were present in both fractions the relative levels varied. For example, almost all of the HPAase activity was present in the lower density fractions in contrast to the AZCase activity which was present at relatively similar levels in both the high and low density fractions. These two fractions indicate the presence of at least two different lysosome populations in *T. vaginalis*. Similar analysis of the hydrolases of *Tritrichomonas foetus* does not indicate the presence of more than one major population of lysosomes. Only one peak in hydrolase activity was detected on 20% gradients corresponding to a Percoll density of 1.050 g ml⁻¹, except in the case of β -N-acetylglucosaminidase which was also detected in a lower density fraction (1.035 g ml⁻¹). The distribution of β -glucosidase activity on the gradient was different from that of the other hydrolases since a peak of activity was detected in a fraction corresponding to a Percoll density of approximately 1.042 g ml⁻¹.

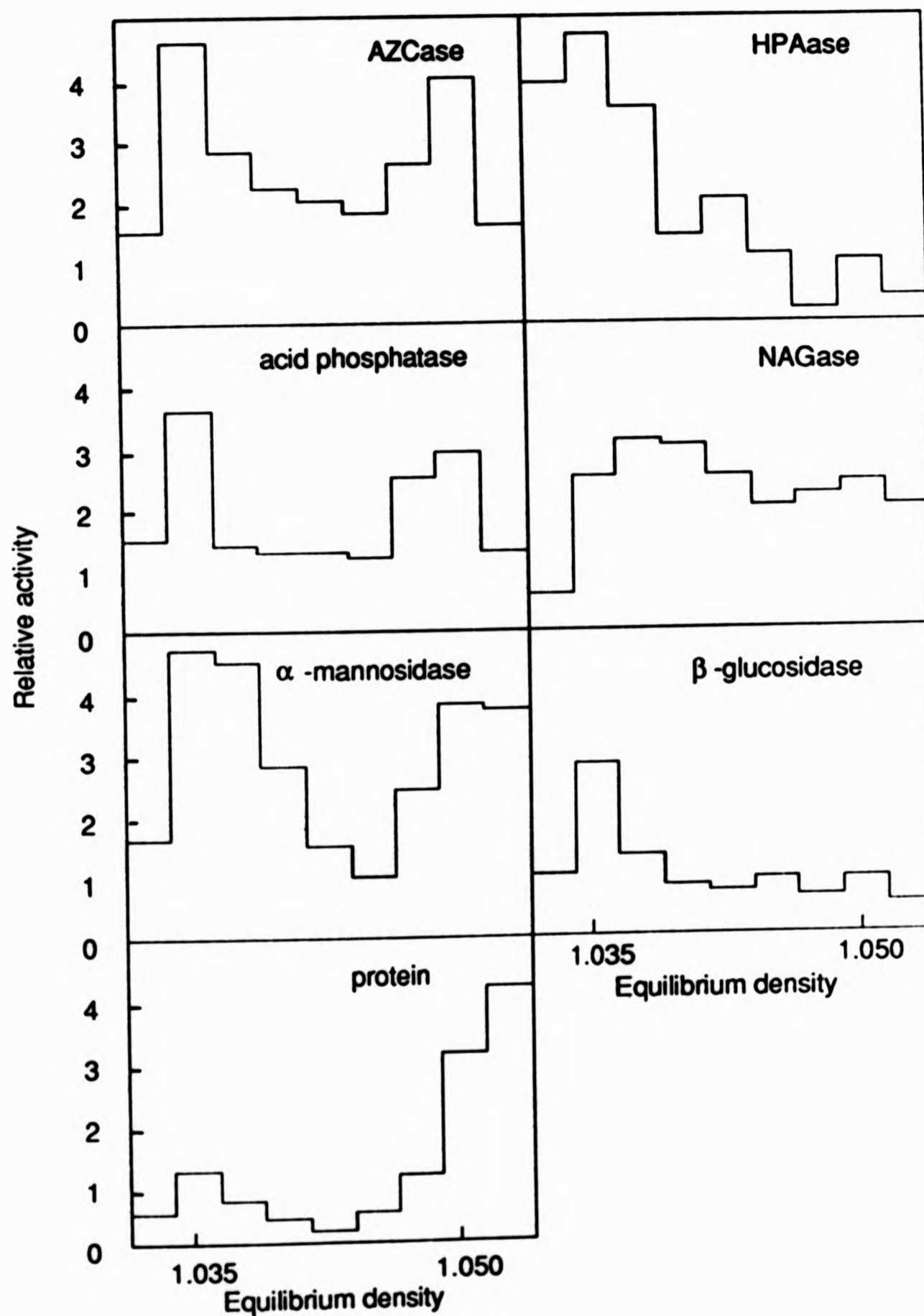


Fig. 20. Distribution of hydrolases after density gradient centrifugation on 20% Percoll gradients.

(a) *Trichomonas vaginalis*. Relative activity is plotted against Percoll density (g ml⁻¹).

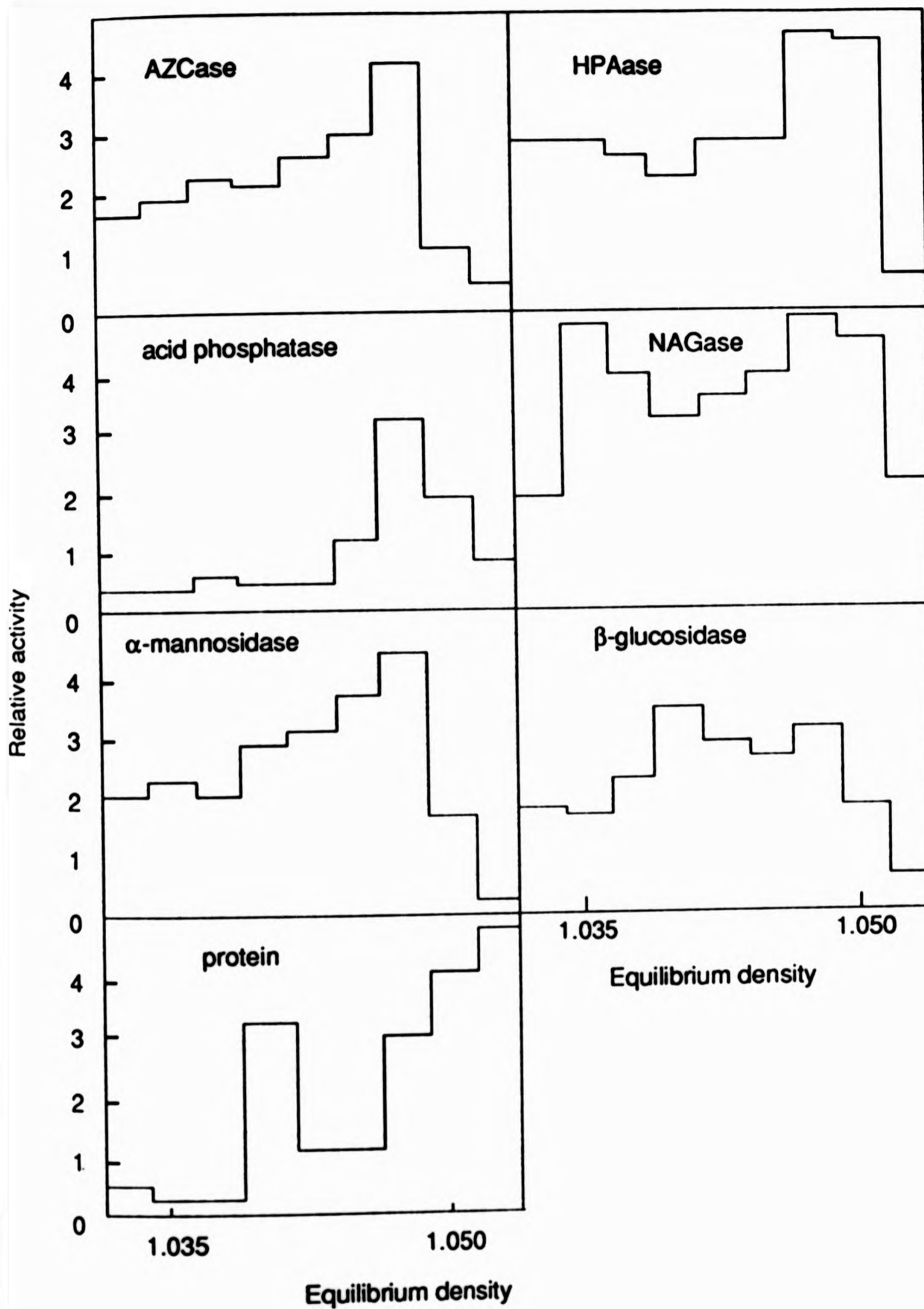


Fig. 20. Distribution of hydrolases after density gradient centrifugation on 20% Percoll gradients.

(b) *Tritrichomonas foetus*. Relative activity is plotted against Percoll density (g ml⁻¹).

Latency was demonstrated in all of the assays on gradient fractions. If the assay was performed without osmotic protection the level of activity was unchanged by the addition of Triton X-100 to a final concentration of 0.2%. In the presence of 0.25 M sucrose, addition of the detergent increased the activity by between 50-80%. Similarly high levels of activity were obtained using samples that had been subjected previously to freezing and thawing.

3.4.3. Summary.

AZCase, HPAase, β -N-acetylglucosaminidase, acid phosphatase, α -mannosidase and β -glucosidase activity is associated with particulate fractions of *Trichomonas vaginalis* and *Tritrichomonas foetus*. Further analysis of the particulate fractions on Percoll gradients show that the activity is present in two distinct lysosomal fractions in *Trichomonas vaginalis* but only in one fraction in *Tritrichomonas foetus*. The lysosome fractions can be separated from another important organelle, the hydrogenosome.

3.5. Partial purification of intracellular proteinases of *Trichomonas vaginalis*.

In order to investigate the properties of the intracellular proteinases of *T. vaginalis* in detail, it is first necessary to obtain purified enzymes. Although a large number of methods are available for separating proteins, when the properties of the required protein are largely unknown, a *trial and error* approach is often necessary. The purification described below represents the techniques that proved to be

most successful in the purification of two intracellular proteinases of *Trichomonas vaginalis*.

3.5.1. Gel filtration.

Cell lysates of *Trichomonas vaginalis* were centrifuged to remove the cell debris and other insoluble particles and the resulting supernatant was used as the starting material for the purification of two intracellular proteinases. Approximately 92-96% of the total proteinase activity present in the cell lysates was retained in the soluble fraction and this was loaded on a column of Sephadex G-75 superfine. Two major peaks of activity were eluted from the column (Fig. 21). Both peaks possessed activity towards hide powder azure, azocasein and the peptide nitroanilide Bz-Pro-Phe-Arg-Nan. The first peak was eluted at the void volume of the column, indicating that the enzymes responsible for the proteinase activity had a molecular weight greater than the exclusion limit of the column (>70 kDa). The second broader peak eluted at a volume corresponding to a molecular weight of between 16 and 24 kDa. Fractions containing either the high or low molecular weight activities were pooled and concentrated prior to further purification. Although this procedure resulted in a four-fold increase in the specific activity of the low molecular weight fractions, it gave a decrease in the specific activity of the high molecular weight activity (high-M_w): therefore the high-M_w activity was further purified by a second gel filtration step using a column of Sephacryl S-300 superfine. Proteinase activity towards hide powder azure and azocasein was eluted as a single peak at a volume corresponding to a molecular weight of approximately 64 kDa (Fig 22).

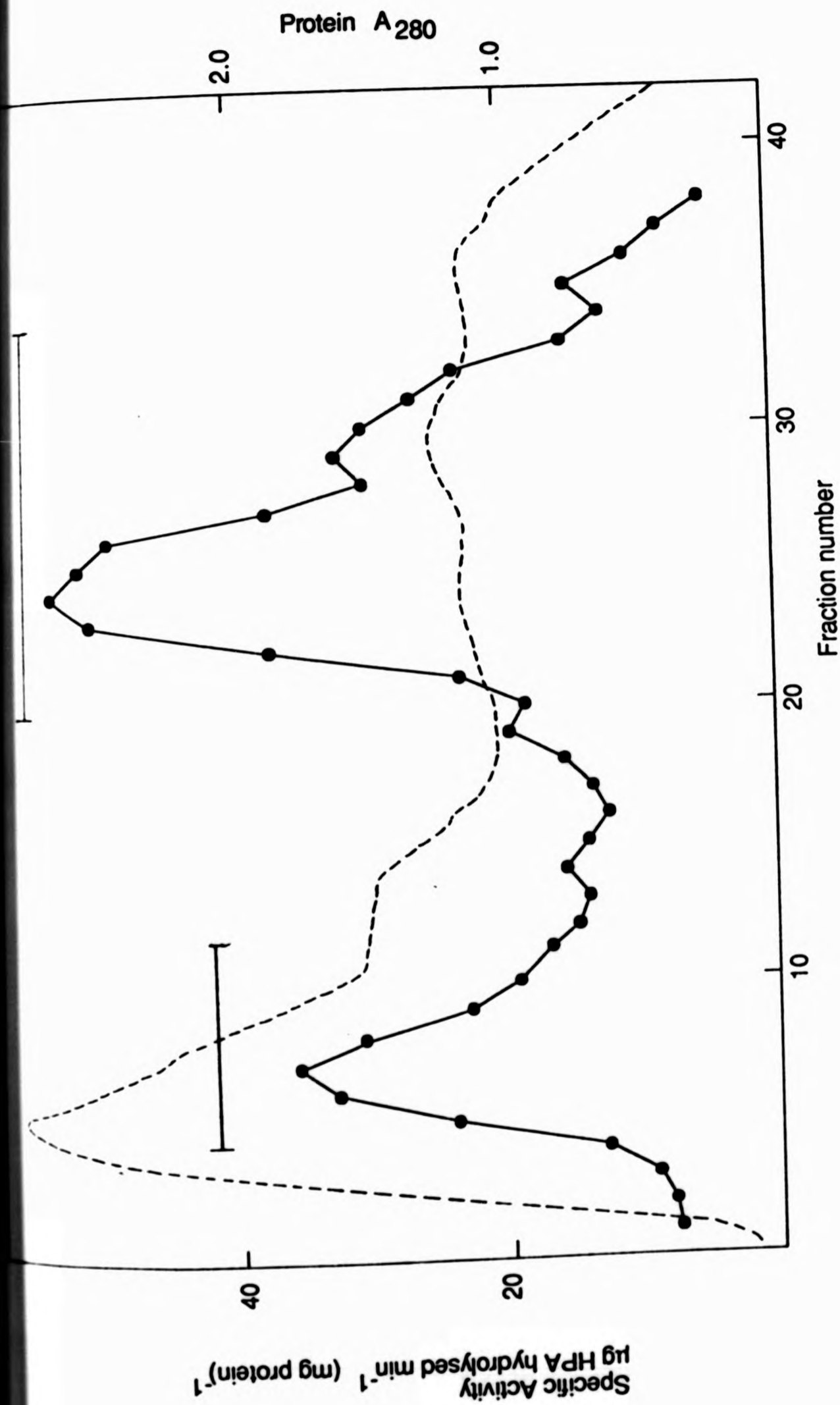


Fig. 21. Gel filtration of trichomonad proteinases on Sephadex G75 superfine. Distribution of proteinases activity towards hide powder azure (●) and protein measured as A280 (----) in the effluent of the column is shown. The fractions combined for further purification are indicated by the horizontal bars.

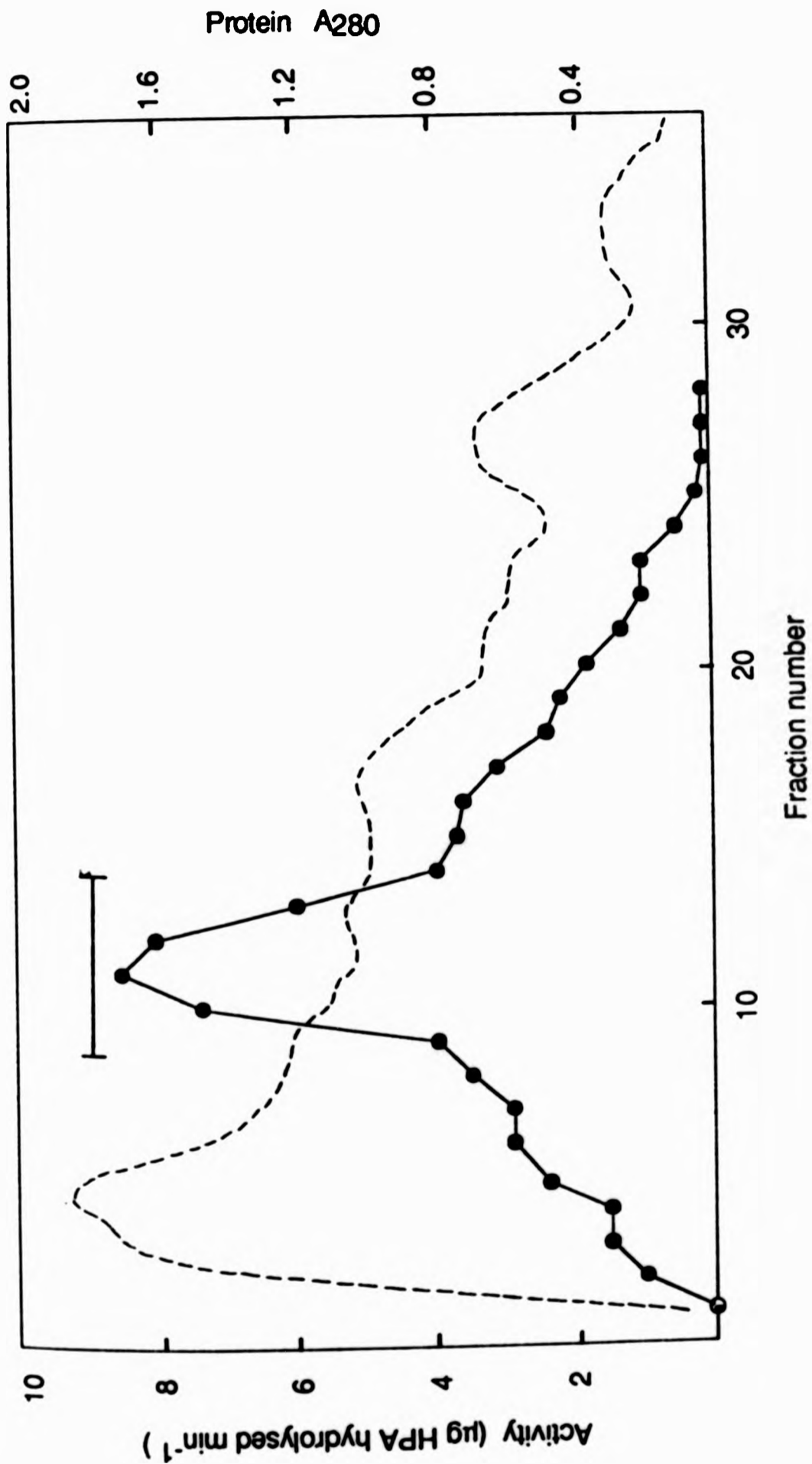


Fig. 22. Gel filtration of trichomonad proteinases on Sephadryl S300. Distribution of proteinase activity towards hide powder azure (●) and protein measured as A280 (---) in the effluent of the column is shown. The fractions combined for further purification are indicated by the horizontal bar.

3.5.2. Ion exchange chromatography.

Preliminary experiments on the pooled and concentrated fractions containing the low M_r proteinase activity demonstrated that the activity bound to anion exchanger at a pH value between pH 6.5 and pH 7.0, and so purification of this activity was continued using a DEAE-cellulose column. The majority of the activity bound to the column, although one peak of activity eluted in the starting buffer. Seven distinct peaks of proteinase activity towards both hide powder azure and azocasein were eluted from the ion-exchange column, using a NaCl gradient (Fig. 23).

Fractions containing the highest level of activity (Peak IV) were pooled and concentrated. Analysis of this activity by PAGE on gels containing denatured haemoglobin gave a single proteinase band, the relative mobility of which was the same as that of proteinase D in the original band patterns produced on the gels by cell lysates of *I. vaginalis* (Fig. 24). Similar analysis of the activities present in the remainder of the peaks eluted from the column did not produce any bands visible on the haemoglobin gels. This may however, be due to this technique being insufficiently sensitive to detect the relatively low levels of activity present in these fractions. The ion-exchange step was very effective in separating the multiple forms of proteinase present in the low molecular weight activity resulting from the preceding gel filtration but resulted in a low recovery of proteinase D, with only 12% of the activity present in the cell lysate retained in peak IV.

3.5.3. Affinity Chromatography.

A number of different affinity chromatography techniques, involving various ligands coupled to CNBr-activated Sepharose 4B, were investigated

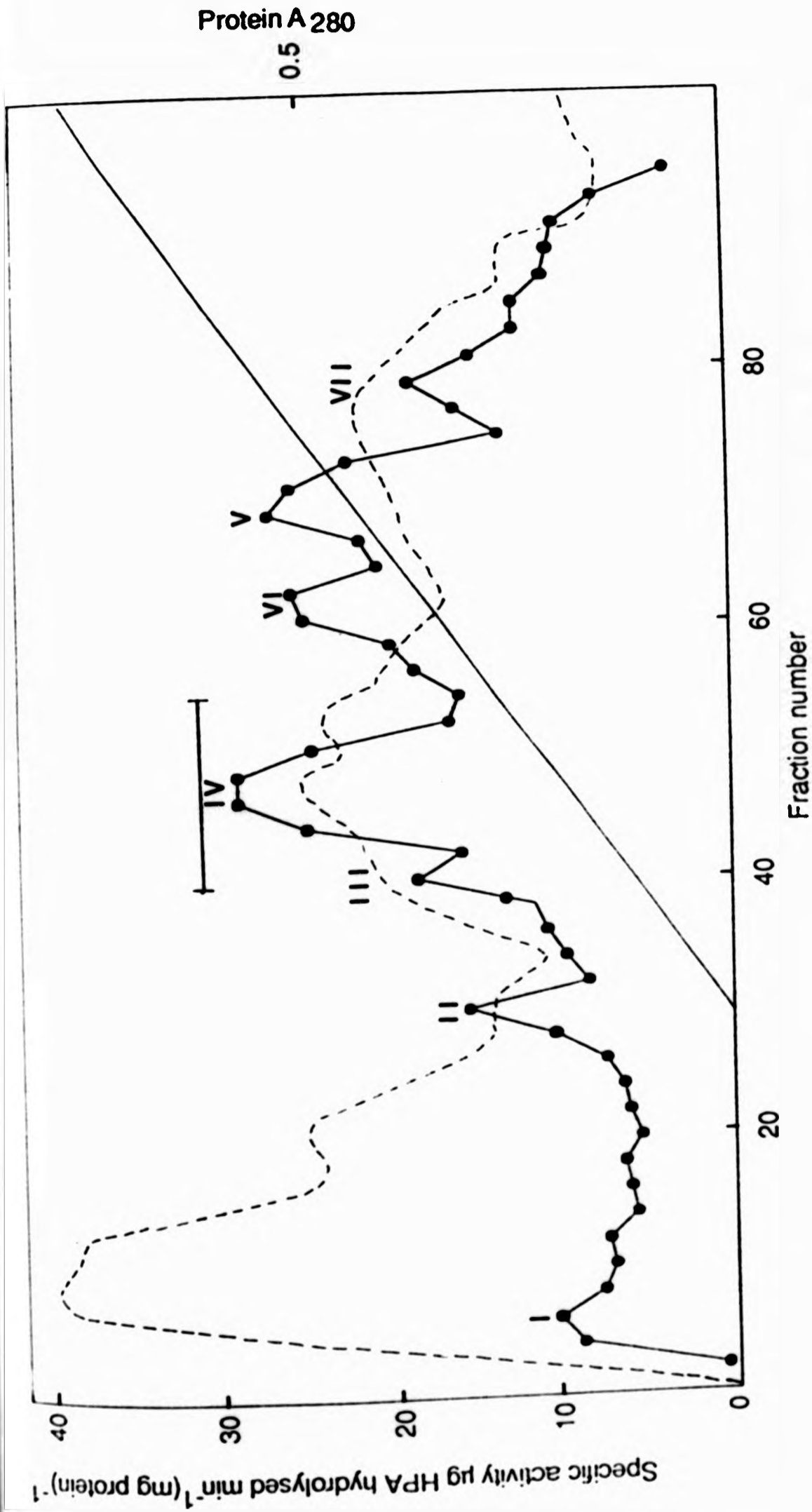


Fig. 23. Ion-exchange chromatography of trichomonad proteinases on DE 52. Distribution of proteinase activity towards hide powder azure (\bullet) and protein measured as A280 (----) in the effluent of the column is shown. The fractions combined for further purification (peak VI) are indicated by the horizontal bar. The sodium chloride gradient (0-300 mM) is represented by the diagonal line.

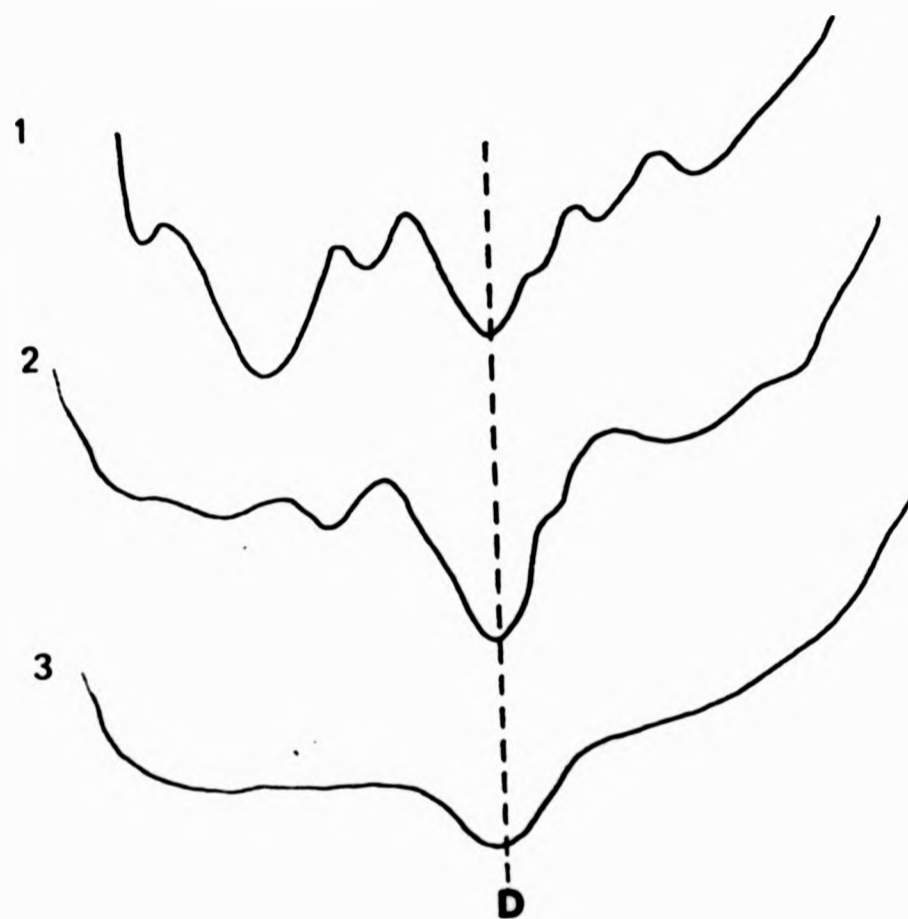


Fig. 24. Analysis of the partially purified proteinase of *Trichomonas vaginalis* on haemoglobin gels. Samples, containing the amounts of protein given were: 1. cell lysate (50 μ g); 2. low molecular weight activity from G75 column (20 μ g); 3. peak IV from DE 52 column. Densitometric scans are shown of gels run from the cathode (left) to the anode (right).

in order to provide further purification of the proteinase activity. Neither the high molecular weight (high-M_r) activity nor proteinase D bound to haemoglobin or α-N-benzoyl-L-arginamide coupled to Sepharose 4B, and although some activities present in the cell lysates bound to activated thiol Sepharose 4B, proteinase D did not. Both the high-M_r and proteinase D did however bind to organomercurial Sepharose. This affinity chromatography step was included in the purification scheme for both of the proteinase activities.

The pooled proteinase D fractions eluted from the the DEAE-cellulose column were dialysed against starting buffer prior to running onto the organomercurial Sepharose column. Unadsorbed protein was eluted first with starting buffer and the adsorbed proteins were eluted using a DTT gradient. The high-M_r activity was treated in exactly the same manner as proteinase D except the activity was eluted from the column using a higher concentration of DTT (Fig 25).

3.5.4. Analysis of purity.

Analysis of the purity of the partially purified enzymes by SDS-PAGE did not produce unequivocal results, since the high levels of proteolysis in trichomonad cell lysates caused streaking and a high level of background staining on the gels. No distinct bands were observed on the gels after electrophoresis of the purified samples.

3.5.5. Summary.

The purification scheme for proteinase D and the high-M_r proteinase is shown in Fig 26. The extent of purification and recoveries of the proteinases are shown in Table 14.

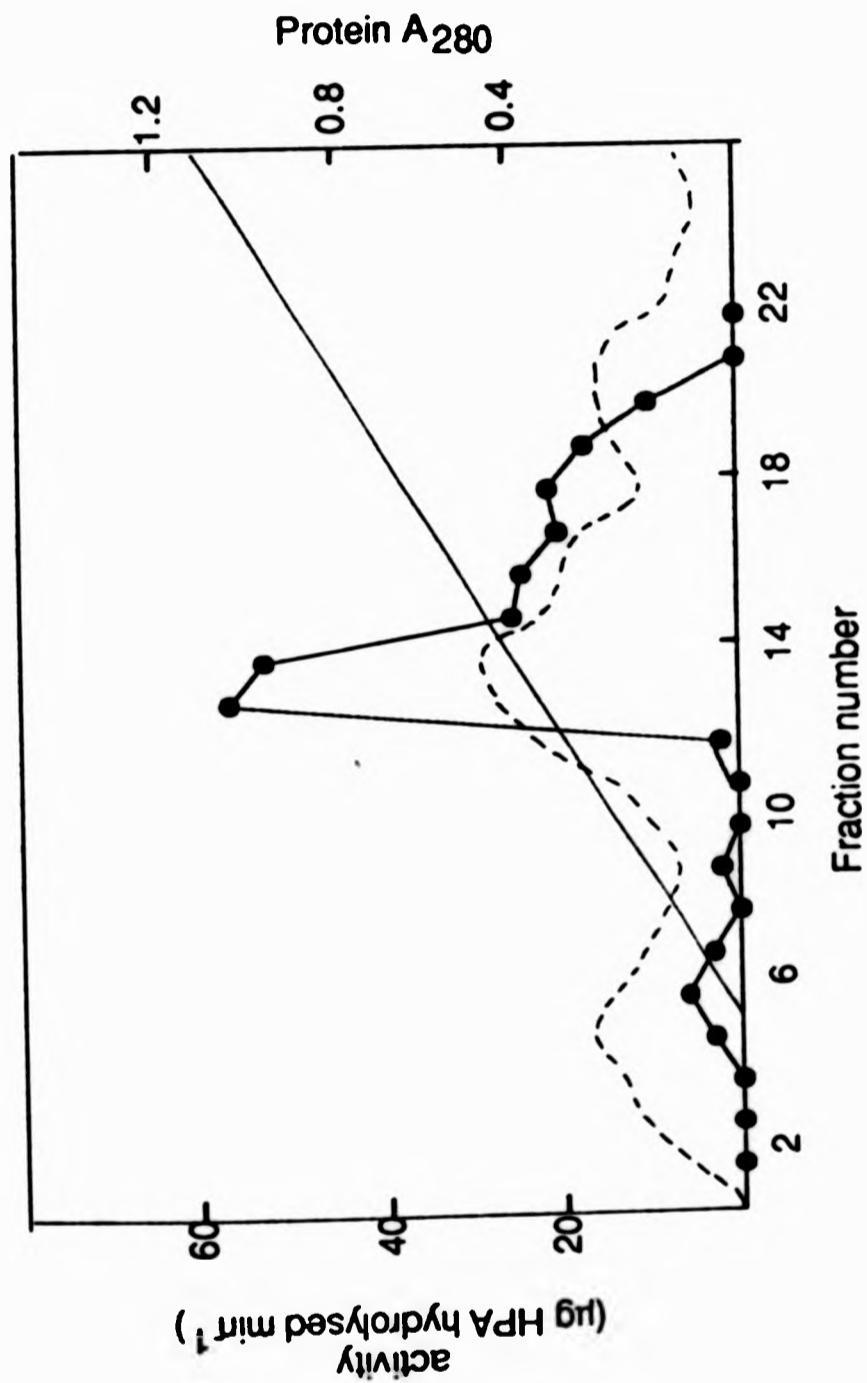


Fig. 25. Affinity chromatography of trichomonad proteinases on organomercurial Sepharose. (a) Proteinase D. Distribution of proteinase activity towards hide powder azure (●) and protein measured as A280 (----) in the effluent of the column is shown. The DTT gradient (0-10 mM) is represented by the diagonal line.

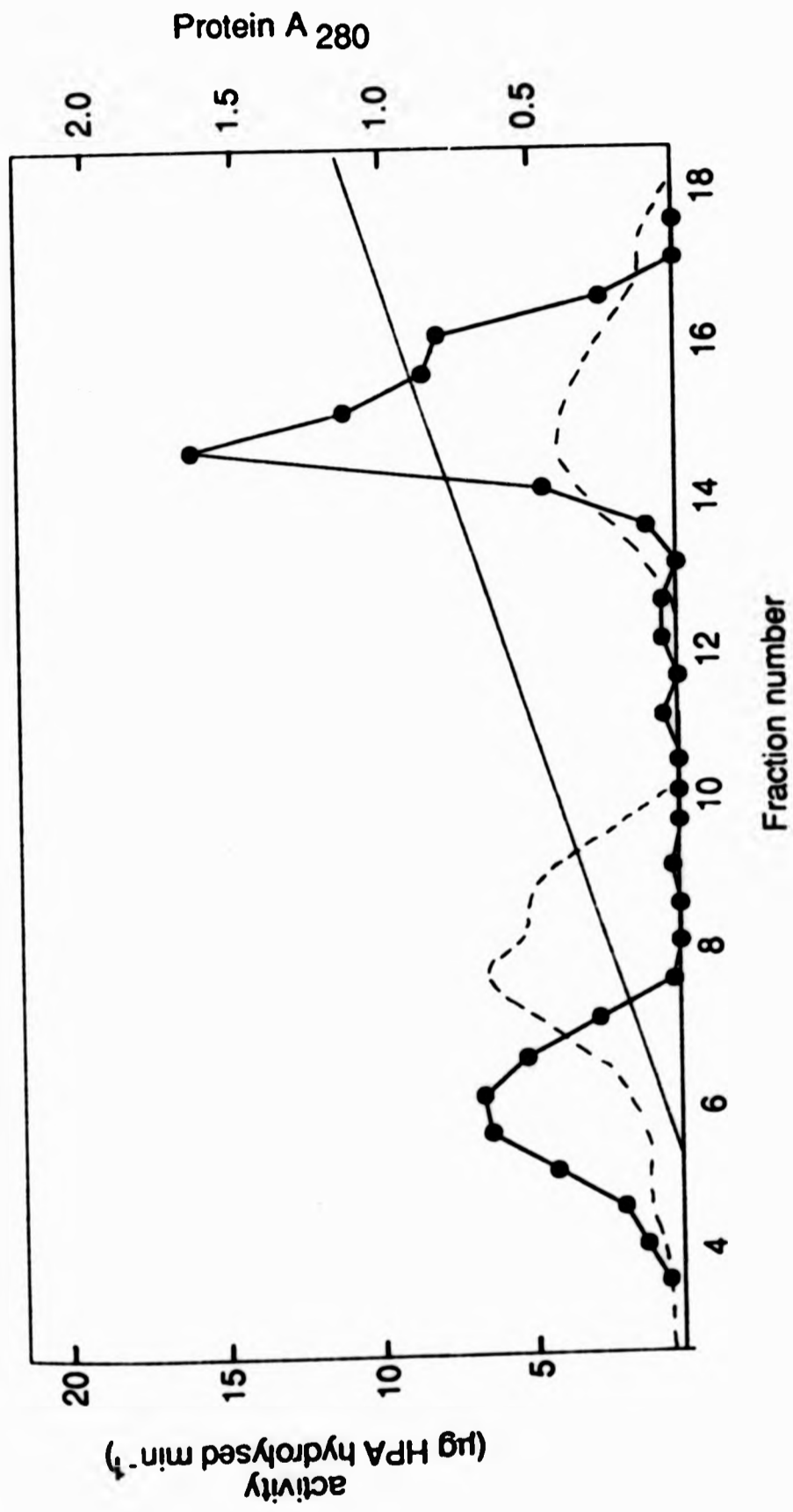


Fig. 25. Affinity chromatography of trichomonad proteinases on organomercurial Sepharose. (b) High-M. Distribution of proteinase activity towards hide powder azure (●) and protein measured as A280 (---) in the effluent of the column is shown. The DTT gradient (0-20 mM) is represented by the diagonal line.

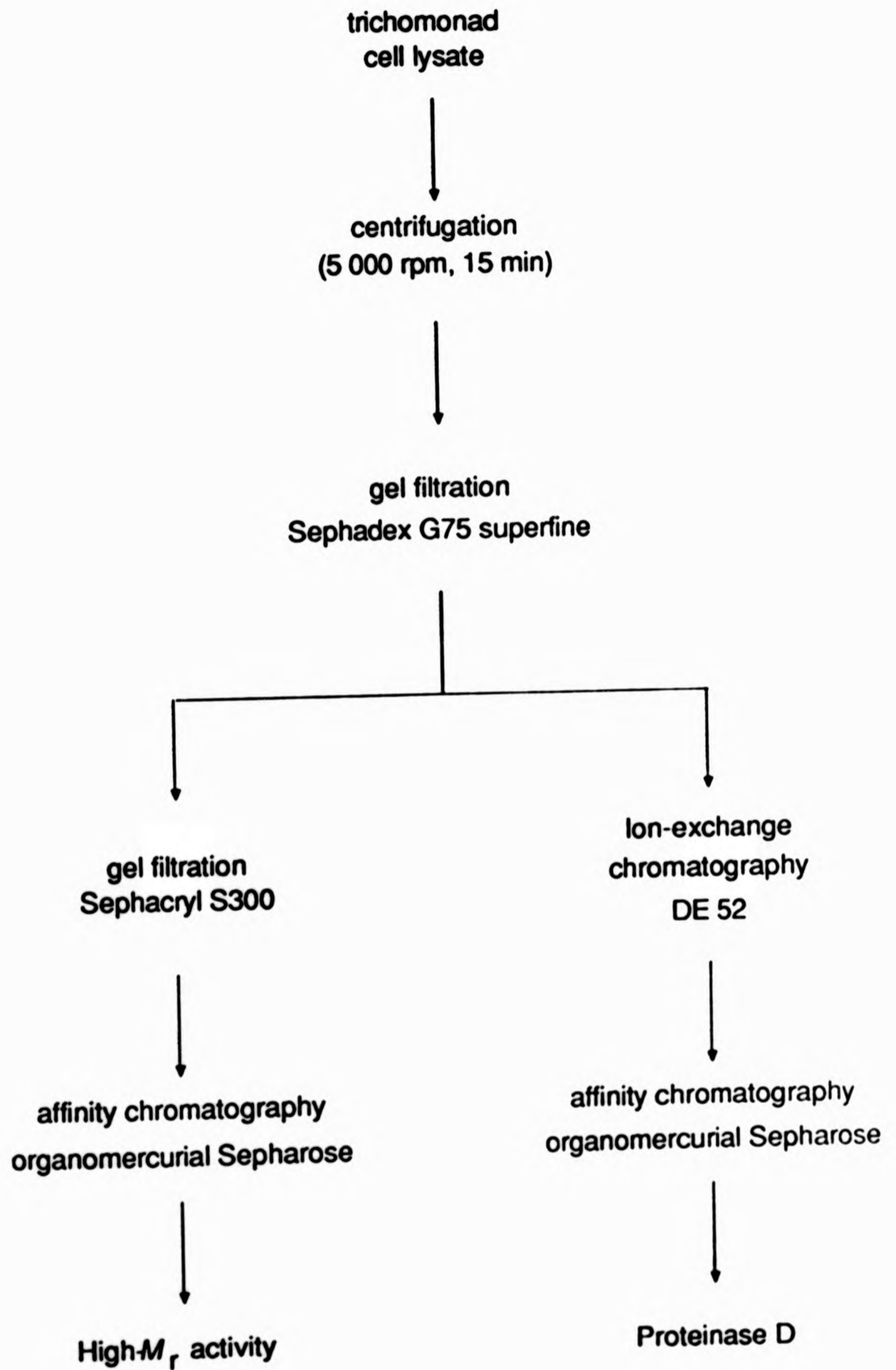


Fig. 26. Purification scheme for proteinase D and high-*M_r* activity.

Table 14. Purification of intracellular proteinases of Trichomonas vaginalis.

	Total Activity	Recovery	Purification	Protein (mg)
Stage of Purification				
Cell lysate	17360	100	1.0	248
supernatant	16665	96	1.2	203
Proteinase D				
gel filtration on Sephadex G75	10332	59	4.4	33
ion exchange chromatography organomercurial Sepharose affinity chromatography	2020	12	5.7	5
	1012	6	24.1	0.6
High-Mr Activity				
gel filtration on Sephadex G75	3710	21	0.7	71
gel filtration on Sephacryl S300 organomercurial Sepharose affinity chromatography	1711	10	1.4	18
	878	5	1.6	7.9

Activity was assayed using hide powder azure as described in section 2.2.1 and given in units of μg substrate hydrolysed min^{-1} .

3.6. Characterisation of the partially purified proteinases.

3.6.1. Molecular weight.

The molecular weights of the partially purified proteinases were determined by gel filtration. Samples of proteinase D and the high-*M.* were loaded onto previously calibrated columns of G-75 superfine and Sephacryl S-300 respectively (see section 2.6.1.). The activities were eluted from the columns at volumes corresponding to molecular weights of 18 kDa for proteinase D and 64 kDa for the high-*M.* proteinase. Analysis of the partially purified enzymes using gelatin-PAGE demonstrated that the high-*M.* activity possessed two distinct proteinases of different molecular weight. The major activity had an apparent molecular weight of 66 kDa the other had a molecular weight of approximately 96 kDa. The molecular weight of proteinase D was 38 kDa when determined by gelatin-PAGE.

3.6.2. Activity towards protein substrates.

Proteinase D hydrolysed 0.63 mg of azocasein and 1.7 mg of hide powder azure min^{-1} $(\text{mg protein})^{-1}$ and the high-*M.* hydrolysed 0.04 mg azocasein and 0.11 mg hide powder azure min^{-1} $(\text{mg protein})^{-1}$. All of the assays were performed in the presence of 1 mM DIT which enhanced the activity of both the purified proteinases, although the extent to which the activity increased differed considerably. The proteinase D activity was increased by between 300-400% whilst the high-*M.* activity was only enhanced by approximately 50%. For both of the enzymes the degree of enhancement was similar with both of the substrates. All of the activities were inhibited by all of the cysteine proteinase inhibitors

tested (Table 15) and were unaffected by the inhibitors of other classes of proteinase. In general the proteinase D activities were more sensitive to the inhibitors than the high-*M.* activity.

The pH optima of the two partially purified proteinases were determined using a variety of different buffers (Fig 27). Proteinase D was optimal towards azocasein at pH 6.5 and hide powder azure at pH 5.5 whereas the high-*M.* activity was optimal at pH 5.5 and 6.5 towards hide powder azure and azocasein respectively

3.6.3. Activity towards peptide nitroanilides.

The two purified proteinases showed similar specificities for the range of peptide nitroanilide substrates used (Table 16) and the specific activity towards all of the substrates was greater for proteinase D than the high-*M.* proteinase. Both of the activities were optimal at pH 7.0 and were significantly increased by the presence of 1 mM DTT at neutral pH, however, only proteinase D was enhanced by DTT at acid pH. At pH 5.0 DTT had no effect on the high-*M.* activity (Fig 28). Both activities towards Bz-Pro-Phe-Arg-Ian were inhibited by cysteine proteinase inhibitors and not by inhibitors of other classes of proteinase (Table 17). As with the activities towards protein substrates, proteinase D was more sensitive to the inhibitors than the high-*M.* activity.

3.6.4. Determination of isoelectric point.

Isoelectric focusing of the partially purified proteinases on polyacrylamide gels revealed that in both cases the activity towards hide powder azure could be detected in the gels at pH 5.0-6.5. More accurate determination of pI was carried out using chromatofocusing. The pIs of

Table 15. Effect of proteinase inhibitors on the activity of the partially purified proteinases towards protein substrates.

	Activity Remaining (%)			
	Proteinase D		High-Mr activity	
	HPA	AZC	HPA	AZC
Pepstatin	103.3 ± 5.4	98.2 ± 7.2	87.4 ± 9.9	85.8 ± 4.6
TLCK	16.2 ± 2.9	21.4 ± 11.3	28.6 ± 4.5	24.8 ± 2.2
TPCK	12.2 ± 3.6	18.3 ± 4.4	41.1 ± 8.6	37.1 ± 6.3
Iodoacetic acid	33.4 ± 7.1	36.7 ± 8.2	30.1 ± 4.2	25.9 ± 9.7
Chymostatin	5.3 ± 1.8	19.6 ± 3.9	24.9 ± 6.1	31.6 ± 6.5
Antipain	13.1 ± 3.6	10.4 ± 6.7	23.2 ± 5.9	33.5 ± 4.9
Phenanthroline	61.9 ± 24.4	97.6 ± 9.3	63.3 ± 14.4	95.0 ± 6.7
PMSF	92.7 ± 5.9	99.3 ± 4.6	100.7 ± 1.7	101.7 ± 9.1
Leupeptin	10.5 ± 7.1	17.1 ± 3.2	35.4 ± 6.0	38.8 ± 5.5

Activities are given as a percentage of that in control samples (given in section 3.6.2). The results are the mean ± standard deviation from at least three independent determinations.

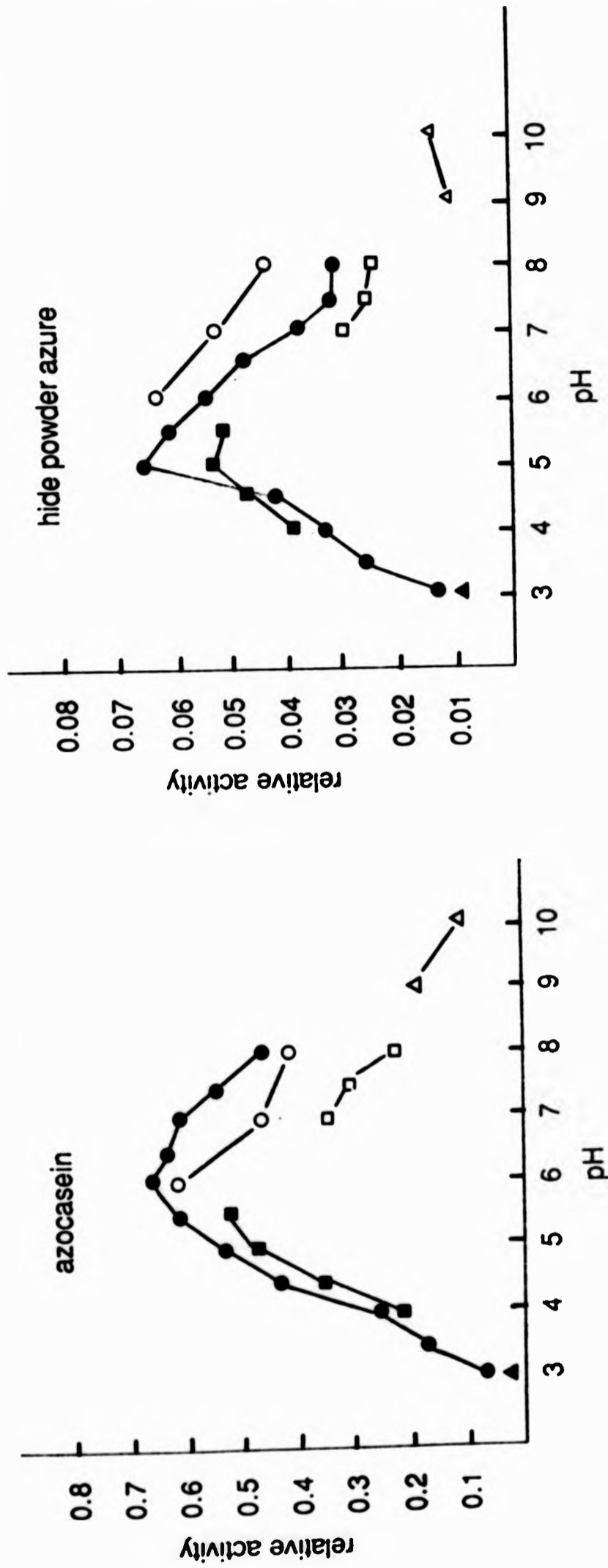


Fig. 27. pH dependence of the partially purified protease activity towards protein substrates (a) Protease D. Buffers used were citrate phosphate (●), glycine-HCl (▲), sodium acetate/acetic acid (■), phosphate (○), Tris-HCl (□), glycine-NaOH (△).

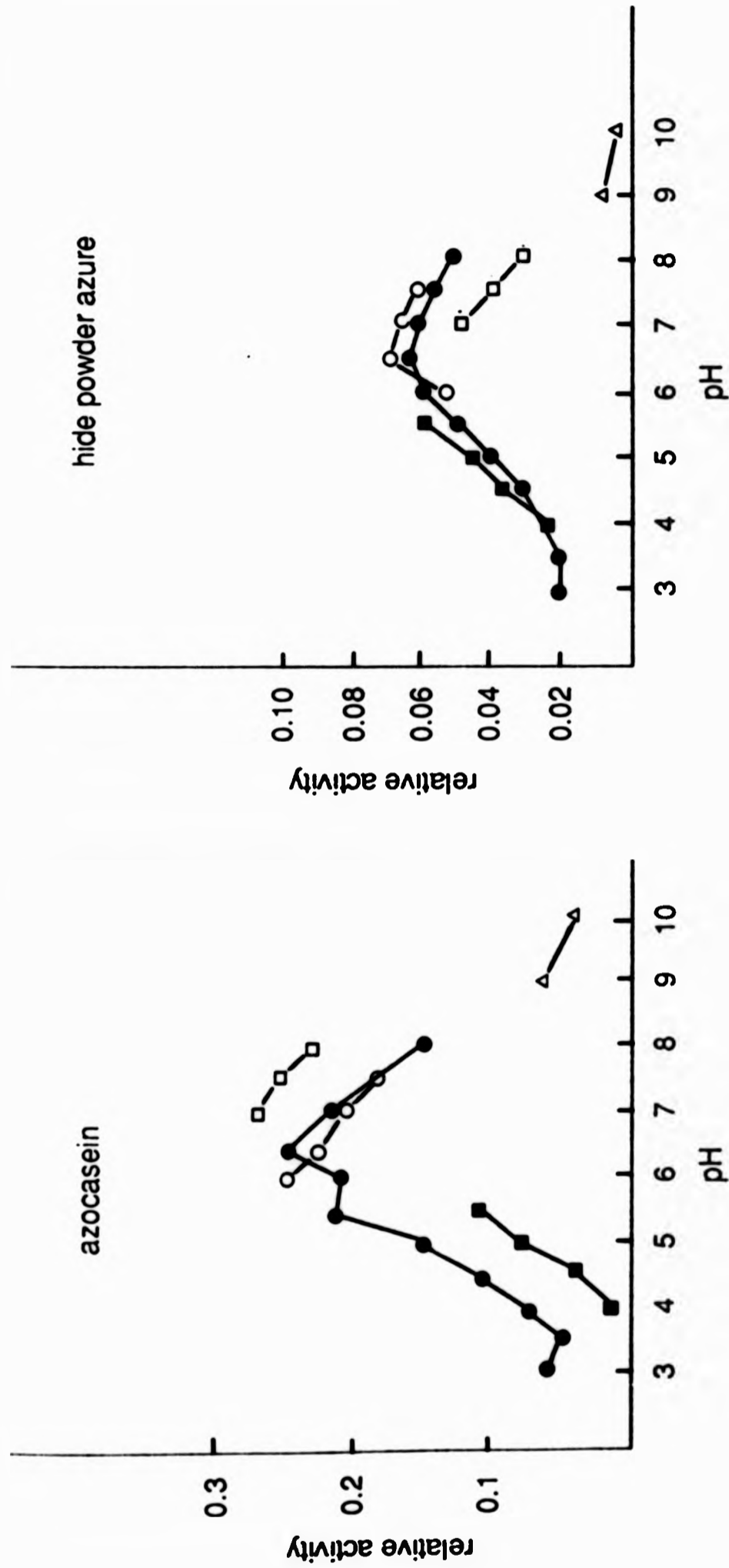


Fig. 27. pH dependence of the partially purified proteinase activity towards protein substrates (b) High-M-activity. Buffers used were citrate phosphate (●), sodium acetate/acetic acid (□), phosphate (○), Tris-HCl (◻), glycine-NaOH (△).

Table 16. Activity of the partially purified proteinases towards peptide p-nitroanilides.

Substrates	Specific Activity					
	High-Mr			Proteinase D		
	pH 7.0	pH 5.0	pH 7.0	pH 5.0	pH 7.0	pH 5.0
DTT concentration	0 mM	1 mM	1 mM	0 mM	1 mM	1 mM
Bz-Pro-Phe-Arg-Nan	0.088	0.182	0.089	0.104	0.050	0.320
Tos-Gly-Pro-Arg-Nan	0.040	0.107	0.039	0.052	0.027	0.147
Bz-Phe-Val-Arg-Nan	0.018	0.065	0.032	0.037	0.033	0.099
Bz-Val-Gly-Arg-Nan	0.005	0.007	0.009	0.008	0.019	0.089
Bz-Arg-Nan	0.009	0.012	0.009	0.004	0.009	0.040
Bz-Ile-Glu-Gly-Arg-Nan	0.004	0.003	0.003	0.001	0.010	0.019

Activity is given in units of $\text{nmol min}^{-1} (\text{mg protein})^{-1}$.

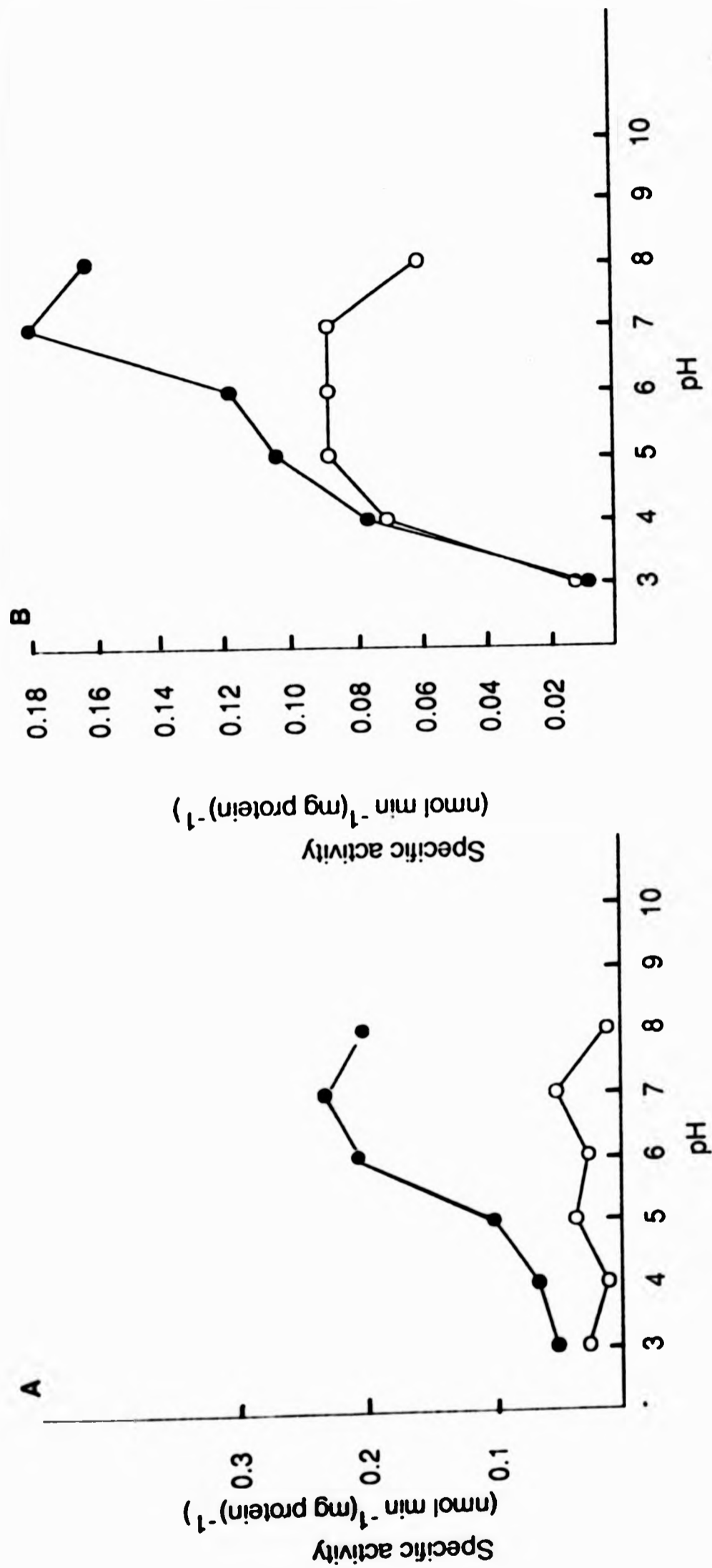


Fig. 28. pH dependence of the activities of the partially purified proteinases towards Bz-Pro-Phe-Arg-Nan. The activities of proteinase D (A) and high-M. activity (B) were determined in the presence (●) and absence (○) of DTT.

Table 17. Effect of proteinase inhibitors on the activity of the partially purified proteinases towards BzPPA.

	Activity Remaining %	
	Proteinase D	High-Mr
Pepstatin	98.7 ± 2.3	89.3 ± 4.9
TLCK	8.1 ± 1.9	26.1 ± 2.4
TPCK	12.2 ± 2.0	33.0 ± 9.2
Iodoacetic acid	8.9 ± 1.1	14.3 ± 3.6
Chymostatin	21.4 ± 3.6	18.6 ± 3.7
Antipain	18.4 ± 5.2	23.3 ± 4.0
Phenanthroline	68.8 ± 11.4	96.2 ± 14.4
PMSF	94.7 ± 3.9	101.9 ± 1.2
Leupeptin	24.4 ± 8.8	31.1 ± 6.9

Activities are given as a percentage of that in control samples (given in Table 16) assays were carried out at pH 7.0. The results are the mean ± standard deviation from at least three independent determination.

the two were similar, with values of 5.2 and 5.9 for proteinase D and the high-*M.* activity respectively.

3.6.5. Summary.

The results show that both of the partially purified proteinases are of the cysteine type, as indicated by their sensitivity to cysteine proteinase inhibitors and enhancement of their activity by DTT. A comparison of the properties of the two enzymes is shown in Table 18. The specificity towards peptide nitroanilide substrates is similar to that identified in the whole cell lysates. This specificity and other properties of the enzymes indicate a high degree of similarity to cysteine proteinases isolated from a large number of other organisms.

3.7. Extracellular activities produced by *Trichomonas vaginalis* and *Tritrichomonas foetus*.

In addition to having information on the intracellular proteinase activity, it is clearly important to know whether trichomonads also release proteinase activity. The presence of extracellular activity would not only allow the enzymes responsible to have a role in the digestion of extracellular protein for the growth of trichomonads in axenic culture but would also have important implications regarding proteolysis the host-parasite interaction.

3.7.1. Extracellular proteinase activity.

Proteinase activity towards azocasein and hide powder azure was detected in samples of the medium in which *Trichomonas vaginalis* and

Table 18. Comparison of the properties of proteinase D and the high-Mr activity.

	Proteinase D	High-Mr activity
1. Specific activity ¹		
HPAase	1.7	0.11
AZCase	0.63	0.04
2. Inhibitors	TLCK ² TPCK ² Iodoacetic acid ² Antipain ³ Leupeptin ³	TLCK ² Antipain ³ Leupeptin ³ Chymostatin ³
3. DTT Activation ⁴		
HPAase	4.33	1.72
AZCase	3.51	1.46
4. pH optimum		
HPAase	5.5	6.5
AZCase	6.0	7.0
5. Molecular weight (by gel filtration)	18000	64000
6. pI	5.2	5.9

- 1 Activities are given in units of g protein hydrolysed min⁻¹ (mg protein)⁻¹ at the optimum pH
2 Tested at 1 mM
3 Tested at 100 g ml⁻¹
4 DTT was included in the assay at a concentration of 1 mM. The activity is expressed relative to that of a control (= 1.0) which contained no DTT

Tritrichomonas foetus had been grown. The levels of activity present in the cells and in the medium in cultures grown to mid-log phase were determined (Table 19). Over half of the total AZCase activity present in these cultures was found to be extracellular. Interestingly, less of the total HPAase activity was extracellular with more than two thirds of the total being retained in the cells.

3.7.2. Extracellular hydrolase activity.

In addition to proteinase activity, acid phosphatase, α -mannosidase, β -glucosidase and β -N-acetylglucosaminidase activities were all found to be present in samples of medium in which *Trichomonas vaginalis* and *Tritrichomonas foetus* had been grown. (Table 19). Comparison of the levels of hydrolase activity in the cells and the medium shows that as for the HPAase and AZCase a significant proportion of the activity present in mid-log phase cultures was present in the medium. The levels of the hydrolase activities in the medium were measured at different stages of trichomonad growth. All of the hydrolase activities could be detected at all stages of growth (Fig. 29). During the logarithmic growth phase the increase in activity was proportional to the increase in viable cell number. When the stationary phase was reached (after 42 h) the level of hydrolytic activity increased significantly, suggesting a possible relationship between cell death and release of activity at late stages of growth. Cell lysis alone would be unlikely to account for the high levels of extracellular activity detected in log-phase cultures, but to eliminate this possibility two exclusively intracellular enzymes were investigated, lactate dehydrogenase, a cytosolic marker enzyme, and malate dehydrogenase (decarboxylating), a hydrogenosomal marker enzyme. Neither

Table 19. Extracellular and intracellular hydrolase activity in *Trichomonas vaginalis* and *Tritrichomonas foetus*.

	Specific Activity			
	<i>Trichomonas vaginalis</i>		<i>Tritrichomonas foetus</i>	
	cells	medium	cells	medium
AZCase	11.7 ± 2.1	15.9 ± 2.3	9.6 ± 4.1	12.4 ± 5.9
HPAase	68.2 ± 11.9	27.6 ± 4.1	30.4 ± 12.5	18.9 ± 7.0
β-N-acetylglucosaminidase	10.7 ± 3.1	9.8 ± 1.7	71.4 ± 15.0	50.2 ± 9.3
acid phosphatase	62.4 ± 9.8	49.6 ± 8.2	31.1 ± 3.2	24.4 ± 3.9
α-mannosidase	4.4 ± 1.9	3.8 ± 0.9	3.9 ± 1.8	4.3 ± 1.9
β-glucosidase	5.2 ± 1.3	3.2 ± 0.2	6.1 ± 0.7	2.9 ± 1.2

Activity is expressed in units of μg protein hydrolysed min^{-1} (mg cell protein) $^{-1}$ for HPAase and ACZase and nmol min^{-1} (mg cell protein) $^{-1}$ for other assays + standard deviation from three separate determinations. All samples were obtained from 30 h cultures.

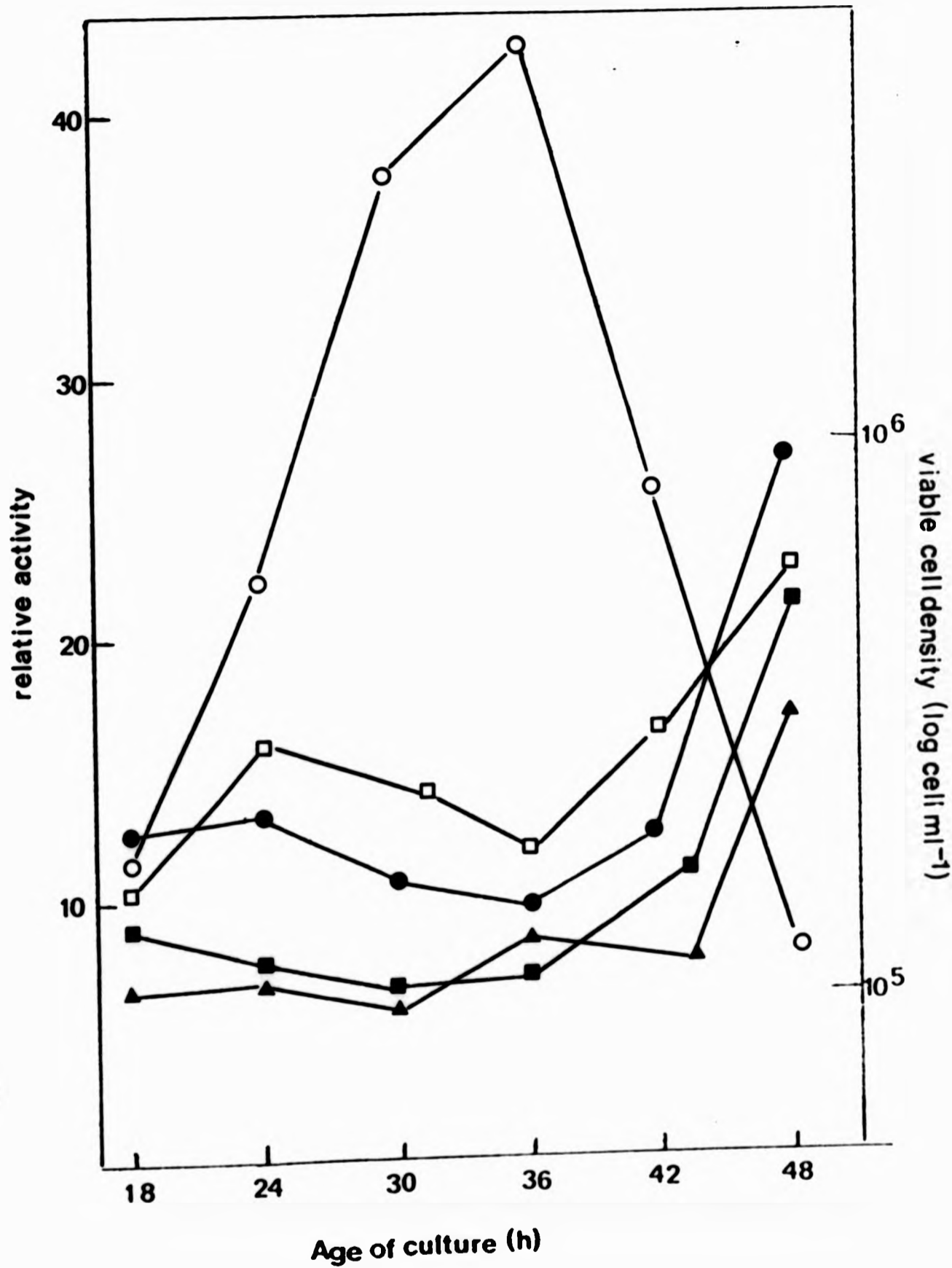


Fig. 29. Release of extracellular hydrolase activity during axenic growth (a) *Trichomonas vaginalis*. Viable cell number (O) was determined using a haemocytometer. Activities detected were: β-N-acetylglucosaminidase (●), acid phosphatase (■), β-glucosidase (□), and α-mannosidase (▲).

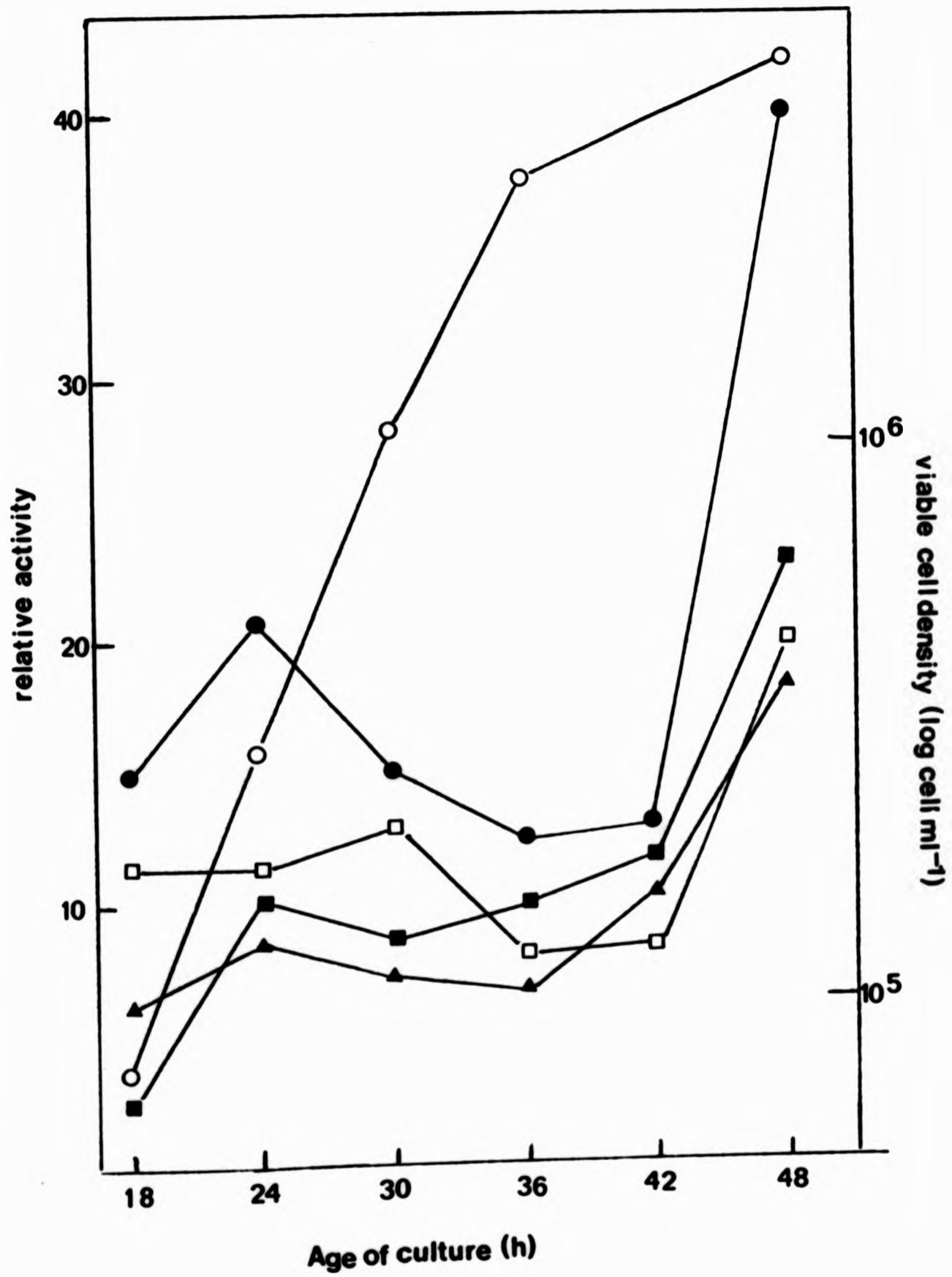


Fig. 29. Release of extracellular hydrolase activity during axenic growth (a) *Tritrichomonas foetus*. Viable cell number (O) was determined using a haemocytometer. Activities detected were: β -N-acetylglucosaminidase (●), acid phosphatase (■), β -glucosidase (□), and α -mannosidase (▲).

of these enzymes could be detected in samples of medium at any stage of *Trichomonas vaginalis* growth, although both were detectable in medium samples containing cells lysed with Triton X-100 which demonstrated that neither enzyme was inactivated in the presence of the medium. Taking into account the limit of detection of the lactate dehydrogenase assay, less than 0.25% of the cells at mid-log phase could have lysed.

3.7.3. Detection of extracellular proteinase activity by gelatin-PAGE.

Proteinase activity was also detected in samples of medium using the gelatin-PAGE technique used to analyse intracellular proteinase activity. In order to demonstrate that the presence of this activity was not just a consequence of cell lysis, proteinase activity was determined at all stages of trichomonad growth *in vitro* (Fig. 30). The earliest medium samples in which activity could be detected were those taken 18 h after initiation of the culture corresponding to a cell density of approximately 1×10^5 cells ml^{-1} (early log-phase) and the activity present increased in proportion to cell number until the stationary-phase was reached. The extracellular activities that were released during early stages of growth were found to have low apparent molecular weights which did not correspond to those of the intracellular activities. At higher cell densities (2.5 and 4.0×10^6 cells ml^{-1} for *T. foetus* and *Trichomonas vaginalis*, respectively) activities corresponding to those present in cell lysates could also be detected in the medium and may be due to cell lysis.

The possibility that the exclusively extracellular proteinases represented released intracellular proteinases which were subsequently modified by factors, e.g. serum proteins present in the medium, was tested

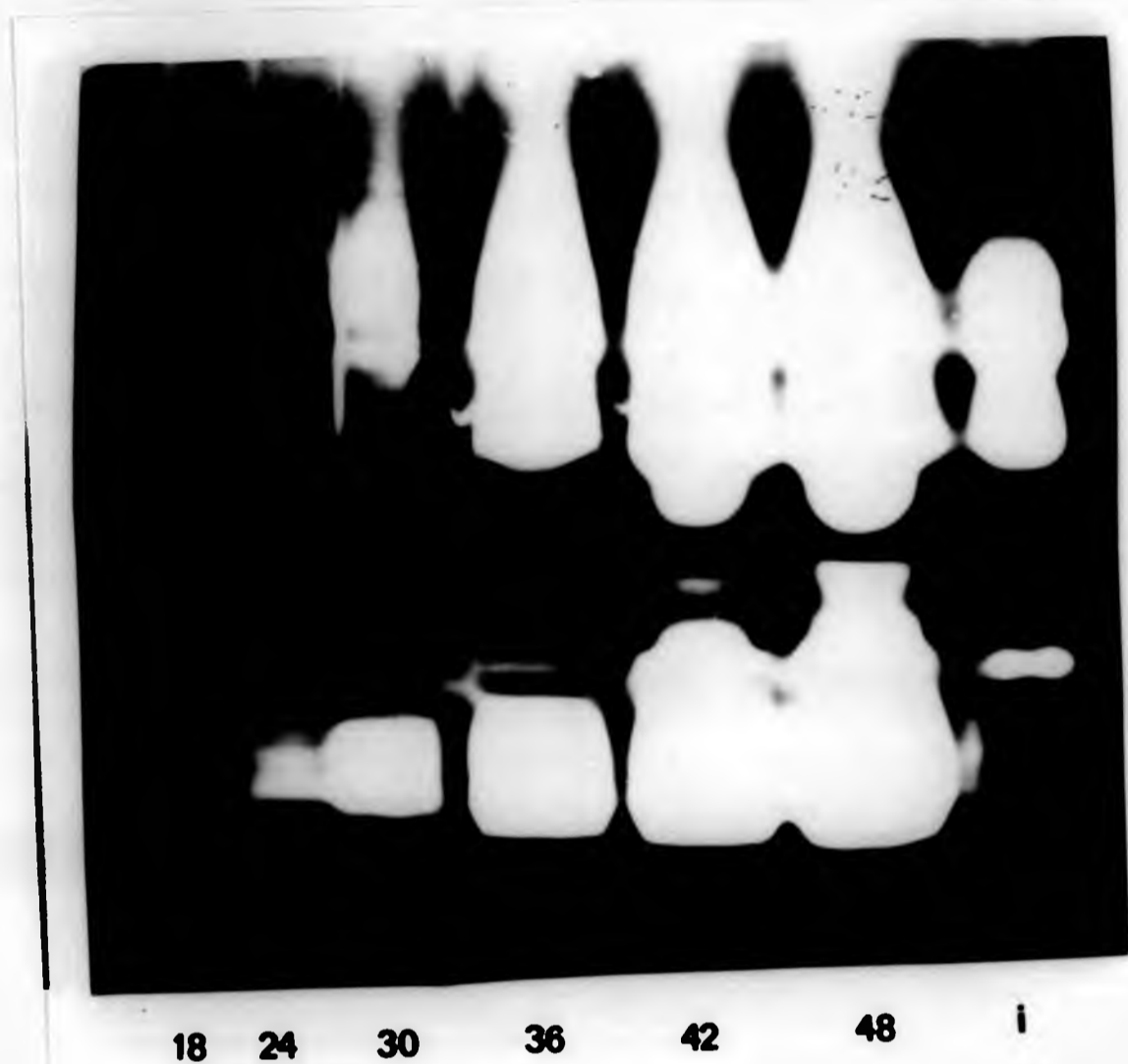


Fig. 30. Extracellular proteinases detected by gelatin-PAGE.
(a) *Trichomonas vaginalis*. Samples of medium (75 μ l), prepared from cultures of different ages, were subjected to electrophoretic analysis as described in section 2.3.2. The numbers refer to the culture age in h. i. intracellular activity: cell lysates (containing 25 μ g protein) prepared from 36 h-cultures. The polyacrylamide concentration was 7.5%.

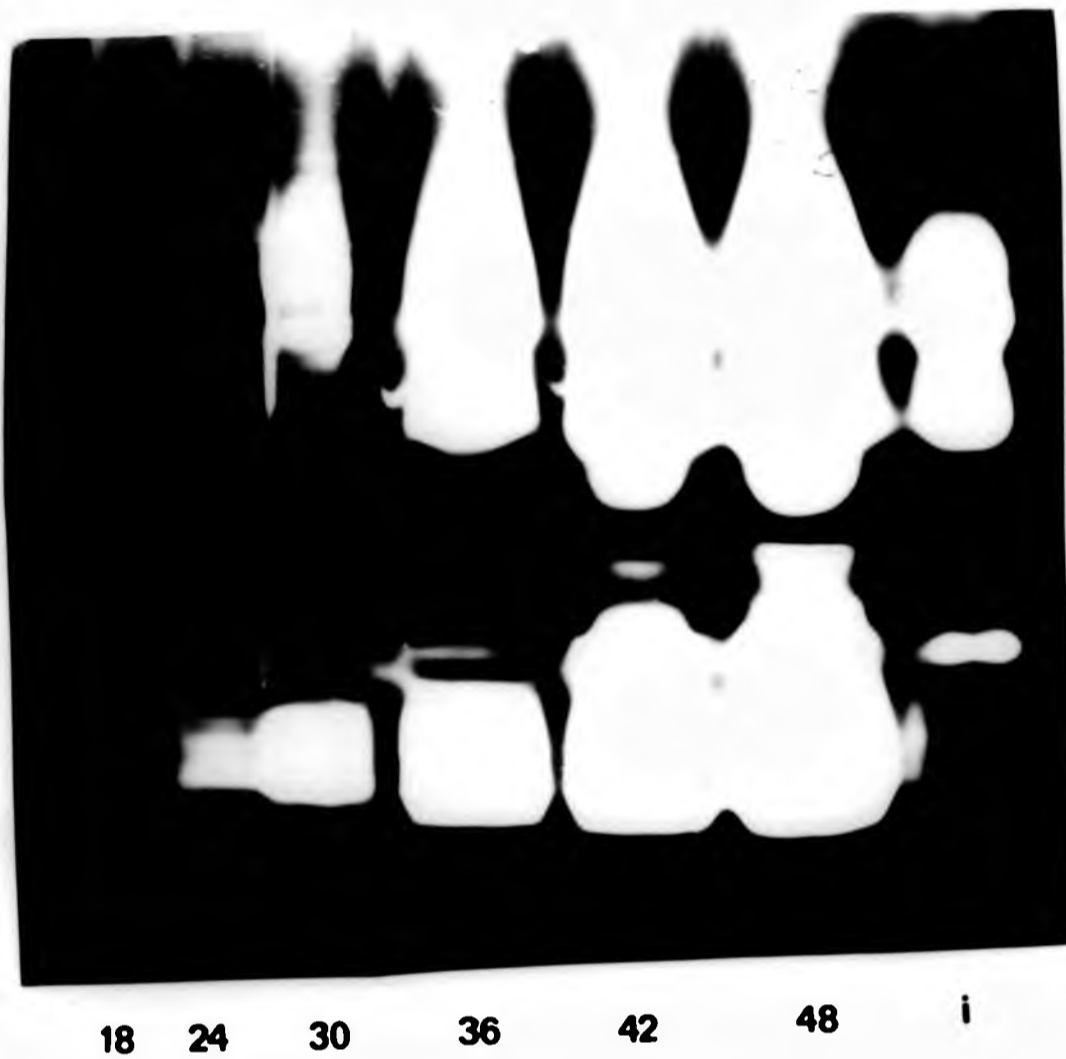


Fig. 30. Extracellular proteinases detected by gelatin-PAGE.
(a) *Trichomonas vaginalis*. Samples of medium (75 μ l), prepared from cultures of different ages, were subjected to electrophoretic analysis as described in section 2.3.2. The numbers refer to the culture age in h. i. intracellular activity: cell lysates (containing 25 μ g protein) prepared from 36 h-cultures. The polyacrylamide concentration was 7.5%.

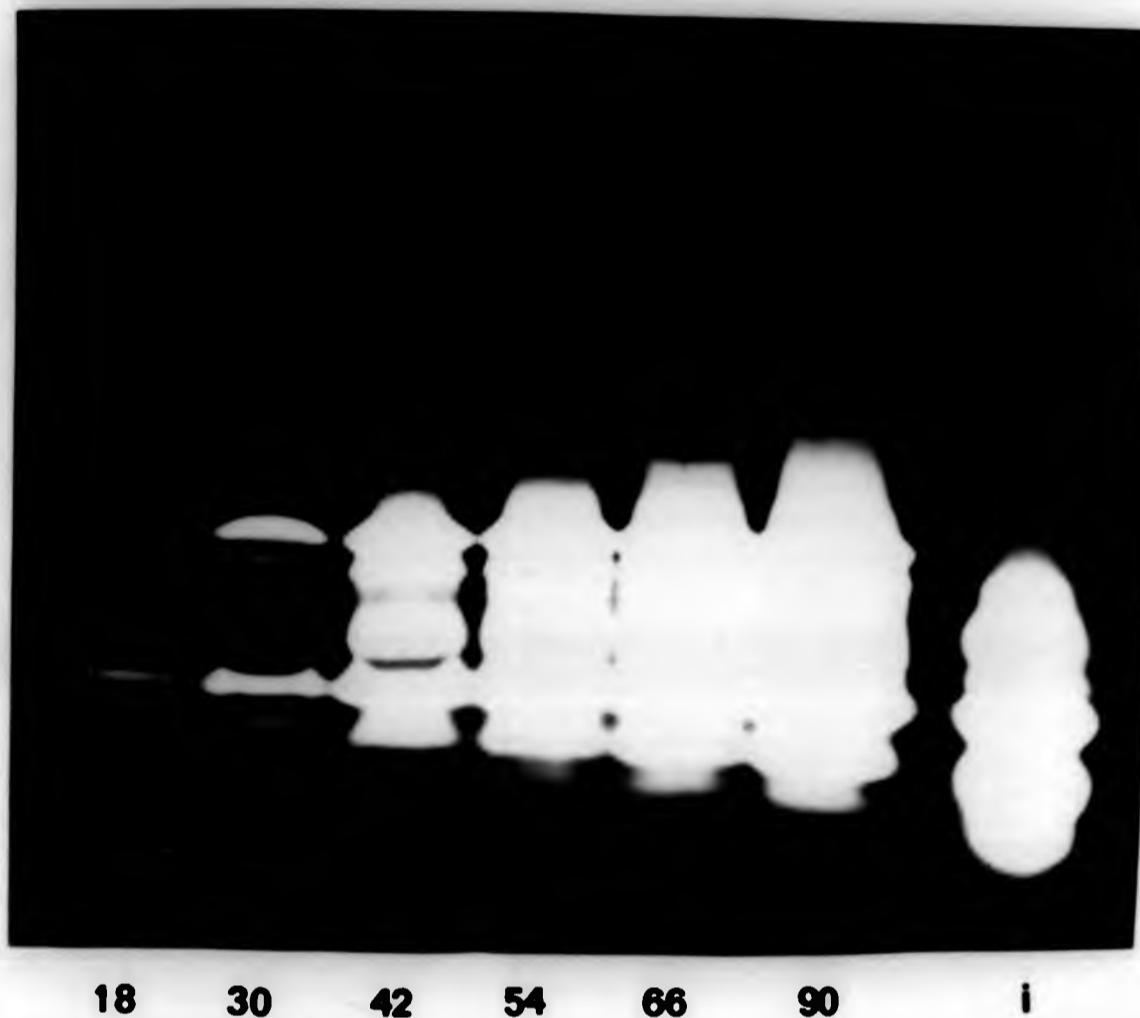


Fig. 30. Extracellular proteinases detected by gelatin-PAGE.

(b) *Tritrichomonas foetus*. Samples of medium (75 μ l), prepared from cultures of different ages, were subjected to electrophoretic analysis as described in section 2.3.2. The numbers refer to the culture age in h. i, intracellular activity: cell lysates (containing 25 μ g protein) prepared from 24 h-cultures. The polyacrylamide concentration was 10%.

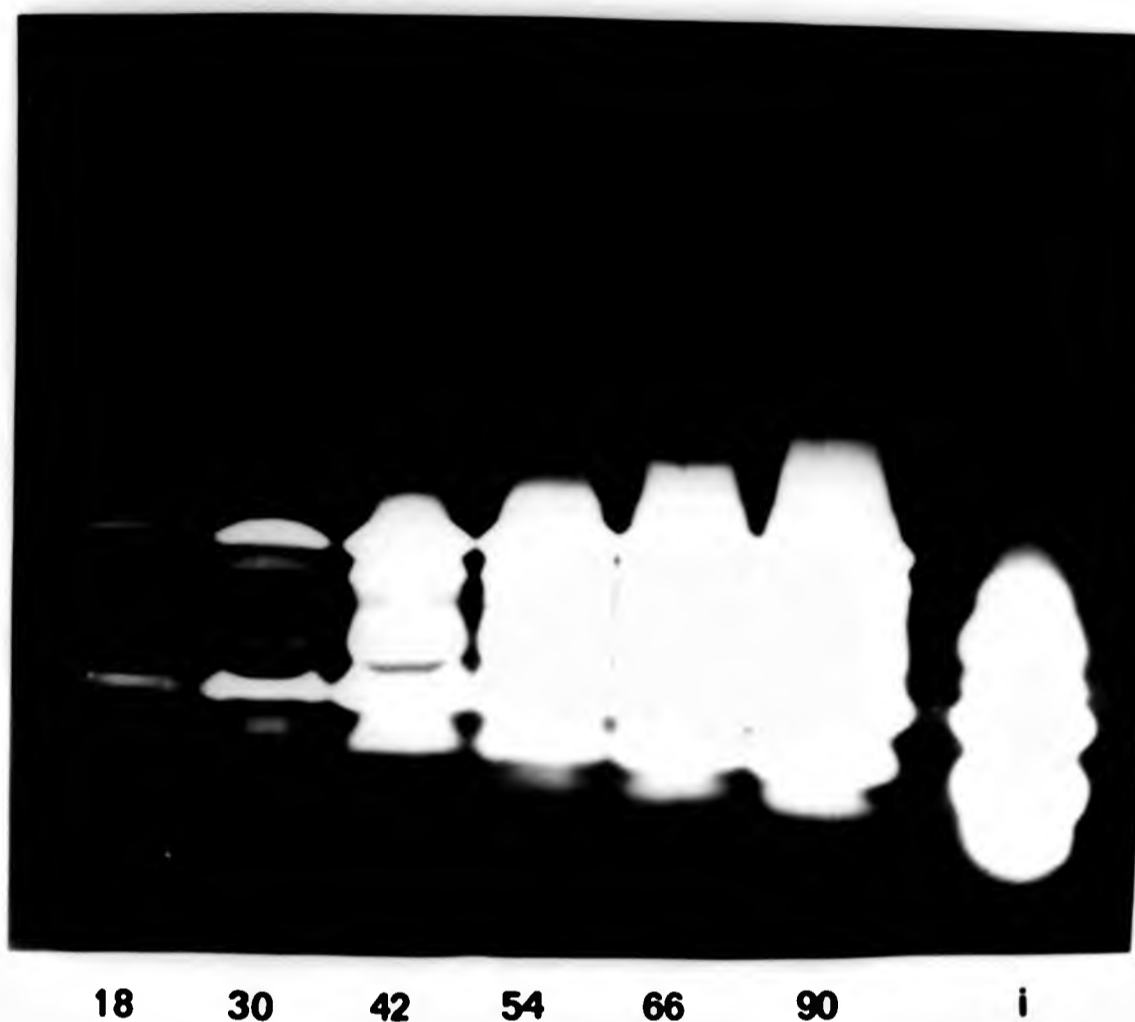


Fig. 30. Extracellular proteinases detected by gelatin-PAGE.

(b) *Tritrichomonas foetus*. Samples of medium (75 μ l), prepared from cultures of different ages, were subjected to electrophoretic analysis as described in section 2.3.2. The numbers refer to the culture age in h. i, intracellular activity: cell lysates (containing 25 μ g protein) prepared from 26 h-cultures. The polyacrylamide concentration was 10%.

by preincubating cell lysates with fresh medium for 3 h at 37°C before electrophoretic analysis. The pattern of proteinases detected was identical to that of untreated lysates and no conversion of the intracellular enzymes to unique extracellular forms was apparent.

3.7.4. Purification of extracellular proteinase activity.

In order to investigate further the relationship between the intra- and extracellular proteinases the low molecular weight (18-24 kDa) extracellular proteinase activities detected on the gelatin-gels were purified from the medium in which *Trichomonas vaginalis* had been grown. Since these activities were present in relatively large volumes and were of significantly lower molecular weight than the other proteinase activities present in medium taken 30 h after initiation of trichomonad growth ultrafiltration was used in an attempt to partially purify and concentrate the activities.

Although the proteinases had apparent molecular weights of 25 kDa, it was found that they did not pass through a PM 30 ultrafiltration membrane (exclusion limit 30 kDa). This could possibly be due to the enzymes binding to serum proteins present in the medium. Since the proteinases could be separated from other proteins under the conditions used for gelatin-PAGE analysis, the medium was made 2% SDS (w/v) and 5% mercaptoethanol (v/v). This allowed the proteinases to pass through the membrane. The filtrate was equilibrated with the starting buffer for organomercurial Sepharose affinity chromatography by ultrafiltration using a PM 10 membrane prior to the affinity chromatography step. The proteinase activities were purified from the 10-30 kDa fraction obtained by ultrafiltration by organomercurial Sepharose affinity chromatography

as previously described (see section 2.6.3.). Fractions eluted from the column using 10 mM DTT were analysed for proteinase activity using the gelatin-PAGE technique and those possessing activity were pooled and concentrated to give a final protein concentration of approximately 1 mg ml⁻¹.

3.7.5. Summary.

HPAase, AZCase, N-acetylglucosaminidase, α -mannosidase, acid phosphatase and β -glucosidase activities were all detected in the medium in which *Trichomonas vaginalis* and *Tritrichomonas foetus* had been grown. The ratio of activity present in the cells to activity in the medium was different for each of the activities. A comparison of these ratios with the levels of the activities demonstrated in the high and low density lysosome-like particles described previously (section 3.3.3) suggests that the high density particles may be involved in the release of these enzymes into the medium during *Trichomonas vaginalis* growth *in vitro*.

The enzymes responsible for the extracellular proteinase activity were demonstrated using the gelatin-PAGE technique. A number of activities were detected that were different from those present intracellularly. A purification scheme for the exclusively extracellular low molecular weight enzymes was devised.

3.8. Characterisation of the anti-proteinase antiserum.

The availability of an anti-proteinase antiserum is clearly advantageous to a study of the proteolytic enzymes of trichomonads. It could be used, for example, in identifying the relationships between

individual proteinase forms, purification of enzymes, and possibly to specifically inhibit proteinase activity in model systems. Although it was not possible to carry out any characterisation of the proteinase purified from *Trichomonas vaginalis* medium, sufficient protein was available to allow antiserum to be raised in a single rabbit.

3.8.1 Western blotting.

Analysis by Western blotting revealed that the antiserum recognised a number of bands in the lysates and samples of medium prepared from cultures of both *T. vaginalis* and *Tritrichomonas foetus*. By loading sufficiently dilute samples of *Trichomonas vaginalis*, however, it was possible to get conditions under which only a single band reacted. This had an apparent molecular weight of 30 kDa (Fig. 31). Surprisingly, purified samples of the extracellular proteinase used to raise the antiserum failed to give a band on the blot, although purified preparations of the high-M_r gave the same band as the cell lysates. Particularly heavy staining of medium samples and the actual positions of the major stained bands suggested that some of the antibodies bound to components of the medium. This was confirmed by the finding that even with medium taken at very early stages of trichomonad growth the staining was just as heavy. Since non-immune serum failed to give any significant staining it was likely that traces of serum protein had been present in the purified proteinase sample causing production of antibodies to these proteins.

3.8.2. Immunoprecipitation.

Although the western blot analysis suggested some specificity to a single protein, it was obvious that a number of proteins could be

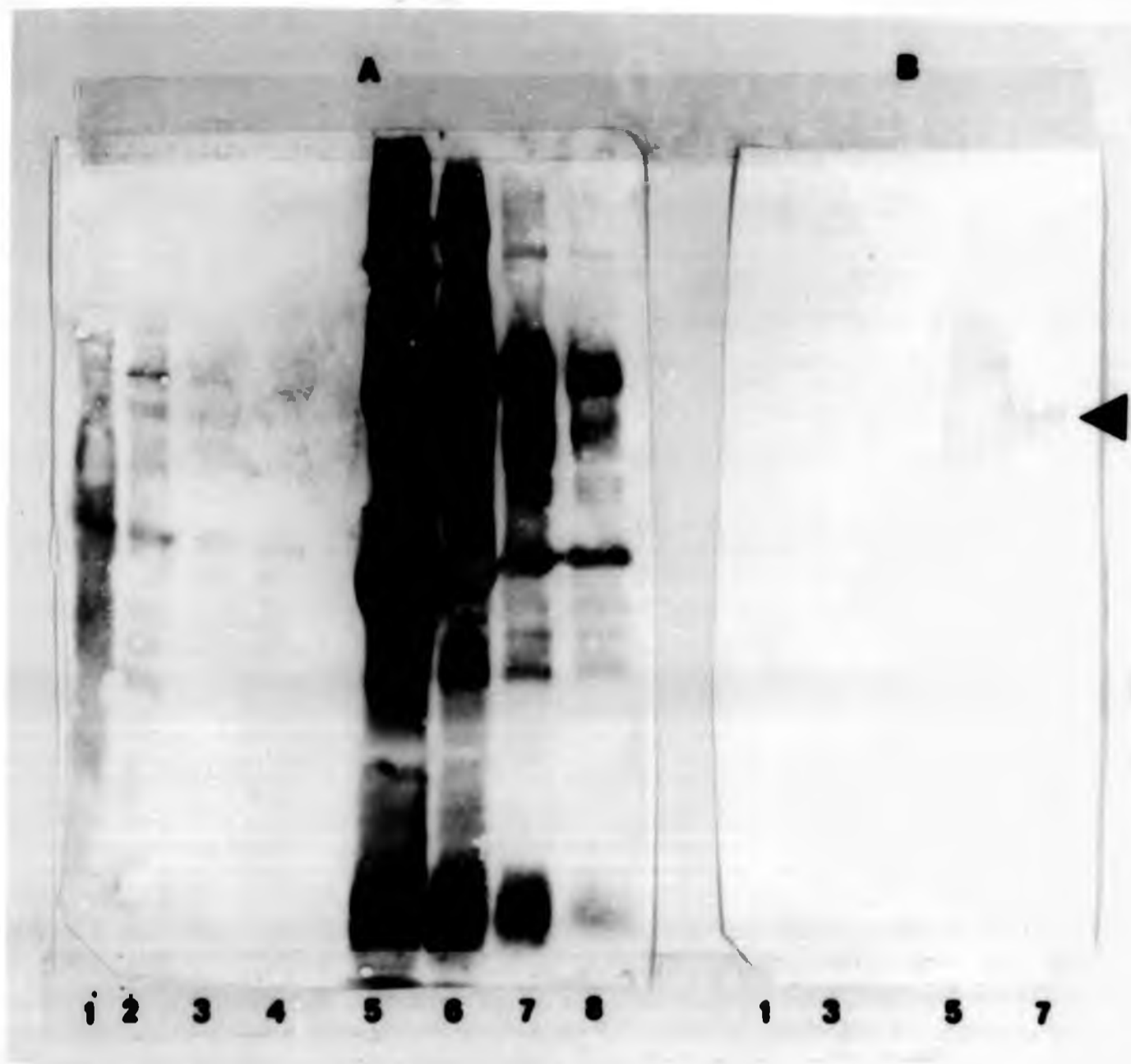


Fig. 31. Western blots of *Trichomonas vaginalis* samples.

Proteinases were separated on a 7.5% acrylamide gel and blotted onto nitrocellulose as described in section 2.9.2. The samples were not heat treated before loading on the gel. Staining was carried out using (A) antiserum raised against the purified extracellular proteinases (1:2 000 dilution) or (B) non-immune serum (1:2 000 dilution). Lanes 1 to 4-cell lysate from 30 h culture; lanes 5 to 8-medium from 48 h cultures. Volumes loaded: (1) 25 μ l, (2) 5 μ l, (3) 20 μ l, 1:20 dilution, (4), 4 μ l 1:20 dilution.

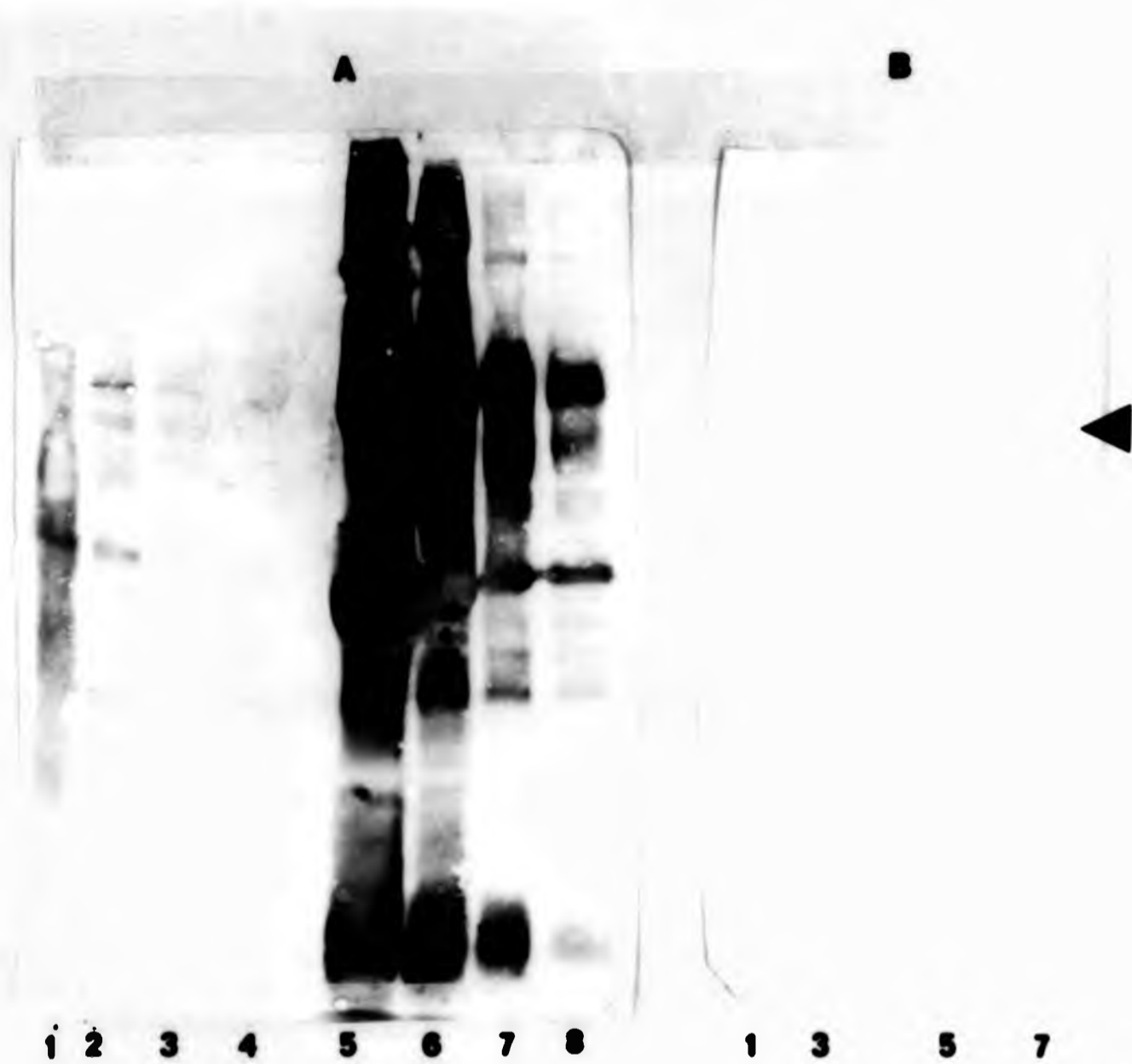


Fig. 31. Western blots of *Trichomonas vaginalis* samples.

Proteinases were separated on a 7.5% acrylamide gel and blotted onto nitrocellulose as described in section 2.9.2. The samples were not heat treated before loading on the gel. Staining was carried out using (A) antiserum raised against the purified extracellular proteinases (1:2 000 dilution) or (B) non-immune serum (1:2 000 dilution). Lanes 1 to 4-cell lysate from 30 h culture; lanes 5 to 8-medium from 48 h cultures. Volumes loaded: (1) 25 μ l, (2) 5 μ l, (3) 20 μ l, 1:20 dilution, (4), 4 μ l 1:20 dilution.

precipitated by the antiserum. This may cause difficulties in interpretation of electrophoretic analyses of immunoprecipitated proteins. The availability of the gelatin-PAGE technique, however, made it possible to analyse the immunoprecipitates specifically for proteinase activity.

The specificity of the antiserum was tested by incubating samples with the antiserum, collecting the immune complexes, re-extracting with sample buffer and applying to gelatin gels which were run and incubated under standard conditions (see section 2.3.2). Proteinase activity was detected in three of the samples tested (Fig 32). The antiserum precipitated proteinase activity from *T. vaginalis* medium, although only from late cultures, which may suggest that the proteinase was released during cell lysis. This was supported by the precipitation of apparently the same proteinase from cell lysates from an earlier stage of growth. In a preliminary experiment it was shown that the antiserum also precipitated an intracellular proteinase from *Tritrichomonas foetus* cell lysates, indicating that proteinases of the two species share common epitopes.

3.8.3. Summary.

Antiserum was raised against extracellular proteinases purified from the medium in which *Trichomonas vaginalis* had previously been grown. Preliminary experiments have shown that the antiserum recognises proteinases in cell lysates of both *T. vaginalis* and *Tritrichomonas foetus* and also in samples of medium in which *Trichomonas vaginalis* had been grown. The antiserum is not, however, totally specific for trichomonad proteins and requires more detailed characterisation to establish its usefulness in further investigations.

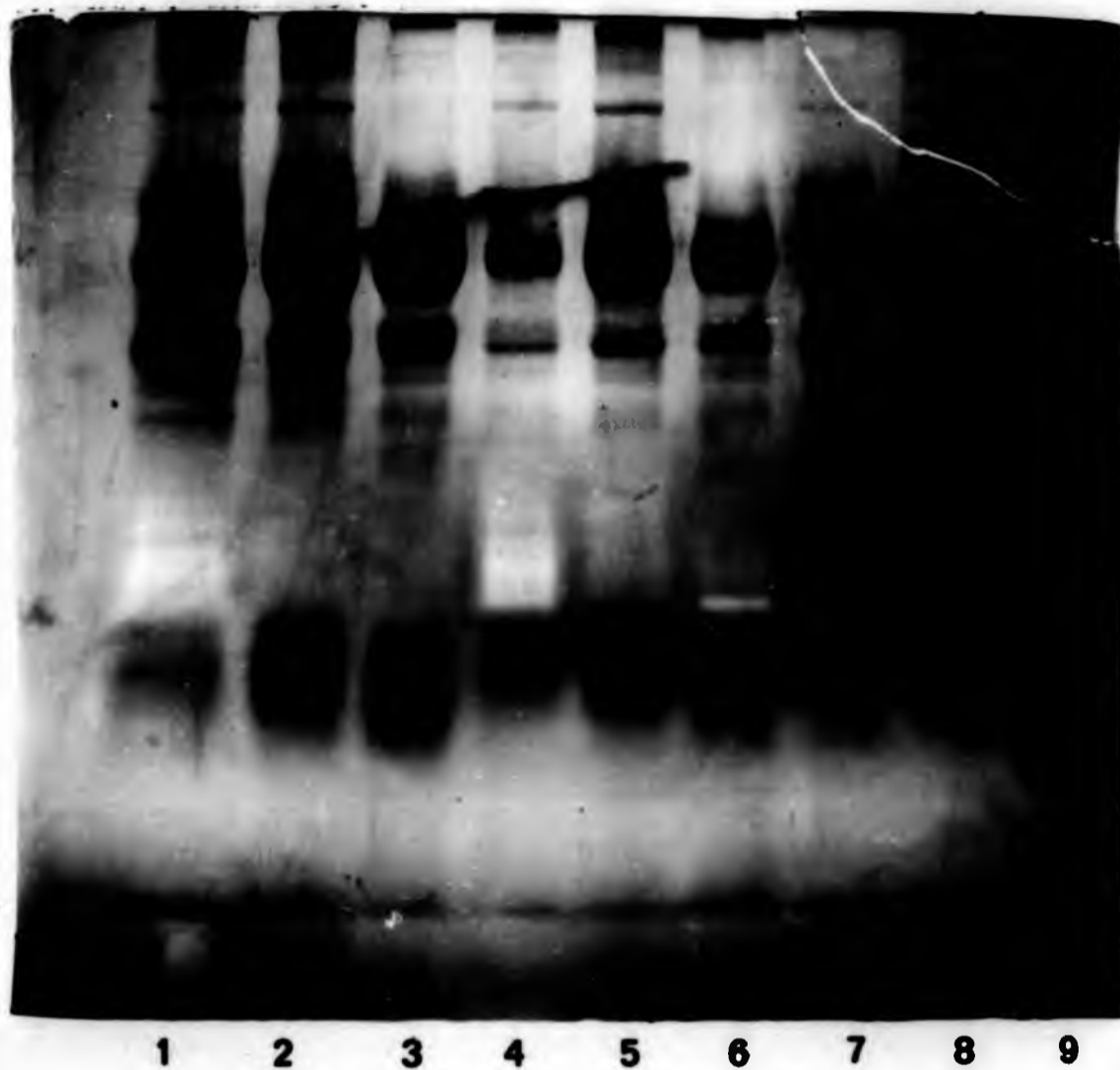


Fig. 32. Analysis of immunoprecipitates by gelatin-PAGE.

Trichomonas vaginalis samples were immunoprecipitated with either 50 μ l (lanes 1-3) or 10 μ l (lanes 4-6) antiserum raised against the purified extracellular proteinases or (lanes 7-9) non-immune serum. Samples were: medium from 48 h cultures (lanes 1, 4 and 7), medium from 30 h cultures (lanes 2, 5 and 8) and cell lysates from 30 h cultures (lanes 3, 6 and 9).

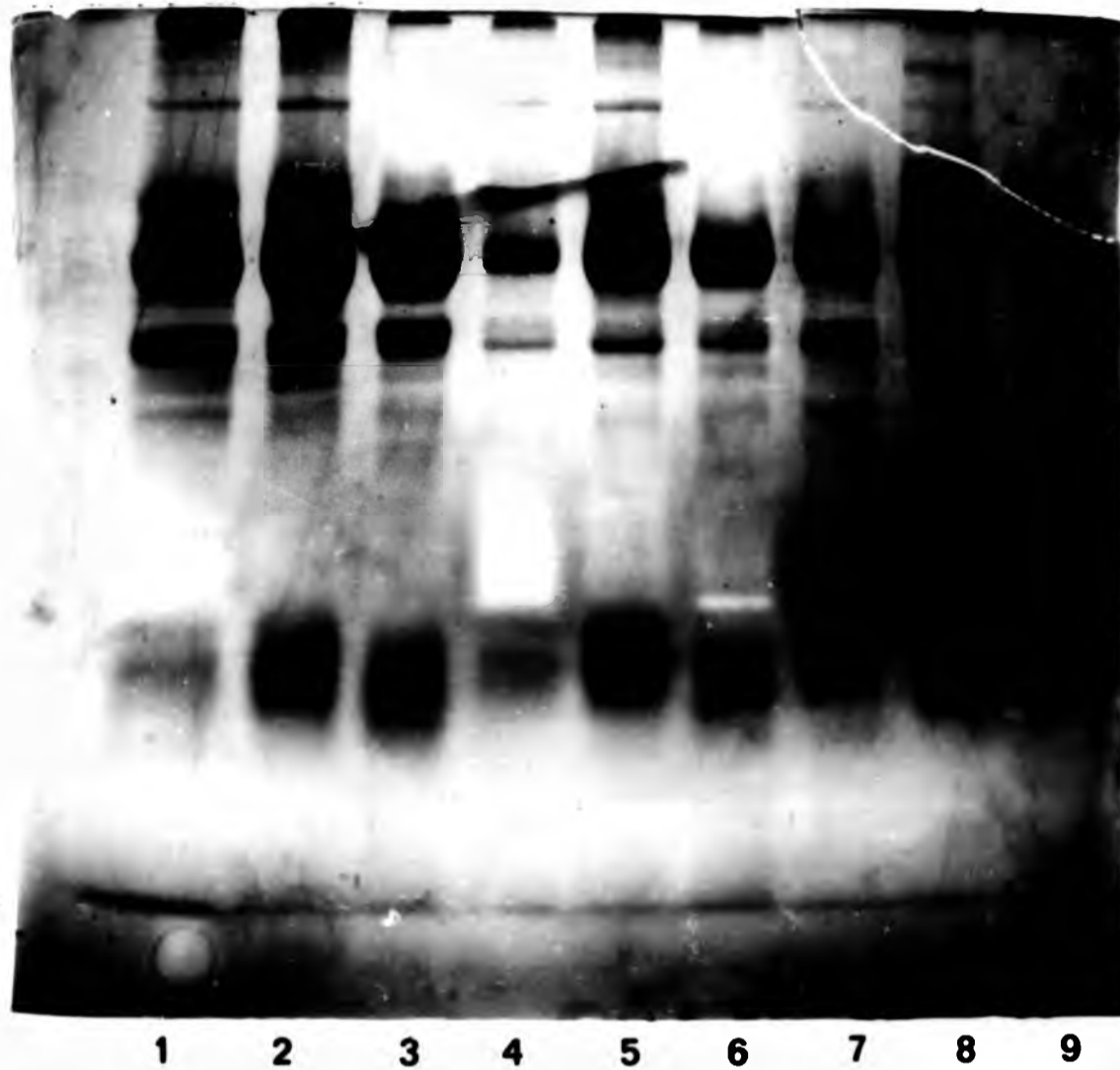


Fig. 32. Analysis of immunoprecipitates by gelatin-PAGE.

Trichomonas vaginalis samples were immunoprecipitated with either 50 μ l (lanes 1-3) or 10 μ l (lanes 4-6) antiserum raised against the purified extracellular proteinases or (lanes 7-9) non-immune serum. Samples were: medium from 48 h cultures (lanes 1, 4 and 7), medium from 30 h cultures (lanes 2, 5 and 8) and cell lysates from 30 h cultures (lanes 3, 6 and 9).

3.9. Proteinases activity in Leishmanias.

The leishmanias are flagellate protozoan parasites that cause a range of diseases in mammals including man. They have been demonstrated to possess relatively high levels of proteinase activity. Two proteinases, a highly active, substrate-specific 31 kDa-enzyme of the cysteine type and a 67 kDa-enzyme of broader specificity, have been purified from *Leishmania mexicana mexicana* (Pupkis and Coombs 1984). The proteinases are subject to differential control during the life cycle, with the former only present in the amastigotes while the latter can be isolated from both the promastigotes and the amastigotes. Detailed investigations of other species of *Leishmania* have not yet been undertaken.

Etges et al. (1986) recently reported that purified preparations of the major cell surface protein of *L. major* (p63) possess proteinase activity. A similar protein appears to be the major surface antigen in other *Leishmania* species (Fong and Chang 1982; Lepay et al. 1983; Gardner et al. 1984; Etges et al. 1985), and so it was of interest to determine whether *Leishmania* species share a common proteinase activity.

Since the gelatin-PAGE technique proved to be extremely useful for comparative studies involving different species of trichomonads, it was used to analyse the proteolytic activities present in several forms of three species of *Leishmania*.

3.9.1. Developmental changes in proteolytic activities.

Proteinases were detected in all the stages of the life cycle of *Leishmania mexicana mexicana*, *L. donovani* and *L. major* that were studied.

At least three independent samples of each stage were analysed for each species and no variation between samples was apparent.

L. m. mexicana amastigotes possessed the most active proteinases. These were low molecular weight enzymes which were absent from the other two species and from log-phase promastigotes of *L. m. mexicana*. This low molecular weight activity could be resolved into four separate bands on the gel by reducing the incubation time to 1 h instead of the 4 h needed to detect the higher molecular weight, lower activity enzymes. Stationary-phase promastigotes also possessed low molecular weight proteinase activity, although these were not identical to any of the low molecular weight activities of the amastigotes with respect to mobility on the gels. The level of activity was considerably lower than in the amastigotes and was somewhat variable between individual samples. All of the other proteinases detected on the gels were of higher molecular weight (60 kDa or greater). *L. m. mexicana* possessed the highest levels of high molecular weight activities, three major bands of activity could be detected in the amastigotes of molecular weight 68, 98, and 150 kDa (Fig 33), however, there were quantitative and qualitative differences detected in different stages of the life cycle of all three species. For example, in *L. donovani* a high molecular weight enzyme (approximately 130 kDa) was much more active in amastigote lysates than in the promastigotes, whereas three other proteinases of lower molecular weight (approximately 60, 90 and 100 kDa) were most active in stationary-phase promastigotes but barely detectable in log-phase promastigotes and absent from amastigotes. The 60 kDa proteinase was not always observed but is apparent in Fig 33. A much simpler pattern of proteolytic enzymes was observed for *L. major*. Two low activity enzymes with apparent molecular

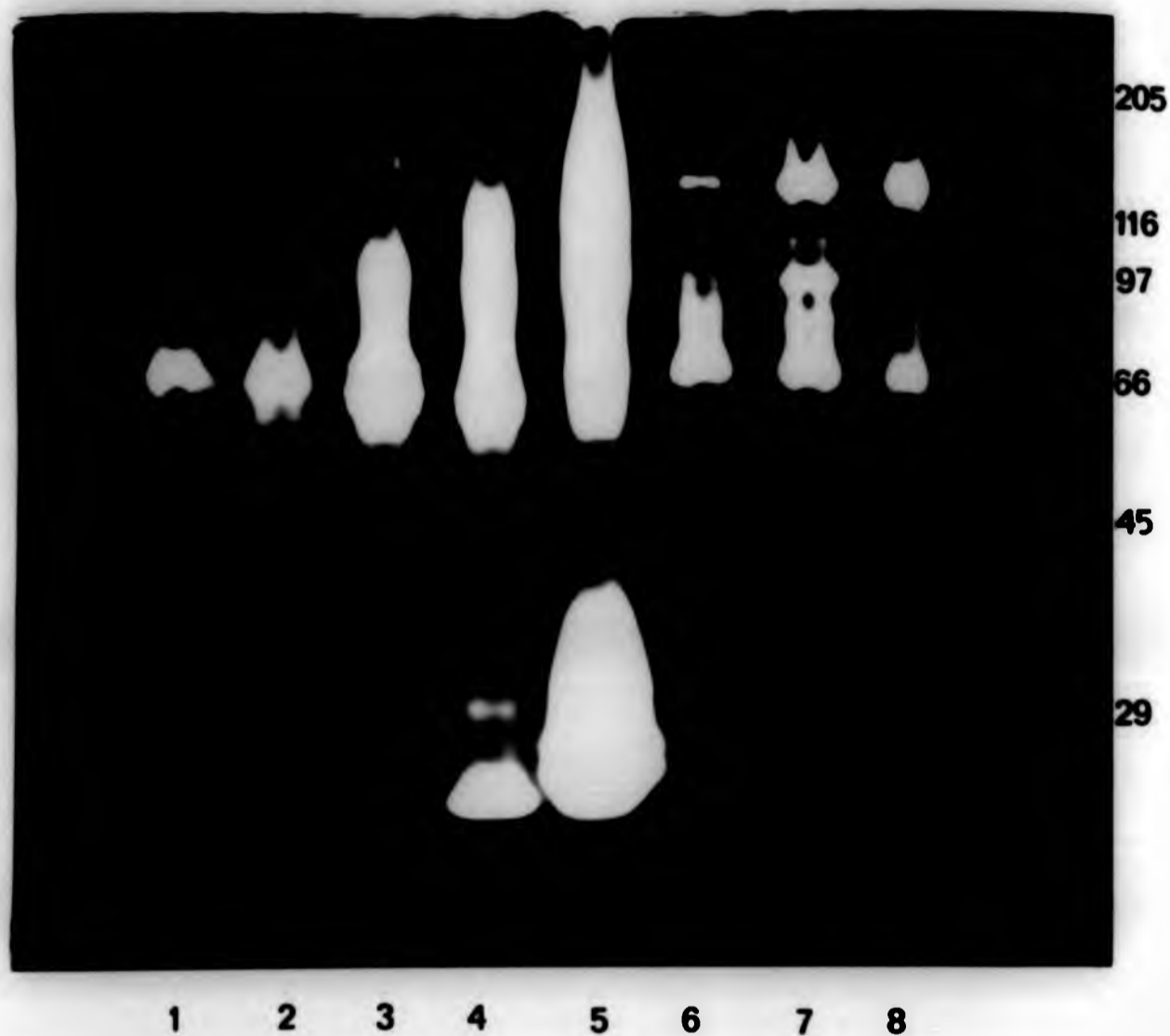


Fig. 33. Gelatin-PAGE analysis of proteinases in *Leishmania* species.

(a) Lanes 1-2, *Leishmania major*: 1, log-phase promastigotes; 2, metacyclic promastigotes. Lanes 3-5, *Leishmania mexicana mexicana*: 3, log-phase promastigotes; 4, stationary-phase promastigotes; 5, amastigotes. Lanes 6-8, *Leishmania donovani*: 6, log-phase promastigotes; 7, stationary-phase promastigotes; 8, amastigotes. All samples contained 50 μ g protein. The position of the molecular weight markers (in kDa) are given on the left of the figure.

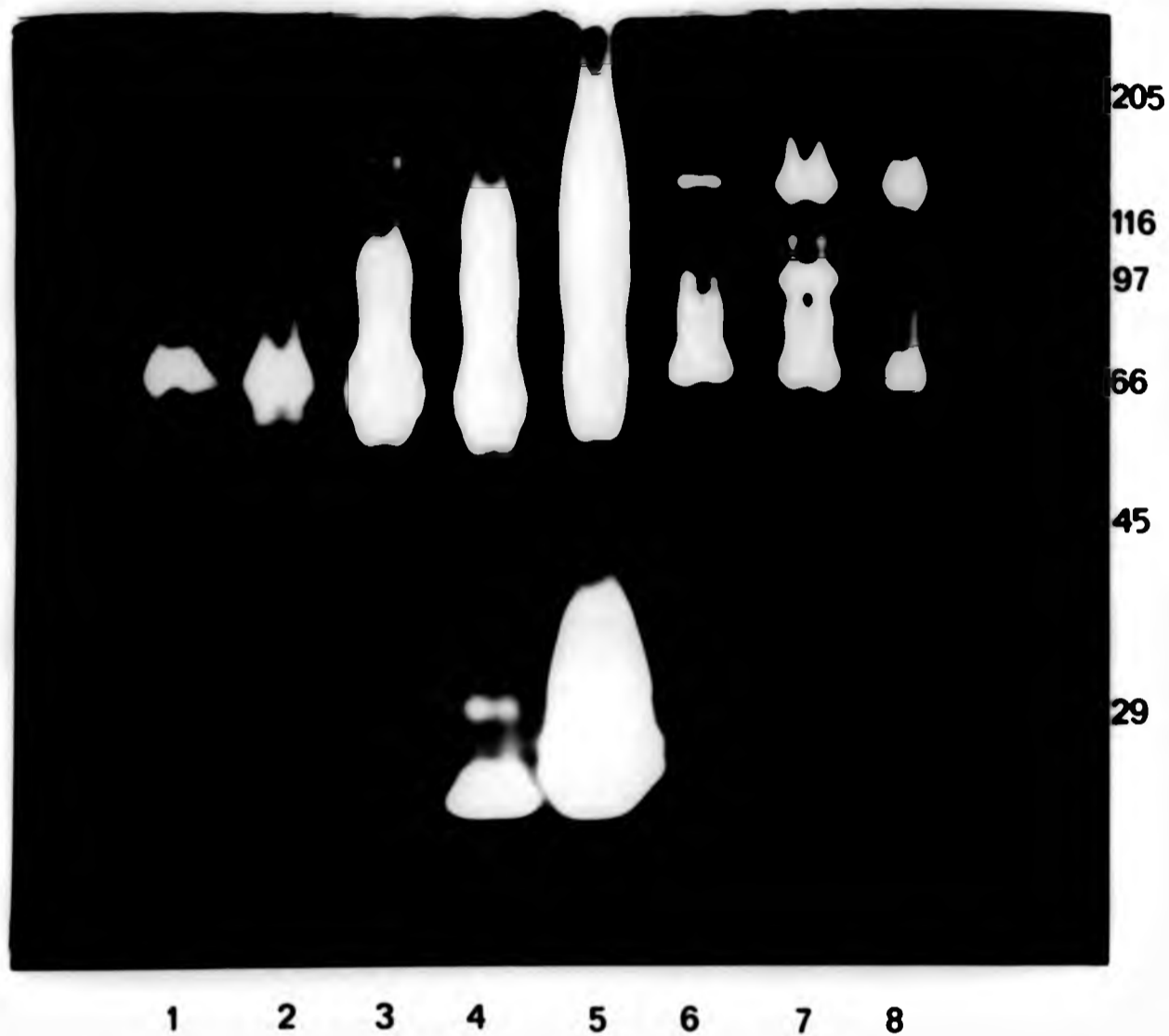


Fig. 33. Gelatin-PAGE analysis of proteinases in *Leishmania* species.
(a) Lanes 1-2, *Leishmania major*: 1, log-phase promastigotes; 2, metacyclic promastigotes. Lanes 3-5, *Leishmania mexicana mexicana*: 3, log-phase promastigotes; 4, stationary-phase promastigotes; 5, amastigotes. Lanes 6-8, *Leishmania donovani*: 6, log-phase promastigotes; 7, stationary-phase promastigotes; 8, amastigotes. All samples contained 50 μ g protein. The position of the molecular weight markers (in kDa) are given on the left of the figure.

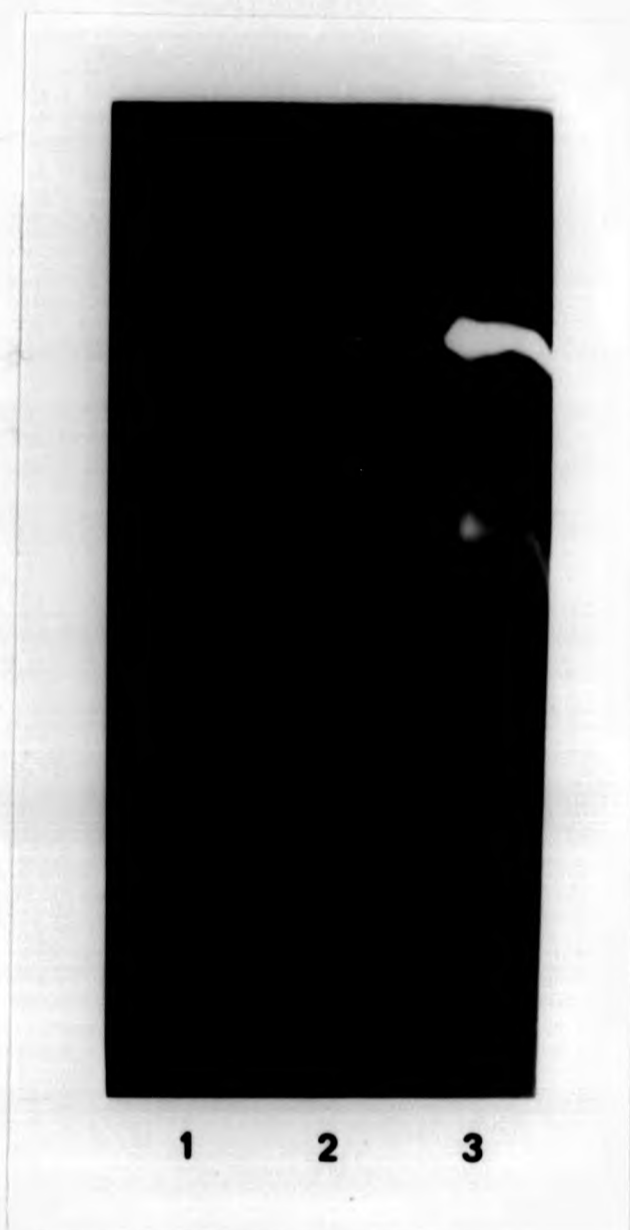


Fig. 33. Gelatin-PAGE analysis of proteinases in *Leishmania* species.
(b) *Leishmania donovani*. 1, log-phase promastigotes; 2, stationary-phase promastigotes; 3, amastigotes. All samples contained 50 μ g protein.



1 2 3

Fig. 33. Gelatin-PAGE analysis of proteinases in *Leishmania* species.

(b) *Leishmania donovani*. 1, log-phase promastigotes; 2, stationary-phase promastigotes; 3, amastigotes. All samples contained 50 μ g protein.

weights of 90 and 120 kDa were detected in the metacyclic (PMA⁻) form and a third enzyme (sometimes seen as a double band) with an apparent molecular weight of approximately 68 kDa was detected in both of the forms examined. Significantly, proteinases of similar mobility were found in all the other lysates irrespective of species or stage.

3.9.2. Properties of *Leishmania* proteinases

Inhibitors have previously been used to show that the soluble proteinases of *L. m. mexicana* are of the cysteine type (North and Coombs 1981; Pupkis and Coombs 1984). It was found however, that the inclusion of DTT was not necessary to detect the activity of any of the *Leishmania* proteinases using the gelatin-PAGE technique. Differential effects were observed with inhibitors of cysteine proteinases. For *L. m. mexicana*, antipain and leupeptin inactivated the low molecular weight proteinases but not the high molecular weight enzymes; neither agent inhibited any of the proteinases of *L. major* or *L. donovani*. Iodoacetic acid also had no effect on the higher molecular weight enzymes. In contrast, phenanthroline, a metalloproteinase inhibitor which also inactivates many cysteine proteinases of protozoa (North 1982), inhibited the higher molecular weight but not the lower molecular weight proteinases of *L. m. mexicana* (Fig 34) and all of the proteinases of the other two species. The aspartic proteinase inhibitor pepstatin, the metalloproteinase inhibitor EDTA and the serine proteinase inhibitor PMSF had no effect on the band patterns of any species.

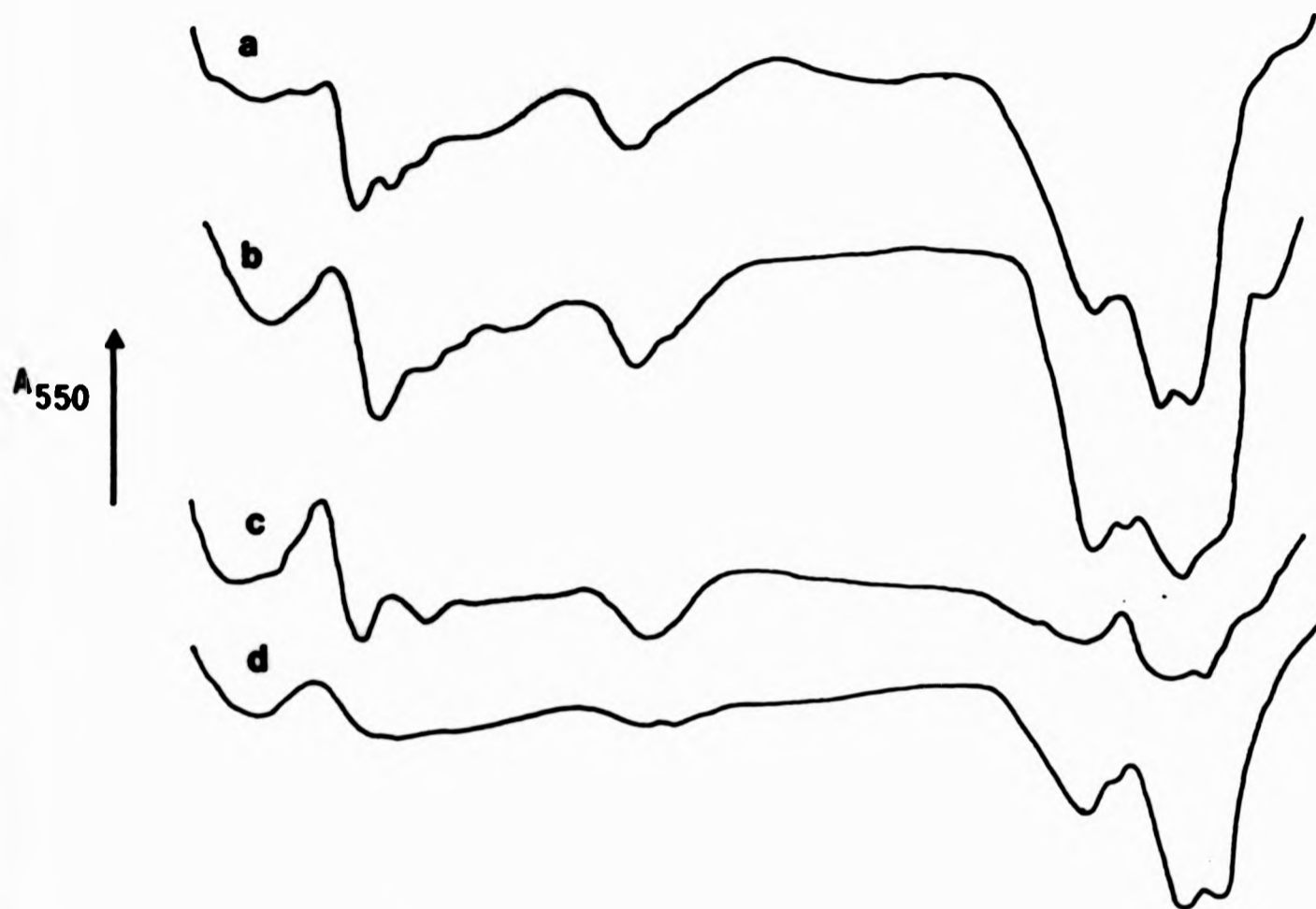


Fig. 34. Inhibition of *Leishmania mexicana mexicana* amastigote proteinase activity. Strips of gel were incubated in (a) buffer alone or buffer containing (b) EDTA (1 mM), (c) leupeptin (20 $\mu\text{g ml}^{-1}$) or (d) phenanthroline (1 mM). Densitometric scans of gels run from the cathode (left) to the anode (right) are shown.

3.9.3. The common 68 kDa proteinase.

A conserved major surface protein is thought to be present in all species of *Leishmania* (Lepay et al. 1983, Bouvier et al. 1985) and has been reported to have proteolytic activity in *L. major* (Etges et al 1986). The common 68 kDa proteinase detected on the gelatin gels resembled this major surface proteinase with respect to its molecular weight, sensitivity to inhibitors and its ability to bind to Con A. A number of the *Leishmania* proteinases were able to bind to Con A conjugated to agarose. This is shown in Fig. 35 for the stationary phase promastigotes of *L. m. mexicana*; the presence of unbound enzyme was probably due to saturation of the Con A.

3.9.4. Summary.

Proteinase activities were detected in three species of *Leishmania* using the gelatin-PAGE technique. In *L. m. mexicana* a number of low molecular weight, high activity proteinases were detected in the amastigotes which were absent from the log-phase promastigotes. Developmental changes were also observed in the proteinases of *L. major* and *L. donovani*.

All of the high molecular weight proteinases were sensitive to phenanthroline and may be of the metalloproteinase type although EDTA which inhibits many enzymes of this class failed to have any effect. In contrast the lower molecular weight activities of *L. m. mexicana* were sensitive to cysteine proteinase inhibitors and therefore may represent a different class.

A proteinase with an apparent molecular weight of 68 kDa was found in all stages of the three *Leishmania* species investigated. Its inhibitor

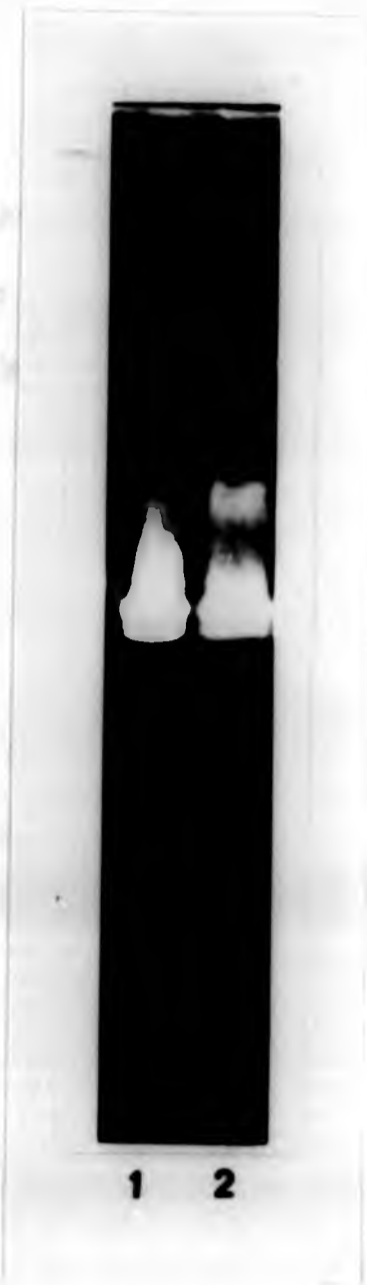


Fig. 35. Binding of *Leishmania* proteinases to Concanavalin A-agarose. A lysate prepared from *Leishmania mexicana mexicana* stationary phase promastigotes was incubated with Con A-agarose. The bound and unbound fractions were analysed by gelatin-PAGE. Lane 1, proteinases eluted after binding to Con A-agarose; lane 2, non binding proteinases.



Fig. 35. Binding of *Leishmania* proteinases to Concanavalin A-agarose. A lysate prepared from *Leishmania mexicana mexicana* stationary phase promastigotes was incubated with Con A-agarose. The bound and unbound fractions were analysed by gelatin-PAGE. Lane 1, proteinases eluted after binding to Con A-agarose; lane 2, non binding proteinases.

sensitivity was the same as that reported for the previously described major surface protein and it also shared the ability to bind Con A. The molecular weight of the major surface antigen has variously been reported to be in the range 63-68 kDa and its mobility can depend on the conditions employed for electrophoresis. Given this similarity in properties and the variability in electrophoretic mobility it is possible that the 68 kDa activity is the same as the major surface protein.

3.10. Proteinase activity in other protozoa species.

Standard incubation conditions were used to assess the usefulness of the gelatin-PAGE technique in detecting proteinase activity in a range of other protozoa. Two species of mammalian trypanosomes were investigated, *Trypanosoma brucei* and *T. rhodesiense*. Similar patterns of proteinase activity were detected in both species in both the procyclic and the bloodstream forms (Fig 36). Three bands of activity were observed, the highest activity proteinase had a molecular weight of approximately 30 kDa, the other two lower activity bands had molecular weights of approximately 36 kDa and 38 kDa. The activity of all the proteinases detected was significantly higher in the bloodstream forms than in the procyclic forms. The pattern of activity was markedly different from those of the non-mammalian trypanosomatids investigated, *Crithidia fasciculata* and *L. tarentolae* (Fig. 37). *C. fasciculata* possessed very high levels of proteolytic activity. Three major bands were observed which had molecular weights of approximately 35, 50 and 180 kDa. In contrast no activity was detected using this method in lysates of *L. tarentolae*.

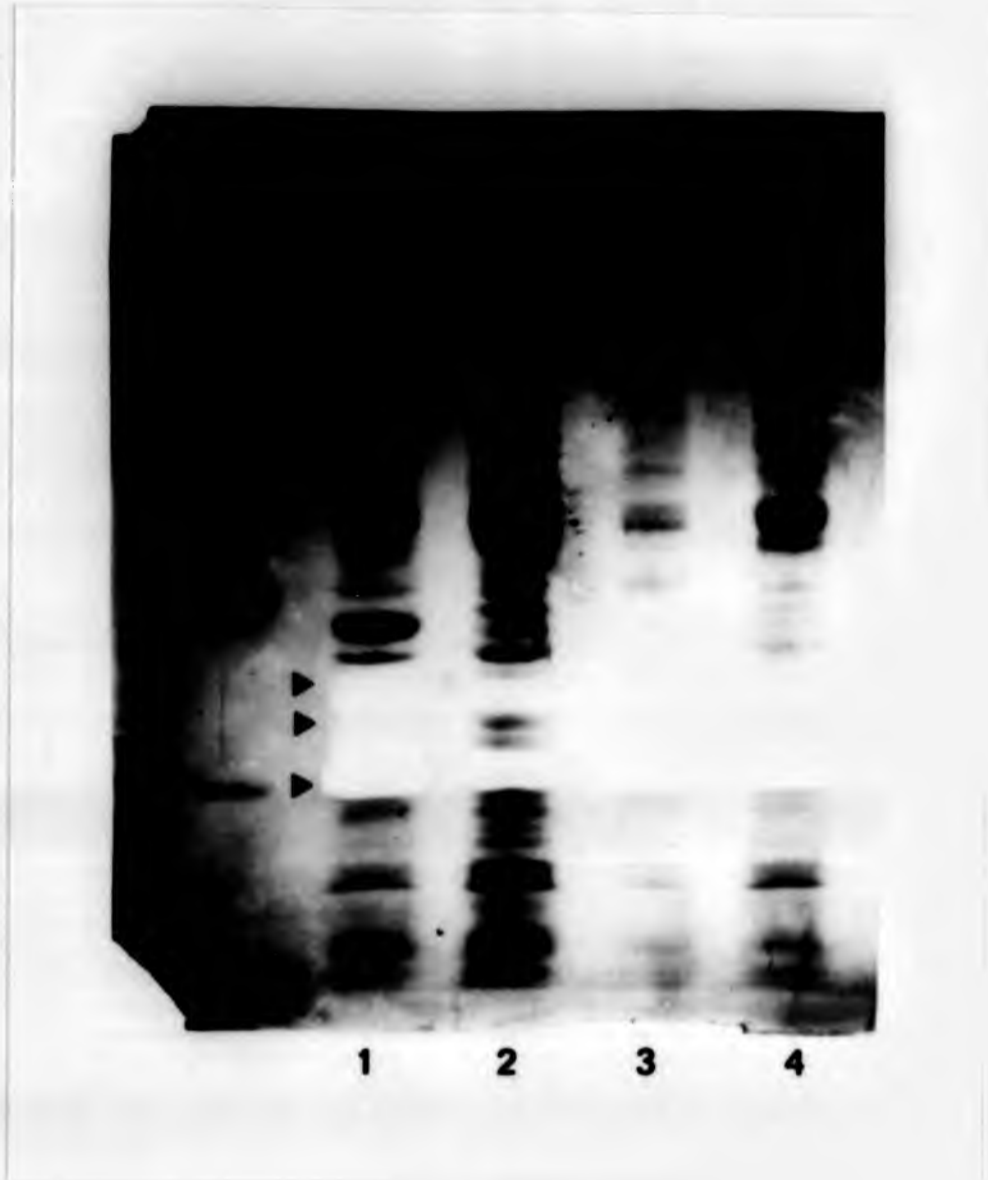


Fig. 36. Gelatin-PAGE analysis of proteinases in *Trypanosomas*.
1, *Trypanosoma brucei*, blood stream form; 2, *T. brucei*, procyclic form; 3, *T. rhodesiense*, blood stream form; 4, *T. rhodesiense*, procyclic form. Cell lysates containing 50 μ g of protein were analysed as described in section 2.3.2.

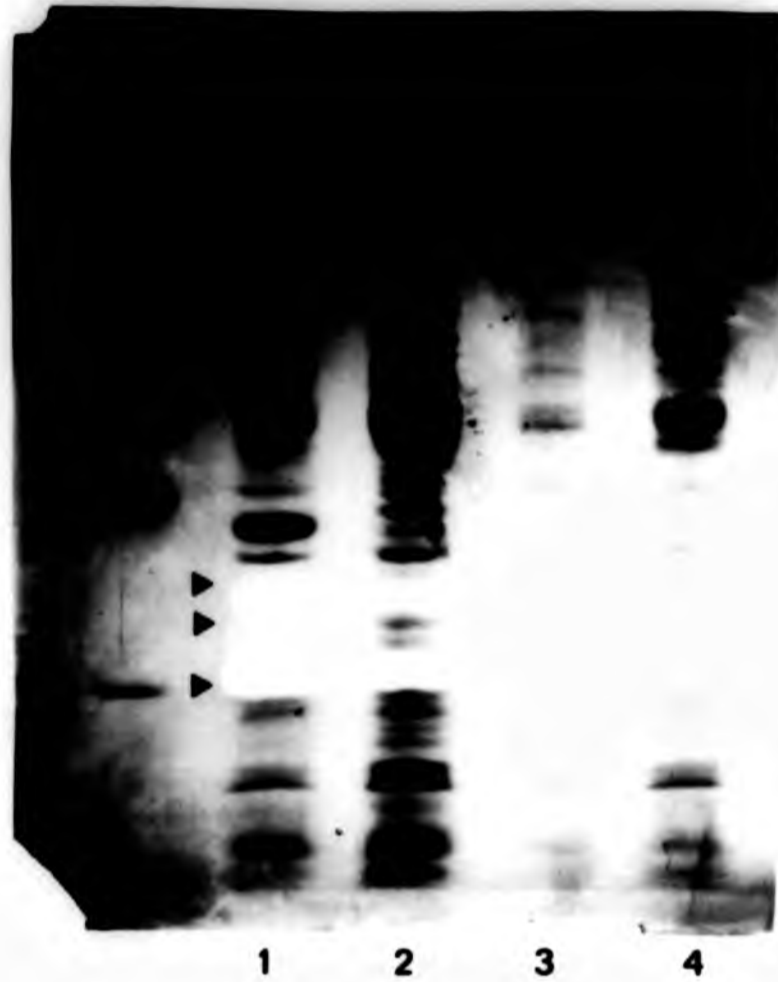


Fig. 36. Gelatin-PAGE analysis of proteinases in *Trypanosomas*.
1, *Trypanosoma brucei*, blood stream form; 2, *T. brucei*, procyclic form; 3,
T. rhodesiense, blood stream form; 4, *T. rhodesiense*, procyclic form. Cell
lysates containing 50 μ g of protein were analysed as described in section
2.3.2.

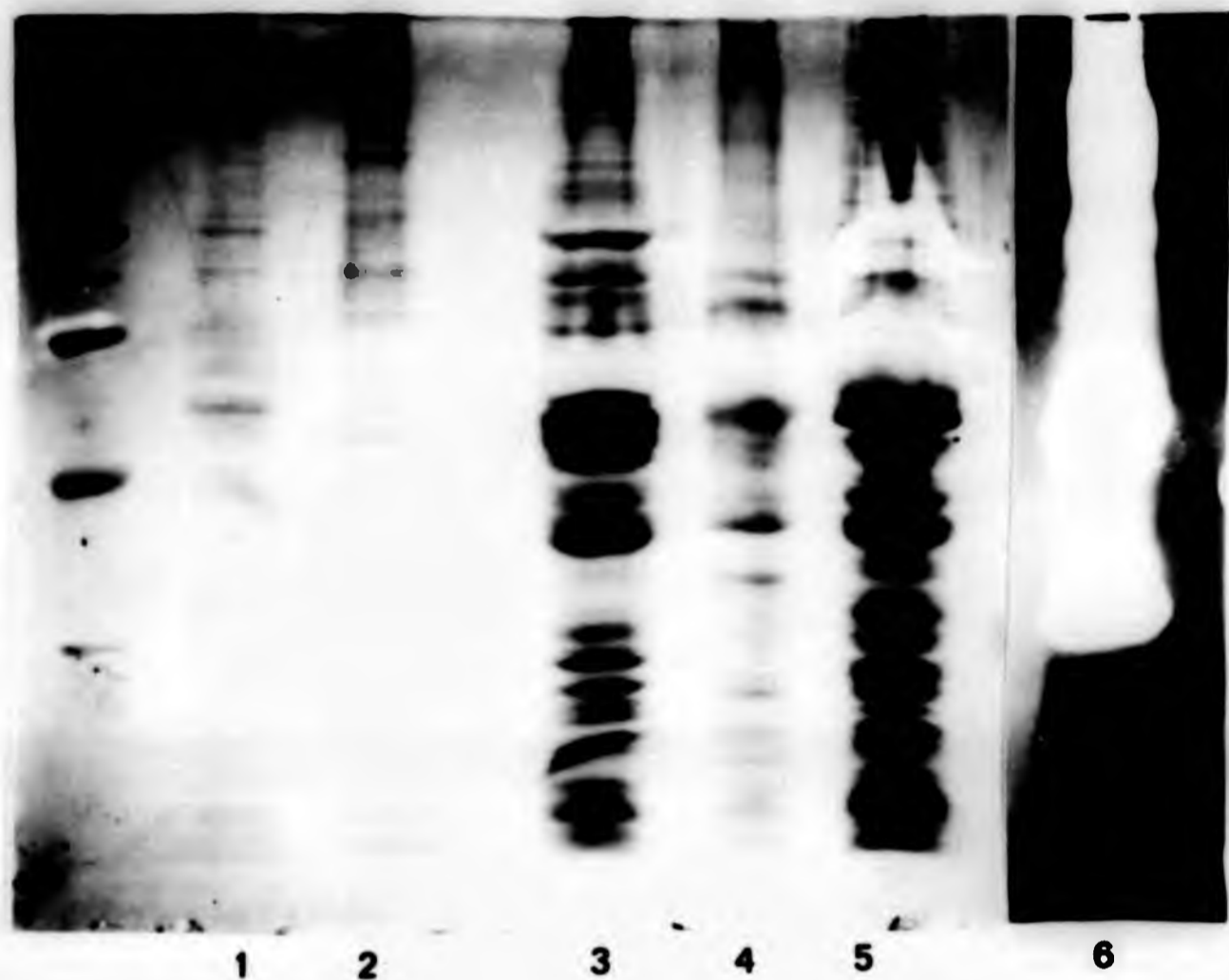


Fig. 37. Gelatin-PAGE analysis in other protozoan species.

1, *Leishmania tarentolae*; 2, *Tetrahymena pyriformis*; 3, *Herpetomonas muscarum muscarum*; 3, *H. m. ingenoplastis*; 5, *Plasmodium chaubaudi*; 6, *Crithidia fasciculata*. Lysates containing approximately 100 μ g of protein were analysed as described in section 2.3.2.

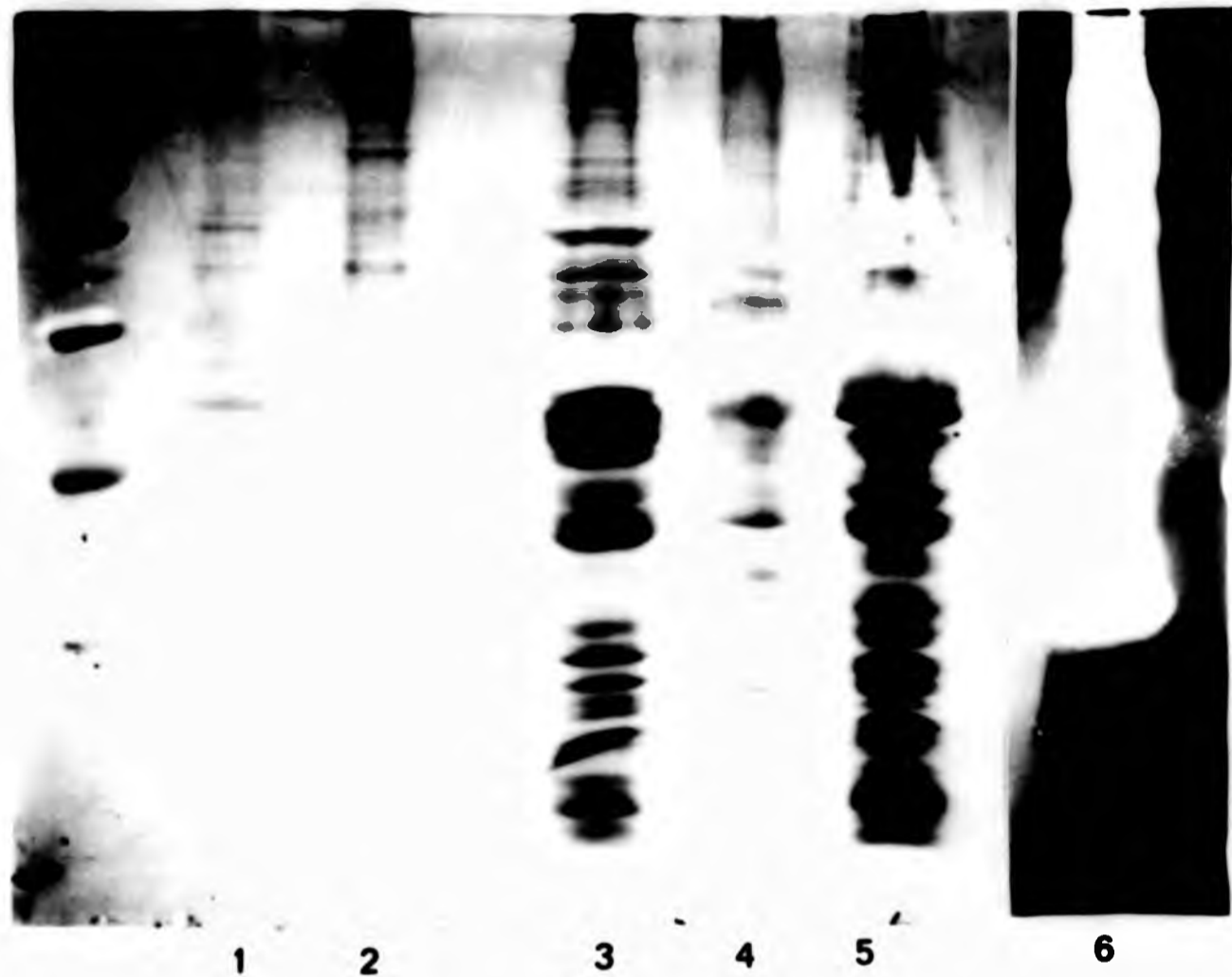


Fig. 37. Gelatin-PAGE analysis in other protozoan species.
1, *Leishmania tarentolae*; 2, *Tetrahymena pyriformis*; 3, *Herpetomonas muscarum muscarum*; 3, *H. m. ingenoplastis*; 5, *Plasmodium chaubaudi*; 6, *Crithidia fasciculata*. Lysates containing approximately 100 μ g of protein were analysed as described in section 2.3.2.

Proteinases were also detected in the lysates of *Herpetomonas muscarum muscarum*, *H. m. ingenoplastis*, *Tetrahymena pyriformis* and *Plasmodium* species, thus demonstrating the usefulness of this technique in separating and detecting the proteinases in a range of different protozoa.

DISCUSSION

4.1. Proteolytic activity in trichomonads.

Methods that have previously been employed to detect proteolytic activity in other protozoa (North et al. 1983; North and Walker 1984) were used to demonstrate a number of common features in the proteolytic systems of trichomonads. All four species examined possessed activity towards the large protein substrates hide powder azure and azocasein which was shown to be due to cysteine proteinases (section 3.1.1.). The presence of cysteine proteinases is characteristic of many protozoan species (North 1982), especially flagellates, and a number of the enzymes have been purified and characterised, including those from *Trypanosoma congolense* (Rautenberg et al. 1982), *Trypanosoma cruzi* (Bongertz and Hungerer 1978; Rangel et al. 1981; Bontempi et al. 1984) and *Leishmania mexicana mexicana* (Pupkis and Coombs 1984). These latter enzymes show similar properties to the activities detected in the lysates of the trichomonads, all of which are optimal at acid or neutral pH, are activated by thiol containing compounds and are inhibited by thiol reagents.

The use of chromogenic peptide substrates distinguished two types of activity in trichomonad lysates. One was optimal at neutral pH and appeared to be due to the enzymes responsible for the HPAase and AZCase activities since it was DTT-dependent and inhibited by cysteine proteinase inhibitors. The other, active at pH 5 was DTT-independent and less sensitive to cysteine proteinase inhibitors. This activity was not, however, significantly inactivated by inhibitors of other types of proteinases (Table 8) and since the enzyme(s) responsible was not purified nor characterised further it is not yet possible to assign it to a specific class.

The high activity of the trichomonad proteinases towards Bz-Pro-Phe-Arg-Nan, is similar to that noted for cysteine proteinases in other protozoa including *Trypanosoma* species (North et al. 1983), *L. m. mexicana* (Pupkis and Coombs 1984) and *Tetrahymena pyriformis* (North and Walker 1984). This compound is also considered to be a good substrate for cathepsin B, the mammalian cysteine proteinase, owing to the presence of arginine at the P₁ position and a bulky residue, phenylalanine, at the P₂ position.

A comparison of the four species of trichomonad does show that there are some differences in their proteolytic systems. For example the activities towards protein and peptide nitroanilide substrates that were detected in *Trichomonas vaginalis* and *Trichomitus batrachorum* were similar to one another whereas a number of differences from the activities of *Tritrichomonas foetus* and *Pentatrichomonas hominis* were apparent. For example, *T. foetus* had considerably lower HPAase activity, particularly at neutral and alkaline pH. In addition, although the DTT-independent activity of *T. foetus* was higher than that of *T. vaginalis*, the related activity towards Bz-Pro-Phe-Arg-Nan at pH 5 was lower and differed in that it showed a greater sensitivity to cysteine proteinase inhibitors and was not affected by pepstatin (Table 8). The activities detected in *P. hominis* were more similar to those of *T. foetus* than those of the other two species. Since these analyses were carried out on unfractionated cell lysates it was possible that endogenous inhibitors may have been present and that differences in the levels of activity detected between the species could have been due as much to the presence of different inhibitors as to different proteinases. Electrophoretic analysis of the proteinases, which would result in separation of any such

inhibitors from the proteinases, demonstrated clear differences between the proteolytic enzymes themselves, and no evidence for the presence of endogenous inhibitors was apparent.

Electrophoretic analysis using gels containing denatured haemoglobin has previously been used to analyse the proteinases of *Leishmania mexicana* (North and Coombs 1981), *Trypanosoma brucei*, *T. equiperdum*, *T. evansi*, *T. vivax*, *L. tarentolae* and *Crithidia fasciculata* (North et al. 1983), *Tetrahymena pyriformis* (North and Walker 1984) and *Trichomonas vaginalis* (Coombs and North 1983). Analysis of *T. vaginalis* demonstrated that the high level of proteinase activity detected in lysates was due to multiple enzyme forms. The results of the present investigation confirm these observations. A similarly complex pattern of proteinase activity was demonstrated for *Tritrichomonas foetus* (Fig. 13). McLaughlin and Muller (1979) proposed that the proteolytic activity of *T. foetus* was due to a single major cysteine proteinase. However, the results of this study do not support this view. Electrophoretic analysis of the two intestinal species *Trichomitus batrachorum* and *P. hominis* demonstrated much simpler proteinase band patterns. It is possible that the lower number of bands detected in the latter two species was due to instability of some of the proteinases during electrophoresis. There is, however, no evidence to support this suggestion. The results obtained using the gelatin-PAGE technique also clearly demonstrated a difference between the urogenital species and the intestinal species with respect to both the number and specific activity of the proteinases present (section 3.2.2.). The observed requirement of all the proteinases detected on the gels for DTT supports the idea that the proteolytic activity in trichomonads is due primarily to cysteine proteinases. This is confirmed by the results of

inhibitors from the proteinases, demonstrated clear differences between the proteolytic enzymes themselves, and no evidence for the presence of endogenous inhibitors was apparent.

Electrophoretic analysis using gels containing denatured haemoglobin has previously been used to analyse the proteinases of *Leishmania mexicana* (North and Coombs 1981), *Trypanosoma brucei*, *T. equiperdum*, *T. evansi*, *T. vivax*, *L. tarentolae* and *Crithidia fasciculata* (North et al. 1983), *Tetrahymena pyriformis* (North and Walker 1984) and *Trichomonas vaginalis* (Coombs and North 1983). Analysis of *T. vaginalis* demonstrated that the high level of proteinase activity detected in lysates was due to multiple enzyme forms. The results of the present investigation confirm these observations. A similarly complex pattern of proteinase activity was demonstrated for *Tritrichomonas foetus* (Fig. 13). McLaughlin and Muller (1979) proposed that the proteolytic activity of *T. foetus* was due to a single major cysteine proteinase. However, the results of this study do not support this view. Electrophoretic analysis of the two intestinal species *Trichomitus batrachorum* and *P. hominis* demonstrated much simpler proteinase band patterns. It is possible that the lower number of bands detected in the latter two species was due to instability of some of the proteinases during electrophoresis. There is, however, no evidence to support this suggestion. The results obtained using the gelatin-PAGE technique also clearly demonstrated a difference between the urogenital species and the intestinal species with respect to both the number and specific activity of the proteinases present (section 3.2.2.). The observed requirement of all the proteinases detected on the gels for DTT supports the idea that the proteolytic activity in trichomonads is due primarily to cysteine proteinases. This is confirmed by the results of

inhibitor studies: all of the effective compounds have been shown to inhibit cysteine proteinases (Barrett and McDonald 1980).

The variation between species, revealed by this type of analysis, may be related to the different environments that the parasites inhabit. Since all trichomonads are likely to require a source of exogenous amino acids, and host proteinases are not known to be present in the urogenital tract, the parasites would have to rely on their own intracellular and extracellular proteinases to supply these nutritional requirements. In contrast, host proteinases present in the intestine would ensure a supply of readily available amino acids to those trichomonads present. Not all trichomonads that parasitise the alimentary canal inhabit the large intestine, for example *Trichomonas tenax* infects the mouth where there would be insignificant proteolysis due to host enzymes, and it would be of interest to know whether this and other species of trichomonad also have high levels of proteinase activity

Multiple proteinase activities are not a unique feature of trichomonads and have been reported for several other species of protozoa. There was a possibility, however, that the complexity in the proteinase band patterns observed using the methods employed in this study could have resulted from autolysis during sample preparation or electrophoresis. Although the exact relationship between the individual proteinases identified in each species will not be fully established until each has been purified and characterised, the observations made to date rendered this possibility unlikely. Neither the inclusion of proteinase inhibitors during sample preparation, preincubation of the samples at 37°C nor incubation of mixtures of different samples affected the patterns. It might be anticipated that proteinases would have to be

inherently more resistant to proteolytic modification than most proteins in order to avoid self-digestion within the cell. The autodegradation of cellular proteinases has received little attention although a recent report on rat cathepsins has indicated that turnover does occur. (Kominami et al. 1987).

The development of the gelatin-PAGE technique for the analysis of complex mixtures of intracellular proteinases was an important advance resulting from this study. Furthermore, it was subsequently found that this technique was a highly effective method for the detection of extracellular proteinases (section 3.7.1.). Novel forms of proteinase that were not detected in the cell lysates were found to be present in the medium. The mechanism of their release is not yet known, but irrespective of whether it is due to cell lysis, which seems unlikely from the data available, or from a specific release mechanism, the results clearly demonstrate that both *Trichomonas vaginalis* and *Tritrichomonas foetus* have proteinases present in their surrounding environment. This is the first report, to my knowledge, of extracellular proteinase activity in trichomonad cultures.

The high sensitivity of this PAGE method for detecting extracellular enzymes has made a number of analytical applications possible. In preliminary experiments high levels of trichomonad proteinases have been detected in medium taken from mixed cultures of trichomonads and mammalian cells, a system previously used to demonstrate trichomonad cytotoxicity. It should now be possible to determine whether purification of cytotoxic factors present in these cultures parallels proteinase purification and to establish whether or not proteinases are responsible for any aspect of the pathogenicity of trichomonads. Furthermore,

Trichomonas vaginalis proteinases have been detected in washouts taken from the vaginas of mice with trichomonad infections. Due to the low numbers of trichomonads normally present in the mouse vagina the gelatin-PAGE technique was only sensitive enough to detect proteinases in wash outs from mice with unusually high levels of parasites. It is possible however that this technique could be of use in diagnosis of human and bovine trichomoniasis where significantly higher numbers of parasites are usually present. The value of this type of analysis is not only that proteinases can be detected but that the unique pattern of the *T. vaginalis* proteinases confirms that they are of trichomonad and not of host origin.

The investigation of isolates of *T. vaginalis* has shown that differences do exist in the proteinase band patterns they produce on gelatin gels (section 3.3.3.). A more detailed study involving a larger number of isolates is now merited in order to determine if any correlation between particular proteinases and virulence exists. The high specific activity of the proteinases of clone G3, which has been maintained in axenic culture for a number of years, may be related to the extent to which this clone has had to rely on its own proteolytic enzymes to derive amino acids during growth in axenic medium. Under these conditions a clone capable of high levels of proteolysis may have a selective advantage. The availability of isolates with different proteinase contents may prove to be of particular value in elucidating the role of individual enzymes in the host-parasite relationship.

A complete understanding of the proteolytic systems of trichomonads must take into account the presence not only of endopeptidase activities but also of exopeptidase activities (aminopeptidases, carboxypeptidases

and dipeptidases). Carboxypeptidase activity towards three substrates (section 3.1.3) and aminopeptidase activity towards five substrates (section 3.1.4.) has been detected. In general the activities were higher in the urogenital species than in the intestinal species of trichomonads. It is impossible, however, to speculate on the roles of the enzymes responsible until more information is available. At least two aminopeptidase activities towards Gly-Phe-2Nap were previously found to be present in the large particle fraction of *Tritrichomonas foetus* which suggested that they are lysosomal (McLaughlin and Muller 1979). The exopeptidase activities detected in the present study were also shown to be active at acid pH but at present there is no direct evidence for their localisation within the cell.

Two proteinase activities were partially purified from the cell lysates of *Trichomonas vaginalis* using a combination of gel filtration, ion exchange chromatography and affinity chromatography (section 3.5.). Subsequent analysis of the intracellular localisation of the proteinase activity now suggests that the particulate fraction obtained by differential centrifugation of *T. vaginalis* homogenates may have proved a more appropriate starting material for the purification. Gel filtration using Sephadex G75 gave good separation of the high and low molecular weight proteinases but the resolution achieved was not sufficient to separate the proteinases of closely related molecular weight detected using the gelatin-PAGE technique, whereas ion exchange chromatography gave a much better separation of the multiple forms of proteinase. Organomercurial-Sepharose affinity chromatography used in the final purification of both activities has previously been used to purify a number of other cysteine proteinases including the low molecular

weight cysteine proteinase purified from *Tritrichomonas foetus* (McLaughlin and Muller 1979).

Many of the properties of the two partially purified proteinases were found to be similar to those of cysteine proteinases isolated from other organisms. They are particulate in origin, and thus in this respect resemble the mammalian lysosomal cathepsins B, L, or H. Proteinase D has a low molecular weight in the same range as those of the best characterised cysteine proteinases (mammalian cathepsins, papain and actinidin) although the values obtained using gel filtration and PAGE did differ (section 3.6.1). A similar anomaly has also been reported for the low molecular weight cysteine proteinase of *L. m. mexicana* (Pupkis and Coombs 1984). Proteinase D was optimally active at acid pH, although retains significant activity at higher pH (Figs. 27 and 28). This is in accord with a recent report by Villenbrock and Brocklehurst (1985) that both cathepsin B and cathepsin H can exhibit considerable activity at pH values as high as 8.0, and is in contrast to earlier reports of cathepsin stability which claimed a marked loss of activity above pH 7.5 and a complete loss at pH 8.0 (Barrett 1972; Suhar and Marks 1979). A similarly broad pH range has been described for cysteine proteinase activity detected in African trypanosomes (Lonsdale-Eccles and Mpimbaza 1986). The high-*M_r* activity was optimal at neutral pH and was similar in this respect to the neutral cysteine proteinases purified from *Entamoeba histolytica* (McLaughlin and Faubert 1977) and *Tetrahymena pyriformis* (Levy et al. 1985), although both these latter enzymes are of lower molecular weight. Both of the purified proteinases showed the same specificities for peptide nitroanilide substrates as was found in the cell

lysates. These proteinases would be expected to make a major contribution to the activity detected in the lysates.

4.2. Roles of trichomonad proteinases.

In mammalian cells the equivalent enzymes to the trichomonad proteinases are lysosomal, and the investigations of the intracellular localisation of the proteolytic activity of *Trichomonas vaginalis* and *Tritrichomonas foetus* have shown that the majority of the proteinase activity is contained in particulate fractions which include lysosome-like organelles. The lysosomes of trichomonads appear to be similar to those of many other cell types (Muller et al. 1966) in containing a number of hydrolases with acidic pH optima and thus the trichomonad proteinases may have a role in intracellular protein breakdown and nutrition similar to that found in other organisms.

Trichomonads take up nutrients in solution by the formation of pinocytic vacuoles (Kulda et al. 1985) but little is known about the subsequent stages of the nutritional process. In other organisms the pinocytic vacuoles coalesce to form the equivalent of a food vacuole and degradation of engulfed material begins when this vacuole fuses with a the hydrolase containing primary lysosome. The resulting nutrient products permeate the cytosol and the indigestible residue is egested. A similar process may occur in trichomonads but although, the properties and localisation of the proteinases of *T. vaginalis* and *Tritrichomonas foetus* are consistent with this it does not, however, explain why the levels of activity found in trichomonads are so high compared to many other flagellates (Coombs 1982) and alternative or additional functions must be considered.

During this work it was noted that trichomonad proteins are highly susceptible to proteolysis by the endogenous proteinases in cell lysates. It was also observed that trichomonads can survive for quite long periods in non-nutrient medium and therefore, it is possible that proteinases may be involved in a process related to autophagy which would allow survival of the parasite under certain adverse conditions in a manner equivalent to that found during differentiation of a number of eukaryotic microorganisms (North 1982).

In *Trichomonas vaginalis* there appear to be at least two distinct populations of lysosomes with different densities (section 3.4.2.), and it is possible that the enzymes contained in the different types of lysosomes fulfil different roles in the cell. Smith (1961) found that culture medium obtained after brief incubation of *Tetrahymena* contained a number of hydrolytic enzymes. This work was extended by Muller (1970,1972) who showed that the hydrolases that were secreted into the culture medium were derived from a separate population of lysosomes which contained these hydrolases in approximately the same proportion in which they appeared in the medium. The results of this study on *Trichomonas vaginalis* indicate that a similar mechanism for the release of hydrolases may occur. Furthermore, recent work on rat tissues has also indicated at least two lysosomal populations which can be separated on Percoll gradients, that contain different levels of cysteine proteinase activity. One subpopulation is thought to be involved in autophagy whereas the other is involved with heterophagy (Kominami et al. 1986). It now seems likely that heterogeneous lysosome populations are a feature of a number if not most eukaryotic systems.

The extracellular proteinases may also have a role in nutrition, for example in the digestion of host proteins to release amino acids required by the trichomonads but the release of degradative enzymes makes it possible that the proteinases may be involved in the pathogenic effects on host cells exhibited by the parasite. Even if the proteinases are released primarily for nutritional purposes their presence could consequentially cause damage to host cell tissue. A more specific role in pathogenicity, however, cannot be ruled out at this time. Some insight into this should be gained from further studies using different isolates in which virulence can be compared with the type and level of proteinase(s) released. Proteinases have been implicated in the pathogenicity of a number of other parasitic protozoa most notably in the formation of tissue lesions associated with invasive amoebiasis due to *Entamoeba histolytica* which might be caused by hydrolytic enzymes including proteinases (McGowan et al. 1982). Indirect evidence for the involvement of proteinases in the cytotoxicity of trichomonads comes from the finding that the proteinase inhibitor, leupeptin, which has been shown to inhibit the trichomonad proteinases, (Tables 15 and 17) has an inhibitory effect on the pathogenicity of the parasite towards mammalian cell lines *in vitro*. This effect is specific as leupeptin is not active against *Trichomonas vaginalis* in axenic culture (Bremner et al. 1986). This indicates that proteinase inhibitors might be of value as antitrichomonal drugs.

4.2. Proteolytic activity in *Leishmania*.

Gelatin-PAGE proved to be an appropriate technique for analysing not only trichomonad proteinases but also those of other protozoa especially

Leishmania (section 3.9.). Unlike the trichomonads *Leishmania* species exist in different developmental forms and the technique not only allows species differences to be analysed but also developmental changes within individual species.

The results provide confirmation of the great differences in proteinase activity between amastigotes and promastigotes of *L. m. mexicana* described previously (North and Coombs 1981). In amastigotes the most active proteinases have high electrophoretic mobility, but that there are also slower moving low activity enzymes present. The low molecular weight amastigote enzymes were in the 20-31 kDa range, and so included the 31 kDa-enzyme characterised previously (Pupkis and Coombs 1984). The latter enzymes were absent from *L. major* and *L. donovani* and from log-phase promastigote populations of *L. m. mexicana* but were present in the stationary phase promastigotes. *Leishmanias* occur in more than one promastigote form when in the midgut of the fly is thought to be equivalent to the promastigotes that predominate in log-phase *in vitro* cultures. The smaller form that subsequently develops in the fly is thought to be the main infective form (the metacyclic) (Mallinson and Coombs 1986). This form also occurs in *in vitro* cultures, although in quantity only in the stationary phase. These forms of *L. major* can be purified and analysed (Mallinson and Coombs 1986), as in this study. There is no satisfactory method available for *L. m. mexicana* or *L. donovani*, however, so studies of the metacyclics of these species are restricted to the cells of stationary phase cultures containing a mixed population of promastigotes. Nevertheless, the use of such material in this study has provided an

insight into the changes in proteinase activity that accompany the transition from multiplicative promastigotes to amastigotes. The presence of low molecular weight enzymes in stationary-phase, but not in log-phase promastigotes suggests that synthesis of this particular type of proteinase may represent a necessary preparation for the intracellular stage of the life cycle. The high mobility promastigote enzymes described previously (North and Coombs 1981) were probably due to a significant proportion of metacyclics in the preparations used.

No detailed analysis of *L. major* or *L. donovani* proteinases had previously been reported, although total proteinase activities determined previously (Pupkis et al. 1986) are in accord with the levels of activity detected on gelatin gels. Developmental changes were apparent in both species. These included increases in enzyme activities which paralleled to some extent the changes observed for *L. m. mexicana* low molecular weight proteinases. The presence in *L. donovani* of proteinases specific to stationary-phase promastigotes (Fig. 33) was particularly interesting.

It is not yet known whether the higher molecular weight enzymes represent an entirely different group from the high activity lower molecular weight forms present in *L. m. mexicana* amastigotes. The lack of sensitivity of the former to various inhibitors suggests that they are not cysteine proteinases. The inhibitory effect of phenanthroline, which was also reported by Pupkis and Coombs (1984) for the 67 kDa proteinase purified from *L. m. mexicana*, suggests that they may be metallo-proteinases, although EDTA, an inhibitor of many enzymes of this class, failed to have any effect. Whether distinctions between types of proteinase reflect other differences such as intracellular location must await further investigation. The finding, however, that at least one of

the high activity enzymes of *L. m. mexicana* amastigotes is located within vacuolar lysosome-like organelles (megosomes), which do not apparently occur in other species or developmental forms (Pupkis et al. 1986), shows that there must be some difference.

An important finding from this study relates to the recent report that the major cell surface protein of *L. major* (p63) possesses proteolytic activity (Etges et al. 1986). A proteinase of a similar molecular weight was present in all leishmania lysates analysed (section 3.9.3.). Its inhibitor sensitivity was the same as that reported for p63 and it also shared the ability to bind to Con A. The molecular weight of the major surface antigen has variously been reported to be in the range 63-68 kDa (Fong and Chang 1982; Lepay et al. 1983; Gardiner et al. 1984; Etges et al. 1985; Etges et al. 1986), and its mobility can depend on the conditions employed for electrophoresis (see Bouvier et al. 1985). In the gelatin-PAGE method used here, samples are reduced but not heated before electrophoresis. Hydrophilic and amphiphilic forms of p63, which can differ from one another in electrophoretic mobility under some conditions, have been described (Bouvier et al. 1985), and this could account for the double proteinase band sometimes observed. All the gelatin-PAGE observations are at present consistent with p63 being identical to the 68 kDa-proteinase. The detection of a similar activity in all forms of all three species suggests that this enzyme may be highly conserved.

4.3. Future work.

The results of this study have shown that the proteolytic systems of trichomonads are exceedingly complex. All four species investigated

contain multiple proteinase forms and additional forms were detected extracellularly in cultures of *T. vaginalis* and *Tritrichomonas foetus*. Although the relationship between these proteinases remains to be elucidated, the availability of anti-proteinase antiserum will now allow further investigation of these multiple forms. For example, preliminary observations suggest that there may be some relationship between the low molecular weight proteinases purified from the medium and a higher molecular weight intracellular proteinase. There are, however, some reservations about the specificity of the antiserum and further characterisation is essential.

In order for the proteolytic systems of trichomonads to provide a suitable target for chemotherapeutic attack it would be expected that they would have to differ in some way from those of the host. This study has shown, however, that the proteinases purified from *Trichomonas vaginalis* share a number of properties with the well characterised mammalian cysteine proteinases. To define essential differences it will now be necessary to investigate these enzymes in more detail using the procedures developed in this work to provide purified preparations and form the basis of the characterisation.

The high levels of proteinase activity and the complexity of the proteolytic systems of trichomonads imply that proteolysis is an important feature of the parasite's growth and survival. Although information on the properties and localisation of the enzymes permits some speculation as to the roles of the proteinases as yet there is no direct evidence as to function of any of the enzymes detected. If specific inhibitors to individual proteinases can be developed, these together with

the availability of specific antibodies would allow the roles of particular enzymes to be investigated.

The gelatin-PAGE technique has opened up a large number of possibilities for the investigation and comparison of the proteinases in a number of species of protozoa. In addition to its use in the separation and detection of intracellular proteinases this study has indicated that it may be of considerable value in identifying proteinases in experimental systems. Preliminary experiments have shown that the technique can be used to identify trichomonad proteinases in medium taken from mixed cultures of trichomonads and mammalian cells and in experimental infections in mice and it is very likely that similar studies could be used to identify proteinase activity in experimental models involving other protozoan parasites.

The application of the gelatin-PAGE technique to the study of changes in proteolytic activity in different developmental stages of *Leishmania* proved particularly useful. Identification of individual proteinases at particular stages suggests specific roles for certain enzymes. This in turn suggests possibilities for the production of antileishmanial drugs, for example inhibitors that act against proteinases present at specific stages or that prevent proteolytic events necessary for the parasite's development. It would now be of interest to investigate developmental changes in proteinase activities in other species of protozoa.

The work on both the trichomonads and *Leishmania* species has highlighted the complexity of the proteolytic systems that are present in protozoa. It is certainly not possible to consider any of the systems

investigated here in terms of single major enzymes. In order to understand fully the roles played by proteolytic enzymes and to exploit them as potential targets for chemotherapeutic attack it will be essential to continue to employ techniques that allow the component parts of the proteolytic systems to be separated and characterised individually. This work has provided details on both the proteolytic enzymes and some of the techniques that may prove useful in future investigations.

Investigations
conducted by
the FBI
in the
case of
the
murder of
Dr. Martin Luther King, Jr.

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