A Study of Hydrolases and their Release from Trichomonads

Submitted for the degree of
Doctor of Philosophy

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November 1992

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ACKNOWLEDGEMENTS

I would like to thank my supervisor Dr M. J. North for his advice, support and friendship throughout this project. I am also indebted to the members of staff and postgraduates in the Department of Biochemistry, University of Stirling, with special thanks to Jacqueline Nairn, Kay Nicol and to Sharon Kelly for her help with the Circular Dichroism work.

I would also like to specially mention my Mum and Dad for their unfailing support in every way, and say thank you to Michael Leaver and the rest of my family.

This investigation received financial support from The University of Stirling.

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ABBREVIATIONS

Con A	:	Concanavalin A
DEAE	:	diethylaminoethyl
DNA	:	deoxyribonucleic acid
DTT	:	dithiothreitol
Endo F	:	endo-ß-N-acetylglucosaminidase F
MDM	:	modified Diamond's medium
NADH	:	nicotinamide adenine dinucleotide (reduced)
NADP	:	nicotinamide adenine dinucleotide phosphate
NADPH	:	nicotinamide adenine dinucleotide phosphate (reduced)
NAHase	:	ß-N-acetyl-D-hexosaminidase
NAGase	:	ß-N-acetyl-D-glucosaminidase
Naphthol AS-BI	:	6-Bromo-2-hydroxy-3-naphthoic acid 2-methoxyanilide
PAGE	:	polyacrylamide gel electrophoresis
PBS	:	phosphate buffered saline
SAM	:	S-adenosyl-L-methionine
SDS	:	sodium dodecyl sulphate
Tris	:	2-amino 2(hydroxymethyl) 1,3-propandiol

Abstract

The acid hydrolase N-acetyl-β-D-glucosaminadase (NAGase) is a high activity lysosomal enzyme in trichomonads. *Tritrichomonas foetus* (strains F2, KV1 and CA84-2), *Trichomonas vaginalis* and *Tritrichomonas augusta* all contained and released NAGase. Analysis of NAGase using electrophoretic techniques demonstrated in all the *Tritrichomonas foetus* strains the same four major forms, named NAGase 1-4, whose mobility suggested apparent M_rs of 54 000, 89 000, 100 000 and 158 000 respectively. *Trichomonas vaginalis* and *Tritrichomonas augusta*, however, each contained a single form of this enzyme of apparent M_rs 138 000 and 107 000 respectively. In all cases the intracellular and extracellular forms of NAGase appeared to be identical.

Growth of *Tritrichomonas foetus*, F2, in the presence of the glycosylation inhibitor, tunicamycin, resulted in an extra NAGase band with higher mobility assumed to be a non-glycosylated but active enzyme form. NAGase 3 mobility was simultaneously diminished. Extra NAGase forms were also observed after incubating this parasite in serum free media containing the two proteinase inhibitors, leupeptin and Z-Phe-Phe-CHN_2, indicating that proteolytic processing may be involved in the production of NAGase forms.

NAGase 1, the enzyme with an apparent M_r of 54 000, was purified from *Tritrichomonas foetus*, F2. Preparative isoelectric focusing was used to separate the four NAGase forms and as the first step in its purification. Total denaturation of NAGase 1 before electrophoresis produced a decrease in mobility from an apparent M_r of 54 000 to a doublet of apparent M_rs of 68 000

and 70 000. This transformation was found to be complete within 2 min at 50°C but was dependent at this temperature on the presence of both SDS and ß-mercaptoethanol. This transformation also occured without sample buffer at 100°C and may relate to the breaking of disulphide bridges. Treatment of NAGase 1 with endoglycosidase reduced its apparent M_r by 2000, indicating the enzyme to be an N-linked glycoprotein.

Trichomonas vaginalis and *Tritrichomonas foetus* were also found to contain and release acid phosphatase. SDS-PAGE analysis of this hydrolase found one form with an apparent M_r of 126 000 in *Tritrichomonas foetus*, strains F2 and KV1, *Tritrichomonas augusta* also had one form of apparent M_r 135 000 while *Trichomonas vaginalis* contained and released two forms with apparent M_rs of 155 000 and 160 000.

Despite several immunisation attempts purified NAGase 1 was found to be non-antigenic in rabbits and so it was not possible to analyse the release processes in the detail originally proposed. However, although the results have not allowed details of the release mechanisms and physiology, they have confirmed release and provided more information on the properties of the hydrolases, especially NAGase.

1.0 Introduction

1.1 Introduction

The protozoa are a large and diverse group of unicellular eukaryotic organisms. There are now over 30 000 named species of protozoa of which nearly 10 000 are parasitic in invertebrate and vertebrate animals. Many of these are of interest to physicians and veterinarians because they produce disease in man and his livestock.

Protozoa share some of the characteristics of both prokaryotic and higher eukaryotic organisms. In common with the prokaryotes, they are usually microscopic, have short generation times and high rates of reproduction. However, protozoa are undoubtedly eukaryotic cells with organelles and metabolic pathways akin to those of higher organisms.

Infections of parasitic protozoa are, unlike bacterial infections, generally long lived owing to their ability to adapt and hence avoid host defence mechanisms. Parasitic protozoa are highly specialised and their particular adaptations frequently include complex life cycles and individual ways of entering their hosts and maintaining themselves therein. Their nutrition, physiology and biochemistry are largely geared to the parasitic habit and are therefore sometimes highly specialised.

1.2 Classification

Protozoa are now, after some controversy, regarded as a subkingdom of the kingdom Animalia, containing seven distinct phyla. Six from the seven phyla contain parasitic organisms. Most of the more important parasites, however,

belong to the two phyla sarcomastigophora and apicomplexa which are shown in table 1. Trichomonads, the topic of this thesis, belong to the phylum Sarcomastigophora, which is characterised by flagella locomotion or pseudopodia or both, and to the subphylum Mastigophora which is characterised by the locomotion of flagella only. A more detailed classification of trichomonads is shown in table 2.

1.3 Biology of trichomonads

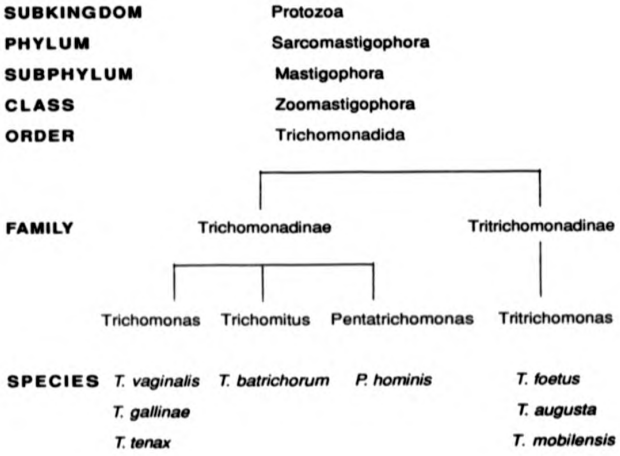
Trichomonads have one developmental form, namely the trophozoite which has an approximate length of 7 - 32 µm and a width of 5 - 12 µm. These organisms have also been reported to occur as cysts (Holtz 1953), pseudocysts (Mattern & Wendell 1980) and giant forms, although none of these states are thought to be significant to the 'normal' biology. Giant forms, which are the most commonly alternative form found, are thought to represent degenerate parasites (Fari *et al.* 1986).

Trichomonads feed mainly by pinocytosis although they are capable of ingesting bacteria by phagocytosis (Francioli *et al.* 1983 ; Juliano *et al.* 1991). Lehker *et al.* (1990) have recently found evidence of specific erythrocyte binding and demonstrated the ability of live parasites to internalise erythrocytes. This could provide the parasites with an additional nutrient source. *In vitro* assays showed that erythrocyte lipids and haemoglobin iron supported trichomonal growth. The ingestion of particulate food is, however, not essential as the organisms can be grown in liquid media.

Table 1. A classification of some important parasitic protozoa adapted from Cox (1987).

SUBKINGDOM	**Protozoa** : single-celled eukaryotic organisms
PHYLUM	**Sarcomastigophora** : Locomotion by flagella, pseudopodia or both
SUBPHYLUM	**Mastigophora** : Locomotion by flagella
CLASS	**Zoomastigophorea**
ORDER	**Trichomonadida** : 4 to 6 flagella
	Tritrichomonas, Trichomonas
ORDER	**Kinetoplastida** : kinetoplast present
	Leishmania, Trypanosoma
SUBPHYLUM	**Sarcodina** : Locomotion by pseudopodia
CLASS	**Lobosea**
ORDER	**Amoebida** : No flagellated stage in life cycle
	Entamoeba, Acanthamoeba
PHYLUM	**Apicomplexa** : Life cycles include feeding stages (trophozoites), asexual multiplication(merogony), sexual stages (gametogony) and formation of spores or sporozoites(sporogany).
CLASS	**Sporozoea** : Infective stages sporozoites resulting from sporogony
SUBCLASS	**Coccidia**
ORDER	**Eucoccidiida** : merogony present
	Toxoplasma, Plasmodium
SUBCLASS	**Piroplasmia**
ORDER	**Piroplasmida** : transmitted by ticks
	Babesia, Theileria

Table 2. The classification of trichomonads adapted from Cox (1987).



Although trichomonad species differ in shape, size, the number of flagella, and in the morphology of subcellular structures (table 3), they have many features in common. The structure of a typical trichomonad cell is shown in figure 1. The most striking features are the flagella, one of which is recurrent and is closely associated with the undulating membrane. Not including the undulating flagella, *Trichomonas vaginalis* has four free flagella while *Tritrichomonas foetus* , as the name suggests, has three. The flagella control the movement of the cell. Extending underneath the undulating membrane, within the cytoplasm, is an organelle called the costa. The presence of this organelle distinguishes the Trichomonadidae from other families in this order. Previously it was assumed that the costa simply acted as a mechanical support but in recent studies it has been found to be motile and may therefore have a more sophisticated function. The axostyle, another prominent feature of these cells, protrudes posterially from the cell with its anterior, middle and posterior parts designated as the capitulum, trunk and caudle tip, respectively. The axostyle and the pelta both consist of sheets of microtubules and form the microtubular structures of the cell. The pelta is a cresent shaped sheet which extends apically, surrounding the walls of the periflagellar channel, and gives support to this channel from which the flagella arise. The prominent nucleus is situated towards the anterior end of the organism between the parabasal body (Golgi) and the capitilum of the axostyle. Rough endoplasmic reticulum surrounds the nucleus. Membrane bound micro-body organelles called hydrogenosomes accumulate in the perinuclear, axostylar and subcostal areas. A detailed description of the trichomonad structure is given in Kulda *et al.* (1986). No morphologically identifiable mitrochondria 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).

TABLE 3. Characteristics of the live trichomonads observed by phase contrast microscopy.

species	shape	size length x width (μm)	locomotion*	'protruding' axostyle	undulating membrane	miscellaneous
Trichomonas vaginalis	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	
Tritrichomonas foetus	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 porterior flagellum	large bright vesicles usually visible at the posterior of the body
Tritrichomonas augusta	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 porterior flagellum	form pseudocysts which occasionally display pseudopodia

* In all cases, whip like action of flagella, wave like movements of undulating membrane and rotation of the cells on their axis can be seen. Table adapted from Thong (1986).

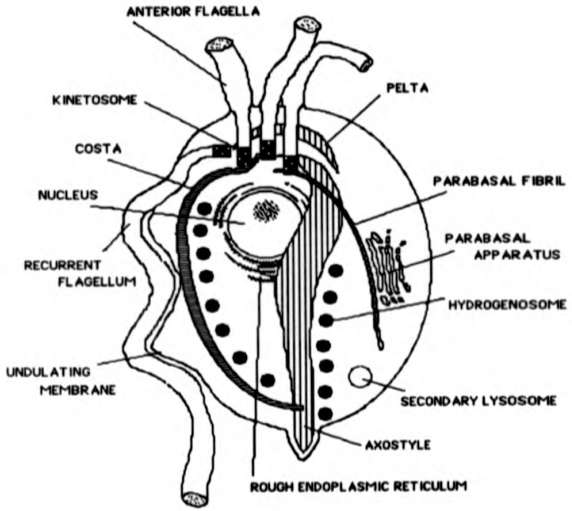


Figure 1. Structure of the trichomonad cell. Information from Kulda (1986) and Honigberg & Brugerolle (1989).

The presence of a virus encoded double-stranded ribonucleic acid in some isolates of *Trichomonas vaginalis* has been reported. It consists of a single linear dsRNA with an estimated size of 5.5 kb and is associated with a major protein of 85 kDa (Wang *et al.* 1987). Flegr *et al.* (1986) reported that the main portion of the dsRNA was found in the large granule fraction of a cell homogenate. More recently the presence of this RNA virus was confirmed in *Trichomonas vaginalis* by Patterson (1990) who also reported a virus, associated with the nuclear fraction, from *Giardia lamblia*, another anaerobic protozoan parasite. Work by Pindak *et al.* (1989) has suggested the possibility that the pathogenic significance of these organisms may be broadened by their potential for viral transmission.

1.4 Pathogenic trichomonads

There are different species of trichomonads which infect the mouth, large intestine, or the urogenital system of a number of vertebrates, most frequently however, they are reported in mammals and birds. As far as it is known very few trichomonad species are pathogenic and those that are generally inhabit the urogenital tract. An exception to this rule is *Tritrichomonas mobilensis* which was discovered in the intestine of the squirrel monkey and is of interest because it is invasive in its natural host as well as in experimental animals (Culberson *et al.* 1988). All species of pathogenic trichomonads contain a spectrum of strains, from very virulent to avirulent, with various levels of intermediate virulence potential. The symptoms of trichomoniasis, which range from mild to severe, may also be due to individual host susceptibility especially where epidemiologically linked hosts are most likely to be infected with the same strain of trichomonad

1.4.1 Pathogenic trichomonads of primates

There are five trichomonad species which have been found in man although only *Trichomonas vaginalis* isolates have been characterised as pathogenic strains. *Trichomonas tenax* has been reported from a variety of sites in, or closely connected with, the oral cavity (Honigberg 1978b) and has been implicated in disease symptoms of the respiratory tract although there is no conclusive proof of its involvement (a more detailed discussion can be found in Honigberg 1989b).

The first clinical description of *Trichomonas vaginalis* was published in 1836 when Donne (1836) identified the protozoan in unstained preparations of vaginal discharge mixed with saline. This parasite causes urogenital trichomoniasis in men, women and children. It has a worldwide distribution and is transmitted sexually by direct genital contact. This disease accounts for about a third of vaginal infections diagnosed in public clinics and it is estimated that approximately 180 million women are infected worldwide. The disease has three stages known as the acute, chronic and latent phases. Symptoms which are associated with vaginal trichomoniasis are yellow discharge, abnormal vaginal odour, vulvar itching, purulent vaginal discharge and vaginal and vulvar inflammation (Wolner-Hanssen *et al.* 1989). In women *Trichomonas vaginalis* principally infects squamous epithelium in the vagina (Nielson & Nielson 1975) although the organisms have been recovered from the urethra and Skene's glands in 90% of infected women (Wallin *et al.* 1981). This extravaginal infection may explain low cure rates in some treatments which may only eradicate organisms from the

vagina. About 9% to 56% of trichomonal infections are asymptomatic in various population groups (Fouts & Kraus 1980). In addition to symptoms varying within populations, symptoms or phases of trichomoniasis may vary over time in the same women (Rein 1989). Symptoms of the disease have been reported by Brown (1972) either to appear or to be exacerbated during or immediately after a menstrual period.

Trichomonas vaginalis infection in men is associated with poorly defined clinical symptoms and uncertain morbidity, although most men have symptoms and signs typical of nongonococcal urethritis which may be associated with considerable morbidity (Krieger 1981 ; McLellan *et al.* 1982). Significant questions remain concerning possible spontaneous resolution of infection and the long term consequences of chronic infection by this parasite in the male.

Trichomonas vaginalis infections also occur in children, including newborn infants on which a detailed report was published by Kurnatowska and Komorowska (1989). Infection in newborns occurs during delivery and young children may acquire the infection indirectly, depending on the family environment, or directly in girls who become sexually active. Girls who have not reached puberty are not so susceptible to infection owing to the inactivity of the genital organs which causes the vagina to have a pH of 7 - 8 rather than 4 - 6.5 as seen in adults and infants.

Because of the extensive variation in symptoms found, diagnosis of trichomoniasis can not be made with certainty on clinical grounds alone and laboratory investigations are essential. Although cultivation is the most sensitive method for this, it can take several weeks and this delays the

appropriate treatment. The most widely accepted routine procedure for making an immediate diagnosis is direct microscopic identification of the parasite in exudate diluted with isotonic saline. Many other diagnostic methods have been investigated ; these involve staining of fixed smears (Cree 1968 ; Thin *et al.* 1975 ; Rodriguez-Martinez 1973 ; Levett 1980) and immunocytochemical procedures (Krieger *et al.* 1985b ; Bennett *et al.* 1980). The detection of specific antibodies in sera (Kuberski 1978 ; Satapathy *et al.* 1988) and secretions (Street *et al.* 1982) has been examined although it should be noted that Corbeil *et al.* (1991) demonstrated that bovine non-specific immunoglobulins bind to *Tritrichomonas foetus* which resulted in false positives. Direct immunofluoresence with monoclonal antibodies (Krieger *et al.* 1988), and most recently a PCR-based diagnostic test, have also been developed (Riley *et al.* 1992).

The discovery of azomycin (Despois *et al.* 1956) and the subsequent synthesis of the 5'-nitromidazole, metronidazole (Cosar & Julou 1959) heralded a new era in the treatment of trichomoniasis. This compound was marketed under the trade name of Flagyl and was 90 - 95 % effective in the systematic treatment of trichomoniasis. Although other derivatives including tinidazole (Bloch & Smyth 1985), ornidazole (Valent *et al.* 1985) and nimorazole (Zurbek & Szymanski 1986) have been successfully used, metronidazole remains the most frequently prescribed and has become the standard treatment for *Trichomonas vaginalis* infections.

Metronidazole enters the trichomonad by a passive diffusion process (Muller 1981). Inside the cell, its nitro group is reduced to a reactive cytotoxic intermediate (to be discussed later) that is thought to react with DNA interrupting nucleic acid synthesis which ultimately results in the death of the

organism (Ings *et al.* 1974 ; Edwards 1977 ; LaRusso *et al.* 1977). It has been reported by Muller & Lindmark (1976) that oxygen inhibits metronidazole uptake by *Trichomonas vaginalis* suggesting that the redox conditions of the drug and the O_2 concentration of the organisms environment may be contributing factors to the efficacy of the drug.

The most common side effects of metronidazole treatment are nausea, emesis and orexia and they are all dose related (Pereyra *et al.* 1980). Although there are reports of the drug having carcinogenic effects (Rustica & Shubik 1972), after over 20 years of widespread use, there is no indication that the standard metronidazole treatment for trichomoniasis is unsafe for humans.

Resistance to metronidazole has now been reported worldwide (Meingassner & Thurner 1979 ; Forsgren & Forsmann 1979 ; Heyworth *et al* 1980 ; Muller *et al.* 1980 ; Kulda *et al.* 1982). Fortunately the resistance is relative rather than absolute and can sometimes be overcome using larger drug doses. Another treatment called SolcoTrichovac, marketed by Solco Basle Ltd in Switzerland, is produced from killed variants of *Lactobacillus acidopholus* isolated from the vaginal secretions of patients with trichomoniasis. Although it is claimed to be highly successful, certain statistics show cure rates as low as 34 % (Hatala *et al.* 1986). Clearly there is still a real need to increase research in order to find alternatives to the nitromidazoles for the treatment of trichomoniasis.

1.4.2 Pathogenic trichomonads of ungulates

Tritrichomonas foetus is an important pathogen of cattle. It has a worldwide

distribution, although its prevalence has now decreased dramatically in areas where artificial insemination is widely practised. However, infection with this parasite results in substantial economic losses throughout those major cattle-rearing areas of the world, such as California, where natural breeding is relied on (BonDurant *et al.* 1990).

The parasite is transmitted venereally and inhabits the reproductive tract. In bulls the infection causes a preputial discharge associated with small nodules on the preputial and penile membranes. Bulls, once infected, remain so permanently, and after the infection is established there are no clinical signs. Transmission to the cow occurs during coitus and from the vagina, the parasite reaches the uterus via the cervix to produce an inflammation of the womb (endometritis). Intermittently, organisms are flushed into the vagina, often two or three days before oestrus. In pregnant cows the infection produces early foetal death which is usually first recognised as an infertility problem. Abortion before the fourth month of pregnancy is the commonest sequel to infection and this is normally followed by recovery. Because of the small size of the foetus the abortions often go undetected and therefore a clinical sign of this disease may simply be one of an irregular oestrus cycle. Occasionally the developing foetal membranes are retained leading to a purulent endometritis and an persistent uterine discharge. Infrequently the glandular tissue of the ovary (corpus luteum) is retained and the cervical seal remains closed, and a massive inflammation of the uterus develops which visually simulates the appearance of pregnancy. The result of purulent endometritis, or a closed pyometra, can lead to the cow becoming permanently sterile (Yule *et al.* 1989). Since the disease is self limiting in the female only symptomatic treatment and sexual rest for three months is normally necessary. For the bull, slaughter is the best policy, although

dimetridazole administered orally or intravenously has been reported to be effective. Clark *et al.* (1984) have, however, found that subcutaneous injections of killed *Tritrichomonas foetus* cells can prevent and eliminate genital infection in most bulls.

Tritrichomonas suis is found in the nasal cavity and digestive tract of swine throughout the world. Pathogenicity of this trichomonad has only been correlated with the strains inhabiting the nasal cavity in pigs where it has been suggested that they cause atrophic rhinitis (Honigberg 1978a). This species has been demonstrated to be similar morphologically (Hibler *et al* 1960), physiologically (Doran 1957) and immunologically (De Carli & Guerrero 1975) to *Tritrichomonas foetus*.

1.4.3 Pathogenic trichomonads of birds

There are two known pathogenic species in birds. *Trichomonas gallinae* is found in the upper digestive tract, primarily in the domestic pigeon. Turkeys and chickens have also been known to become infected. Some strains have the capacity to invade the viscera and the central nervous system of their avian hosts. Symptoms of this disease are found as yellow, necrotic lesions in the mouth, oesophagus and crop of pigeons, and the condition is frequently fatal. Infection is acquired via regurgitated crop contents from adult birds, which, although immune, remain carriers. Dimetridazole is recommended for treatment while control depends on preventing access of wild pigeons to drinking water (Honigberg 1978a).

Tetratrichomonas gallinarum is found worldwide in gallinaceous birds such as the chicken, turkey and guinea fowl where it inhabits the ceca and causes

enterohepititus-like symptoms. It has also been reported in liver lesions (Honigberg 1978a).

1.5 Biochemistry of trichomonads

Biochemical studies on trichomonads began when axenic cultures became available in the 1940s owing to the availability of suitable antibiotics. Trichomonads depend on a large number of preformed metabolites as nutrients, reflecting the absence of major biosynthetic pathways found in the majority of eukaryotic cells. There are significant biochemical differences among trichomonad groups, although detailed information has only been obtained for the two species, *Trichomonas vaginalis* and *Tritrichomonas foetus*, both of which will be discussed in this section. It must, however, be mentioned that firstly, as with all *in vitro* systems, it is not known to what extent the biochemistry of cultured cells differs from that of organisms growing within their hosts and, secondly, there have been no studies on freshly isolated cells.

There are six basic groups of nutrients required by trichomonads: (1) carbohydrates, primarily as an energy source, (2) fatty acids and cholesterol, (3) amino acids or a protein digest as a source of amino acids, (4) purines and pyrimidines as precursors for nucleotide and nucleic acid synthesis, (5) several vitamins and (6) several inorganic salts. Neither the rates nor mechanisms of uptake have been determined for most of these nutrients although it is believed that several transport processes do exist. As indicated earlier, trichomonads are able to phagocytose larger particles such as bacteria, which could provide many of the required nutrients. This process is

obviously not important for organisms multiplying in liquid media and its contribution to the nutrition of the trichomonad in the host remains to be investigated.

1.5.1 Carbohydrate metabolism

Carbohydrate metabolism is the main source of energy production. For *T. vaginalis* maltose, glucose and galactose are utilized most readily (Read 1957). Glycogen and starch can also support growth if added to the medium. Maltose is, however, most commonly used to support the growth of trichomonads in axenic culture. Sucrose and mannose are not utilized. The major nutrient reserve of *Trichomonas vaginalis* is glycogen, which represents up to 20 % of the dry weight of the cell (Michaels & Treick 1962).

Trichomonads possess a glycolytic pathway which is similar to that found in most prokaryotes and eukaryotes. All the enzymes involved in this pathway have been detected in *T. vaginalis*, although very few of the enzymes have been purified or characterised in detail. No unusual properties have yet been found except for an inorganic pyrophosphate (PPi)-linked phosphofructokinase in *Trichomonas vaginalis* and *Tritrichomonas foetus* (Mertens *et al.* 1989). In aerobic eukaryotes this enzyme is ATP-linked. The replacement of ATP by PPi as the phosphoryl donor in this enzymatic step renders the reaction freely reversible. PPi is a 'waste product' of biosynthetic reactions and the energy of the pyrophosphate bond is usually lost by hydrolysis. PPi-linked phosphofructokinase salvages the energy of the pyrophosphate bond and decreases the input of ATP needed for glycolysis, thus it increases the ATP yield (Muller 1991). Hence the use of PPi as a phosphoryl donor is likely to benefit an organism which obtains its ATP

primarily from glycolysis. A detailed discussion of the glycolytic enzymes can be found in Muller (1989).

In *Trichomonas vaginalis* glucose or glycogen is converted to glycerol, lactate, acetate, malate and CO_2 and, under low oxygen concentration conditions, H_2 is also formed (Mack & Muller 1980 ; Chapman *et al.* 1985a). In *Tritrichomonas foetus* carbohydrate metabolism results in formation of glycerol, ethanol, acetate, succinate and CO_2 (Ryley 1955 ; Chapman *et al.* 1985a). H_2 is again formed under low oxygen concentrations. The pathways leading to these end products are shown in figures 2 and 3 for *Trichomonas vaginalis* and *Tritrichomonas foetus* respectively. A large number of minor products have also been detected including traces of butyrate, propionate and isovalerate (Saeki *et al.* 1984) and various gases including methane, ethane, propane, ethylene, carbon monoxide and carbon dioxide together with other unidentified gases (Ishiguro 1985).

As can be seen from figures 2 and 3 some phosphoenolpyruvate is carboxylated to oxaloacetate in both species but it can only be reduced further to succinate in *Tritrichomonas foetus*. The remainder of the phosphoenolpyruvate is converted to pyruvate which enters the hydrogenosomes. In *Trichomonas vaginalis* some of the pyruvate is further reduced to lactate although the production of lactate seems not to be correlated with that of the other end products. Glycerol, acetate and H_2 are produced in nearly equimolar amounts (Steinbuchel & Muller 1986b). The production of lactate helps maintain the redox balance : NAD reduction is coupled to the oxidation of glyceraldehyde-3-phosphate and the reoxidation of NADH is coupled to pyruvate reduction. The redox balance is maintained

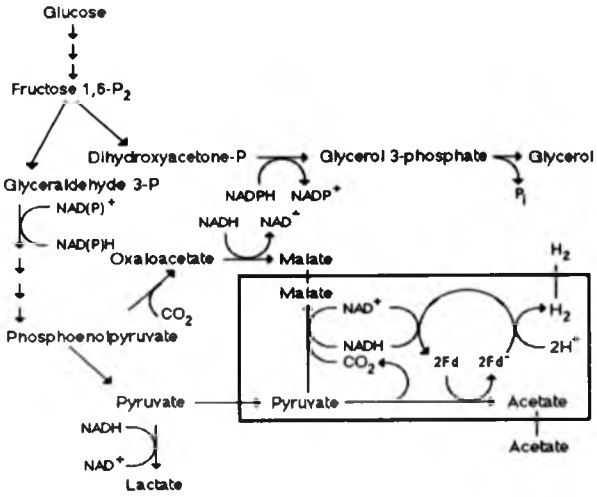


Figure 2. Metabolic map for *Trichomonas vaginalis*. Hydrogenosomal reactions involved in the formation of acetate from acetyl CoA are simplified. Arrows indicate the assumed physiological direction *in vivo*. The direction of flow is not indicated where uncertain. Figure obtained from Steinbuchel & Muller (1986a & b).

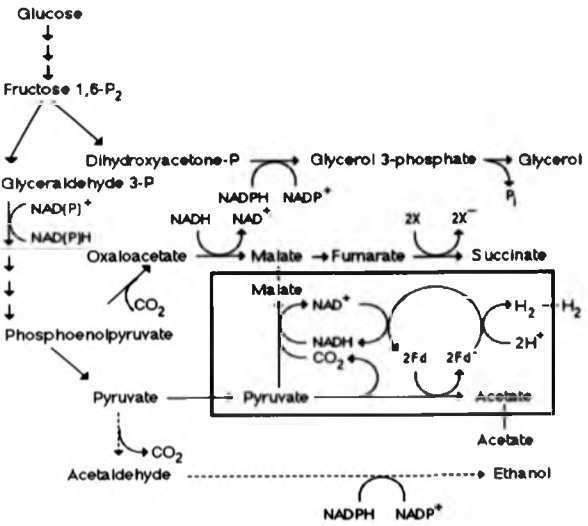


Figure 3. Metabolic map for *Tritrichomonas foetus*. Hydrogenosomal reactions involved in the formation of acetate from acetyl CoA are simplified. Arrows indicate the assumed physiological direction *in vivo*. The direction of flow is not indicated where uncertain. Figure obtained from Steinbuchel & Muller (1986a & b).

independently in the two major compartments of the cell, the cytosol and the hydrogenosomes, and no flux of reducing agents appears to occur across the hydrogenosomal membrane. It can also be inferred from the known data that, although the enzymes potentially involved in the carboxylation of C_3 compounds are present, reactions mediated by such enzymes play a subordinate role in *Trichomonas vaginalis*. This is indicated by the low level of malate production (Mack & Muller 1980 ; Steinbuchel & Muller 1986a) and the absence of secretion of succinate. This is perhaps in contrast to the situation in *Tritrichomonas foetus*, in which succinate is a major end product (Ryley 1955).

Trichomonads appear to be neither strict nor aerotolerant anaerobes, but are microaerophiles. They have a lifestyle that is tuned to the O_2 concentration fluctuations of their habitat. *Trichomonas vaginalis* can be cultured in the presence of a vast excess of O_2, although growth rates decrease by comparison with those achieved anaerobically (Mack & Muller 1978). Optimal growth conditions for *Trichomonas vaginalis* were found to involve high concentrations of CO_2 with only traces of O_2. However, respiration (reduction of O_2) is a significant feature of trichomonads under aerobic conditions (Muller 1976). The concentration of CO_2 and the presence of oxygen have certain effects on the formation of end products of both *Trichomonas vaginalis* and *Tritrichomonas foetus*. In general high CO_2 favours biosynthetic carbon flow and the production of glycerol and lactate by cytosolic pathways whereas low CO_2 favours those pathways in the hydrogenosomes which result in acetate and H_2 formation (Paget & Lloyd 1990). For *Trichomonas vaginalis* some data indicate that aerobic conditions might stimulate glycolysis and lactate formation (Mack & Muller 1980) although other studies have shown that the total rate of acidic end product

formation and the proportions of lactate and acetate formed are not significantly different under aerobic and anaerobic conditions (Muller & Gorrell 1983) and that there is no change in glycerol formation (Steinbuchel & Muller 1986b). In *Tritrichomonas foetus* , oxygen decreases the production of succinate but stimulates the production of acetate (Muller 1976). The decrease in succinate formation may be due to the diversion of reducing equivalents to oxygen (the electron acceptor for cytoplasmic NADH oxidase) which results in less oxaloacetate reduction. The production of H_2 is inhibited in both species in the presence of oxygen probably because oxygen scavenges electrons from reduced ferredoxin. In *Trichomonas vaginalis* the enzymes or enzyme systems capable of transferring electrons to O_2 are present both in the cytosol and within the hydrogenosome. The cytosol contains two oxidases which have been characterised (Linstead & Bradley 1988) and shown to use NADH and NADPH as substrates. The physiological role of the two oxidases is not established, but they may be involved in lowering the intracellular pO_2, thus providing a mechanism for oxygen tolerance. The nature of the hydrogenosomal terminal oxidase is not known.

The presence of O_2 can result in reactive oxygen species which are toxic to all cells and although defensive mechanisms against the toxic products of O_2 reduction are not known, superoxide dismutase, a ubiquitous enzyme which is important in protecting against activated oxygen, has been described in both *Trichomonas vaginalis* and *Tritrichomonas foetus* (Lindmark & Muller 1974 ; Muller 1989). The majority of the enzyme activity is found in the cytosol although 15% is contained in the hydrogenosomes. Catalase has also been detected in *Tritrichomonas foetus* (Muller 1973) but not in *Trichomonas vaginalis* (Ninomiya & Suzuoki 1952 ; Ryley 1955).

1.5.2 The hydrogenosome

An important feature of trichomonads is that they contain no mitrochondria but instead have unique organelles called hydrogenosomes. The main function of these organelles is pyruvate metabolism which yields energy for the cell. Hydrogenosomes are predominantly spherical organelles with a diameter of approximately 0.5 µm and, in trichomonads, they occupy about 6% of the total cell volume (Neilsen & Diemer 1976). They frequently have an electron dense core, a granular matrix and are bound by two closely opposed unit membranes (Honigberg *et al.* 1984).

These organelles were first described by Lindmark and Muller (1973) in *Tritrichomonas foetus* but have now also been demonstrated in *Trichomonas vaginalis* (Lindmark *et al.* 1975) and also in a number of rumen ciliates (Yarlett *et al.* 1983 ; Snyers *et al.* 1982), free living ciliates (Van Bruggen *et al.* 1984) and an anaerobic fungus of the sheep rumen, *Neocallimastix patriciarum* (Yarlett *et al.* 1986). The evolutionary origin of the hydrogenosome is unknown but the variety of the organisms in which they have now been detected, might argue against a single evolutionary line.

The main metabolic function of the hydrogenosome is the oxidative decarboxylation of pyruvate with acetate, malate, CO_2 and H_2, as the end products, and the phosphorylation of ADP to ATP. The metabolic reactions of the hydrogenosome are shown in figure 4. The reducing equivalents generated under anaerobic conditions in the hydrogenosomes are removed as molecular hydrogen. Under aerobic conditions, hydrogenosomes can use molecular oxygen as a terminal electron acceptor. In *Tritrichomonas foetus* hydrogenosomal respiration is not inhibited by cyanide or rotenone, both of

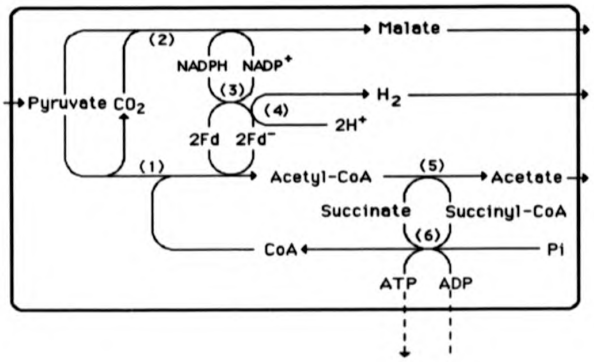


Figure 4. Map of the hydrogenosomal metabolism of *Tritrichomonas foetus* and *Trichomonas vaginalis*. Key to the enzymes : (1) pyruvate:ferredoxin oxidoreductase (2) malate dehydrogenase (decarboxylating) (NAD) (3) NAD^+ ferredoxin oxidoreductase (4) hydrogenase (5) acetate:succinate CoA transferase (6) succinate thiokinase. Arrows indicate the assumed physiological direction *in vivo*. Information obtained from Steinbuchel & Muller (1986).

which are standard inhibitors of mitrochondrial respiration, although the nature of the hydrogenosomal terminal oxidase is not known (Cerkasov *et al.* 1978).

Iron-sulphur proteins are thought to play a major role in the hydrogenosome. Ferredoxin containing a [2Fe-2S] cluster has been shown to be present in *Tritrichomonas foetus* (Marczak *et al.* 1983) and *Trichomonas vaginalis* (Gorrell *et al.* 1984). This protein is the major electron transport component of hydrogenosomes and links pyruvate:ferredoxin oxidoreductase to hydrogenase (Steinbuchel & Muller 1986a). More recently Johnson *et al.* (1990) determined the primary structure of [2Fe-2S] ferredoxin from *Trichomonas vaginalis*. They found the protein to be composed of 93 amino acids and when compared with over 80 other ferredoxins it showed most similarity to [2Fe-2S] putidaredoxin of the aerobic bacterium *Pseudomonas putida* and not the ferredoxins from other hydrogen-producing bacteria. Another hydrogenosomal enzyme succinate thiokinase (STK) was detected in *Tritrichomonas foetus* by Lindmark (1976). In this organism STK was proposed as a key step in the conservation of the energy liberated by the oxidative decarboxylation of pyruvate in the hydrogenosome. However, more recently STK has also been found to be present in *Trichomonas vaginalis* and surprisingly the molecular size of the enzyme was found to differ in the two trichomonad species. A more typical 'small eukaryotic' enzyme (apparent M_r of 75 000) was found in *Tritrichomonas foetus* whereas *Trichomonas vaginalis* contained a larger enzyme (apparent M_r of 150 000) previously found only in Gram-negative bacteria (Jenkins *et al.* 1991). Further work is required to investigate the reason for the occurrence of this anomalous large STK in *T. vaginalis*.

Hydrogenosomal enzyme systems are thought to be involved in the activation of 5'-nitroimidazole derivatives, which are used in the treatment of trichomoniasis (section 1.4.1). Reductive activation of metronidazole occurs in the hydrogenosome where pyruvate:ferredoxin oxidoreductase is the major source of reduced ferredoxin, the electron donor for the reduction of metronidazole and its metabolites (Chapman *et al.* 1985b ; Yarlett *et al.* 1985). It has also been shown that strains of *Trichomonas vaginalis* and *Tritrichomonas foetus* in which a loss of pyruvate:ferredoxin oxidoreductase activity has been induced are no longer susceptible to 5'-nitromidazoles (Cerkasovova *et al.* 1984 ; Cerkasovova *et al.* 1986). Trichomonads with active hydrogenosomes have lowered susceptibility to metronidazole when tests are performed under aerobic conditions. This indicates that there is inhibition by O_2 of reductive drug activation (Muller *et al.* 1980 ; Lossick *et al.* 1986). The precise mechanism of this is not understood although oxygen is thought to play an important role in resistance to metronidazole during clinical *Trichomonas vaginalis* infections.

1.5.3 Lipid metabolism.

De novo biosynthesis of fatty acids or cholesterol does not occur in *Trichomonas vaginalis* (Holtz *et al.* 1985) or *Tritrichomonas foetus* (Beach *et al.* 1990). It is thought that the parasites are unable to carry out any of the oxygen dependent steps of lipid biosynthesis and degradation and cannot convert or retro-convert long chain fatty acids or cholersterol (Lindmark 1983). All the evidence indicates that these components are incorporated unchanged into neutral lipids, phospholipids and other derivatives. The lipid composition of *Trichomonas vaginalis* is as follows. The major fatty acids are palmitic, stearic, oleic, linoleic (50% of the total) and

α-linolenic. Cholesterol is the only sterol detected and the major lipids are 30% neutral lipids, 65% phospholipids and 5% glycolipids (Holtz *et al.* 1985). Holtz *et al.* (1985) reported that trichomonads can convert saturated fatty acids to fatty alcohols, perhaps by a reductase dehyrogenase mechanism, and that the alcohols act as precursors in the synthesis of glycerol ether lipids. More recently Beach *et al.* (1990) have also shown that the trichomonads are able to incorporate cholesteryl esters, to hydrolyse them, and use the resulting fatty acids for phospholipid acylations.

1.5.4 Polyamine metabolism

Polyamines are ubiquitous components of eukaryote and most prokaryote cells and are required for optimal cell multiplication and differentiation. They are biologically active low molecular weight oligoamines which include putrescine, spermidine and spermine. Steps in polyamine synthesis differ between many parasites and their hosts and hence selective inhibition of polyamine metabolism may be an important target for chemotherapy. The predominant polyamine of trichomonads is putrescine although spermidine and spermine are also present. The relative proportions of these polyamines set trichomonads apart from most other eukaryotes where both spermidine and spermine are present, but with only trace amounts of putrescine (Yarlett 1988). *Entamoeba hystolytica*, another amitrochondrial, anaerobic protozoan also contains putrescine as the major polyamine (Gillin *et al.* 1984) although whether this is a characterstic of all anaerobic protozoans has not yet been fully investigated.

Polyamine biosynthesis in *Trichomonas vaginalis* originates from arginine

via the three enzymes of the dihydrolase pathway, arginine deiminase, catabolic ornithine carbamoyl transferase and ornithine decarboxylase, which act to convert arginine to putrescine. Ornithine decarboxylase, the enzyme which produces putrescine from ornithine, and S-adenosyl-L-methionine (SAM) decarboxylase, which provides aminopropyl groups for the conversion of putrescine to spermidine and spermine, have both been shown to be present in *Trichomonas vaginalis* (White *et al.* 1983 ; North *et al.* 1986). SAM decarboxylase activity could not, however, be detected in *Tritrichomonas augusta* and was only present in trace amounts in *Tritrichomonas foetus* (North *et al.* 1986).

1.5.5 Amino acid metabolism

Trichomonads require an exogenous source of amino acids for protein synthesis. In the absence of free amino acids, host proteins are thought to provide the parasites with their amino acid requirements *in vivo* although the mechanism of uptake is not known. *Trichomonas vaginalis* rapidly consumes arginine, methionine, leucine and threonine from its enviroment and catabolizes them through energy-yielding pathways (Lockwood & Coombs 1989).

Aminotransferases have been described. One enzyme catalyzes the transamination of ornithine and lysine, a second enzyme functions as a branched chain amino acid transferase, the third is an unstable alanine aminotransferase and the fourth an aspartate:α-ketoglutarate aminotransferase. Unlike the mammalian enzyme this fourth parasite enzyme can utilize aromatic amino acids as amino donors or phenylpyruvate

as an acceptor in addition to using aspartate and α-ketoglutarate (Lowe & Rowe 1985 ; Lowe & Rowe 1986).

Glutamate dehydrogenase, which generally functions with aminotransferases in transferring amino groups between keto and amino acids, was isolated from *Trichomonas vaginalis* (Turner & Lushbaugh 1988). The amination reaction of glutamate dehydrogenase is probably an insignificant contributor to the parasites supply of NADP, and this is probably satisfied by NADPH-oxidase which is present at high activity in the cytosol. However, in the anaerobic protozoan *Giardia lamblia*, which has both glutamate dehydrogenase and alanine aminotransferase activity, it is suggested that these enzymes cooperate to convert pyruvate to alanine since glutamate is not seen as an end product (Paget *et al.* 1990).

Arginine is broken down by the dihydrolase pathway, which consists of three enzymes, in *Trichomonas vaginalis* (see polyamine metabolism). Ornithine, a product of this pathway, is further converted to putrescine by an active ornithine decarboxylase. This dihydrolase pathway is unusual in a eukaryote, although relatively common in both aerobic and anaerobic bacteria (Lockwood & Coombs 1991).

The sulphur containing amino acid L-methionine is a precursor for the biosynthesis of S-adenosylmethionine (SAM). This compound is involved in the synthesis of polyamines and transmethylation reactions. In *Trichomonas vaginalis* L-methionine is rapidly catabolized to volatile thiols, including methanethiol (Thong *et al.* 1987). The enzyme that catalyses this reaction is methionine γ-lyase which is present at high activity in *Trichomonas vaginalis*.

The same enzyme is also responsible for the homocysteine desulphurase activity that catalyses the breakdown of homocysteine to hydrogen sulphide, α-ketobutyrate and ammonia (Lockwood & Coombs 1989). The presence of this enzyme in *Trichomonas vaginalis* is particularly interesting since it apparently does not occur in any other species of trichomonad or in a range of other protozoan parasites. It is, however, present in *Entamoeba hystolytica* and a number of rumen ciliates, albeit at much lower levels than in *Trichomonas vaginalis* (Lockwood & Coombs 1991).

Unlike *Tritrichomonas foetus*, *Trichomonas vaginalis* has been shown to incorporate exogenous labelled L-methionine into intracellular SAM (Thong *et al.* 1987) and subsequently into a range of macromolecules. This and the presence of methionine γ-lyase accounts for the high rate of consumption of methionine by *T. vaginalis* and may also explain the large difference in consumption between this species of trichomonad and *Tritrichomonas foetus*. Another thiol producing enzyme that is common to all anaerobic protozoa is serine sulphydrase, which catalyses the reversible interconversion of serine and cysteine (Thong & Coombs 1985).

1.5.6 Nucleic acid metabolism.

All parasitic protozoa studied to date lack *de novo* purine nucleotide synthesis and hence are dependent on purine salvage for survival (Berens *et al* 1981 ; Wang & Aldritt 1983 ; Lo & Wang 1985). *Trichomonas vaginalis* and *Tritrichomonas foetus*, as well as two other anaerobes *Giardia lamblia* and *Entamoeba histioytica*, have been shown to be incapable of *de novo* synthesis of both purine or pyrimidine nucleotides (Heyworth *et al.* 1982 ;

Wang *et al.* 1983a ; Wang *et al.* 1983b ; Wang & Cheng 1984). Both the trichomonads lack the enzymes dihydrofolate reductase and thymidylate synthetase and each has unique salvage pathways for nucleosides or bases of purine and pyrimidine.

In the purine salvage pathway *Trichomonas vaginalis* converts guanosine and adenosine to their respective monophosphates through the action of kinases (Miller & Linstead 1983). Although this trichomonad cannot interconvert the two mononucleotides it appears that nucleotide interconversions do occur in *Tritrichomonas foetus* which generates nucleotides by salvaging purine bases with phosphoribosyl transferases (Wang *et al.* 1983a).

Several enzymes of the pyrimidine salvage pathway have been identified in trichomonads. *Trichomonas vaginalis* salvages thymidine by the enzyme thymidine phosphotransferase which generates thymidine monophosphate. Cytidine and uridine are thought to be salvaged in a similar manner to thymidine, despite *Trichomonas vaginalis* containing very high and as yet unexplained activities of cytidine deaminase and uridine phosphorylase. *Tritrichomonas foetus* has been shown to use these enzymes to convert both cytidine and uridine to uracil, via uracil phosphoribosyl transferase. *Tritrichomonas foetus* contains ribonucleotide reductase which is not present in *Trichomonas vaginalis*. *Trichomonas vaginalis* obtains the required deoxyribonucleotides directly from deoxyribonucleosides through the action of deoxyribonucleoside phosphotransferase. The presence of some unique and essential enzymes in trichomonad nucleic acid metabolism may indicate potential targets for antiparasitic drug development.

1.6 Hydrolases of trichomonads

Clearly there is an interest in any factors involved in the host-parasite relationship of trichomonads which may affect pathogenesis. Any enzymes which are released could interact with host cells or other host molecules and may be vital to the establishment of the parasite. Hydrolases are continually released in large amounts by trichomonads and hence a full understanding of this process is essential. A summary of the hydrolases which have been studied is given in table 4.

Watkins (1953) conducted one of the first investigations into the hydrolases of trichomonads when she reported enzymes from *Tritrichomonas foetus* which degraded blood group substances. Since these initial investigations a number of authors have published the results of studies identifying and characterising the hydrolases of trichomonads.

1.6.1 Glycosidases

The hydrolytic enzymes were first identified in studies on human blood group degrading substances. Work by Watkins (1956) on the human B substance degrading enzyme showed that galactose was the sugar liberated in the greatest amount by the B enzyme but that small quantities of fucose and N-acetylhexosamine could also be detected. Tyler & Watkins (1960) characterised an enzyme from *Tritrichomonas foetus* which inactivated the human blood group H substances. After partial purification this enzyme had a slight capacity to hydrolyse low molecular weight ß-galactoside and ß-N-acetylglucosaminide substrates. The results of further investigations were

TABLE 4. Studies on the hydrolases of *Tritrichomonas foetus* and *Trichomonas vaginalis*.

Hydrolase	Activity reported in *Tritrichomonas foetus*	*Trichomonas vaginalis*	Reference
α-D-Galactosidase	•		Watkins (1959)
	•		Harrap & Watkins (1964)
			Yates *et al.* (1975)
ß-Galactosidase	•		Harrap & Watkins (1970)
α-L-Fucosidase		•	Tyler & Watkins (1960)
α-Mannosidase	•	•	Lockwood *et al.* (1988)
ß-Glucosidase	•		Watkins (1959)
	•	•	Lockwood *et al.* (1988)
Neuraminidase	•		Romanovska & Watkins (1963)
	•		Muller & Saathoff (1972)
	•		Muller *et al.* (1974)
	•		Crampen *et al.* (1979)
	•		Silva Filho *et al.* (1989)

β-N-Acetylhexosaminidase	•		Watkins (1959)
	•		Edwards *et al.* (1975)
	•	•	Lockwood *et al.* (1988)
	•	•	North *et al.* (1989)
Acid phosphatase		•	Sharma & Bourne (1963)
		•	Takeuchi *et al.* (1972)
	•	•	Lockwood *et al.* (1988)
	•	•	North *et al.* (1989)
Cysteine proteinases	•		McLaughlin & Muller (1979)
		•	Coombs & North (1983)
	•	•	Lockwood *et al.* (1984, 1985, 1986 & 1987)
		•	Bozner & Demes (1991a & b)
		•	Garber & Lemchuk-favel (1989)
	•	•	North *et al.* (1989 & 1990a & b)
		•	Neale & Alderete (1990)

found to be consistent with the view that the enzymes which destroy blood group specificity act by removing terminal non-reducing sugars. Since the first sugar to be removed by the H-enzyme preparation was fucose the H enzyme of *Tritrichomonas foetus* was characterised as a fucosidase. In 1964 Harrap & Watkins (1964) reported the tentative identification, from chromatographic work, that the A decomposing enzyme from *Tritrichomonas foetus* was an α-N-acetyl-D-galactosaminidase. Later Yates *et al.* (1975) isolated three linkage-specific α-D-galactosidases from this protozoan. Harrap & Watkins (1970) reported two ß-galactosidase activities in extracts of *Tritrichomonas foetus* which could be separated by gel filtration. Neuraminidase was reported to be present in large quantities in *Tritrichomonas foetus* (Muller & Saathoff 1972 ; Muller *et al.* 1974) and two forms were later purified by Crampen *et al.* (1979). The M_rs of the two neuraminidases were determined as 320 000 and 38 000. Edwards *et al.* (1975) found at least two separate ß-N-acetylhexosaminidase activities in *Tritrichomonas foetus* extracts. One form of the hydrolase was partially purified and was found to have an M_r of 150 000 and a pH optimum of 6.2. The hydrolase had a dual specificity for N-acetyl-glucosamine and N-acetyl-galactosamine.

1.6.2 Phosphatases

All the initial investigations of phosphatases in trichomonads were performed using *Trichomonas vaginalis* and there are no data on *Tritrichomonas foetus*. Sharma & Bourne (1963) studied the histochemical distribution of phosphatases from *Trichomonas vaginalis*. They found the parasite to contain strong acid phosphatase activity which was localised mainly in what

they called mitochondrion-like granules, probably lysosomes, and around the nucleus. In 1972 Takeuchi *et al* (1972) reported two kinds of acid phosphatase activities to be present in the sedimentable fractions of *Trichomonas vaginalis*.

1.6.3 Proteinases

Proteinase activity was first reported by Muller (1973) in a study of lysosomal hydrolases in *Tritrichomonas foetus* . In 1979 McLaughlin & Muller purified and partially characterised a low M_r cysteine proteinase from this parasite which they considered to be the single major proteinase present. Lockwood *et al.* (1984, 1987), however, showed that the proteinase activity was due to multiple cysteine proteinases.

An investigation of the proteinase activities of *Trichomonas vaginalis* was performed by Coombs & North (1983) using electrophoresis of polyacrylamide gels containing denatured haemoglobin. Seven bands of activity were detected which differed with respect to pH optima and relative sensitivities to inhibitors. All the activities were thought to be cysteine proteinases. Using this same electrophoresis technique and then using gelatin SDS-PAGE Lockwood *et al.* (1984 ; 1987) identified at least eleven different cysteine proteinases of *Trichomonas vaginalis*. However, an even greater complexity has now been demonstrated by two dimensional gelatin-SDS-PAGE which has resolved as many as 23 distinct proteinases in this organism (Neale & Alderete 1990). Bozner & Demes (1991a) have recently reported two high molecular weight enzymes which are inhibited by EDTA but not by cysteine proteinase inhibitors and are thus considered to be metalloproteinases.

Lockwood *et al.* (1987) compared the proteolytic activities of *Trichomonas vaginalis*, *Tritrichomonas foetus* and *Tritrichomonas augusta*. The proteinases of *Tritrichomonas foetus* with the highest activity towards gelatin were of low apparent molecular weight compared to the *Trichomonas vaginalis* proteinases and in general the gelatin SDS-PAGE proteinase pattern of *Tritrichomonas augusta* was less complex and the enzymes less active towards gelatin.

Further characterisation of purified proteinases from *Trichomonas vaginalis* was performed by Lockwood *et al.* (1988). Two intracellular cysteine proteinases were purified with apparent M_rs 18 000 and 64 000 by gel filtration. Two extracellular cysteine proteinases were also purified by Garber & Lemchuk-Favel (1989) with apparent M_rs of 60 000 and 30 000. Antibody raised against one of these enzymes was specific and did not recognise the second purified enzyme suggesting it is unlikely that proteinase multiplicity is the result only of differential modification of a single gene product. This was also suggested by differences in substrate specificty of the proteinases found in studies using fluorogenic substrates, specific inhibitors (North *et al.* 1990) and recent analysis of the genes (Mallinson & North personal communication).

1.6.4 Release of extracellular hydrolases

Lockwood *et al.* (1988) found that *Trichomonas vaginalis* and *Tritrichomonas foetus* contained, and released, high activities of a number of hydrolases including N-acetyl-ß-D-glucosaminidase (NAGase), acid phosphatase, mannosidase, ß-glucosidase and cysteine proteinases. These hydrolases were released continually during axenic growth *in vitro*. The

release of these hydrolytic enzymes represents a major activity of these parasites. The culture medium, during logarithmic growth, can contain as much as 50% of the total detectable activity in a culture for the majority of hydrolases. The three types of hydrolase, acid phosphatase, β-N-acetylglucosaminidase and proteinase appeared to be released in a similar fashion (North *et al.* 1989). The time courses of release were similar for all the enzymes, and the effects of lower temperature and inhibitors did not differ from enzyme to enzyme. Interestingly in the case of proteinases some of the extracellular enzyme forms were distinct from those detected in the cells apparently suggesting that the various forms are differentially released (Lockwood *et al.* 1987 ; North *et al.* 1990). The metalloproteinases reported by Bozner & Demes (1991b) in *Trichomonas tenax* were not found to be released although it is not yet known if these enzymes are lysosomal. The possibility that metalloproteinases are released, but are then inactivated, cannot, however, be ruled out at present.

Silva Filho et al (1989) have reported that supernatants taken from axenic cultures of *Trichomonas vaginalis* and *Tritrichomonas foetus* contain a neuraminidase activity which was found to be able to release sialic acid from the surface of erythrocytes. This hydrolase was detected in the supernatants by a fluorometric assay using 2'-(4-methylumbelliferyl)-α-D-N-acetyl neuraminic acid as the substrate.

1.6.5 Subcellular distribution of hydrolases

Investigations into the subcellular distribution of the hydrolases in *Tritrichomonas foetus* (Muller 1973), *Trichomonas vaginalis* (Lindmark *et*

al. 1975) and a related flagellate *Monocercomonas sp.* (Lindmark & Muller 1974) have shown these enzymes to be present in particulate fractions. Lockwood *et al.* (1988) have more recently reported that proteinases, ß-N-acetylglucosaminidase, α-mannosidase and ß-glucosidase as well as some acid phosphatase activity are associated with a population of large particles. The rest of the acid phosphatase activity and most of the ß-glucosidase activity were found in a smaller granule fraction. Using shallow Percoll gradients, two subpopulations of the hydrolase containing particles could be resolved from the higher density fraction in *Trichomonas vaginalis*. The major difference between the subpopulations was the lack of one of the proteinase activities and of ß-glucosidase in the higher density fraction. These were also the two activities that were found to be under-represented in the medium in comparison with the other hydrolases. Thus it may be the higher density fraction which is more likely to be associated with enzyme release.

No physiological function for the extracellular enzymes is as yet established. The enzymes may be required specifically for the establishment of the parasite within the host, perhaps by providing a means of countering the host defence systems. The hydrolases would provide an array of degradative reagents which would be active at the acidic pH of the vagina. Even if they are not specifically directed against host targets, their activities are such that they would create a potentially hostile enviroment for host tissues, and so hydrolase release could make a significant contribution to pathogenisis. Alternatively, they may simply be released to allow digestion of extracellular food and provide a supplement to nutrients derived from intracellular digestion. A more detailed discussion of the possible role of these hydrolases can be found in section 4.6.

1.7 Pathogenic mechanisms of trichomonads

Most of the information relating to pathogenic mechanisms concerns *Trichomonas vaginalis*. The pathogenicity of *T. vaginalis* has predominantly been studied using cells grown by tissue culture. Hogue (1943) was the first to report any pathological effects of this parasite on tissue cultures. Since then there have been many studies which are documented in detail by Honigberg (1989). When isolated strains of *Trichomonas vaginalis* and *Tritrichomonas foetus* were added to monolayers of various mammalian cell types a number of investigations have shown that the typical progression of events involves the following stages:

(1) The parasites swim freely over the cell monolayers.

(2) Many strains form aggregates consisting of numerous cells.

(3) The more virulent strains tend to settle on and adhere to the culture cells.

(4) Cell free areas, or lesions, and cytolysis occur underneath or to the side of the adherent flagellates.

Less inherently virulent strains, or strains cultivated for prolonged periods of time, have less tendency for cytadherence (Honigberg 1989). Interestingly Alderete and Pearlman (1984) found that *Trichomonas tenax*, a non-pathogenic trichomonad, produced no measurable cytotoxicity to cell monolayers when compared to *Trichomonas vaginalis* and *Tritrichomonas foetus*.

It is still not fully understood whether the cytopathogenicity of these parasites is contact dependent, requires a released lytic toxin or involves both of these mechanisms. Evidence for contact dependent cytopathogenicity has been

reported by many authors (Heath 1981 ; Alderete & Pearlman 1984 ; Krieger *et al.* 1985 ; Rasmussen *et al.* 1986). The actual mechanism of contact dependent pathogenicity is not known although Rusmussen *et al.* (1986) questioned whether cytotoxicity depended on the mechanical action of the flagella or on the activity of lytic factors bound to the parasite membranes. Alderete & Garza (1986) reported that the adherence of the parasites to monolayer cells was indicative of receptor-ligand type reactions. Results from an investigation by Kreiger *et al.* (1983) suggested that the hypothesis that a released toxin was involved may not be correct owing to the fact that the effects of the trichomonad cells were always focal with some apparently normal cells always present.

There have, however, been some reports which support the involvement of a released cytotoxin (Gentry *et al.* 1985 ; Pindak *et al.* 1986). Neilsen and Neilsen (1975) investigated the interaction between the vaginal epithelium and trichomonads. They found the parasites clustered over microulcerations in biopsies, but since the trichomonads were not present in every area of epithelial disruption they concluded that some damage resulted from cell free cytotoxins and that contact dependent destruction was a secondary phenomenon. Burgess *et al.* (1990) reported that live *Tritrichomonas foetus* cells, whole cell extracts and parasite conditioned medium all caused lysis of bovine erythrocytes. Lytic activity in the conditioned media was reduced by freeze thawing, although proteinase inhibitors which reduced the damage caused by the live parasites, had no effect on the lytic activity of the conditioned media. Interestingly Bremner *et al.* (1986) found that the proteinase inhibitor leupeptin did inhibit cytotoxicity of *Trichomonas vaginalis* towards mammalian cells.

Dailey *et al.* (1990) characterised a haemolytic activity of live *Trichomonas vaginalis* . Data suggested a contact-dependent mechanism for haemolysis which was temperature dependent and was reduced in the presence of metronidazole and cysteine proteinase inhibitors. Although the significance of trichomonal-mediated haemolysis to the biology of this parasite or the host-parasite interface, is not known, erythrocytes may represent a nutrient source. As discussed previously trichomonads are deficient in lipid biosynthesis, and the erythrocytes are rich in cholesterol that could be used by these parasites. Furthermore, iron, which has been shown to be essential to trichomonads, could be potentially acquired from the haemoglobin following haemolysis. It was also reported that factors involved in haemolysis were coordinated independently from surface immunogens and adhesion proteins of trichomonads and these are both discussed later.

Some authors have studied factors affecting adherence of the parasites to the cell monolayers. From studies with inhibitors Juliano *et al.* (1987) concluded that alterations induced in the trichomonal cytoskeleton may affect its adhesiveness and also its *in vitro* cytopathic effect. Sugarman & Mummaw (1988) also found that the presence of oestrogens, at physiological concentrations, decreased parasite growth, and their attachment to mammalian cells which may in turn decrease virulence. Cytadherence in *Trichomonas vaginalis* has been found to be specific as it requires individual receptors to interact with the trichomonad adhesion proteins (Alderete & Garza 1988 ; Roussel *et al.* 1991). Arroyo *et al.* (1992) identified four surface proteins (adhesins) which mediate the interaction of *Trichomonas vaginalis* with epithelial cells. Fresh isolates, when compared with long-term-grown isolates, had greater amounts of adhesins, which correlated with increased levels of cytoadherence. Anti-adhesin antibodies were found to inhibit the

binding of live parasites to epithelial cells and hence were protective from contact-dependent cytotoxicity. Moreover, the epithelial cells possessed molecules which recognized and bound to adhesins on nitrocellulose blots.

Work by Arroyo & Alderete (1989) showed for the first time the involvement of cysteine proteinase activity in parasite attachment to epithelial cells. As discussed above, Burgess *et al.* (1990), has also found proteinase involvement in the lytic mechanism of *Tritrichomonas foetus* and other studies have suggested that *Tritrichomonas foetus* proteinases are likely to play a role in the host-parasite interactions (Talbot *et al.* 1991). It has been reported that *Trichomonas vaginalis* had different levels of recognition and binding to epithelial cells when grown in a medium with varying concentrations of iron. Data from this study indicated that genes encoding the trichomonad adhesion proteins are coordinately regulated by iron (Lehker *et al.* 1991) .

1.8 Immunology of trichomonads

The existence of different serotypes among isolates of *Trichomonas vaginalis* was first reported by Schoenherr (1956) and since then many investigations have demonstrated antigenic heterogeneity. Alderete (1983) reported the characterisation of about 20 surface proteins accessible to antibody with a range of molecular weights between 30 and 300 kDa. Two bands of molecular weights 65 and 92 kDa were particularly prominent. Further work with monoclonal antibodies showed these two proteins to be major surface proteins, one of the monoclonals however only reacted with some of the isolates tested. The absence of this particular antigen, however,

was temporary, and from further work Alderete *et al.* (1986a,b) hypothesised that all strains possess the full complement of antigens and that serotypic differences could be largely explained by failure of vaccinated animals and hosts to respond to all of the antigens present. Connelly *et al.* (1985) have also reported major surface antigens one of which is believed to be a glycoprotein with a molecular weight of 115 kDa. Kreiger *et al.* (1985b) examined the geographic variation among isolates of *Trichomonas vaginalis* from the United States. The patterns of reactivity with the antibodies were significantly different in isolates from the four geographic regions, in contrast with results of Torian *et al.* (1984) which showed four of the more specific antibodies reacted with *Tritrichomonas foetus* . Although it is still not clear whether antigenic differences between strains are due to differences in genotype or to levels of expression, their existence is accepted.

An important point investigated by a number of workers is whether there is a correlation between high or low virulence and antigenic structure. Warton and Honigberg (1980) have shown that differences in exposed sugar residues on *Trichomonas vaginalis* in part correlate with differences in pathogenicity. They found that differences between the pathogenic and mild strains reflected levels of D-lactosyl residues on the cell surface, these residues being more abundant on strains with higher pathogenicity levels. Alderete *et al.* (1986b) went on to evaluate the extent and nature of heterogeneity among *Trichomonas vaginalis* isolates. A surface immunogen with a molecular weight of 267 000 was found to be involved in phenotypic variation and antigenic heterogeneity. Parasites without this surface glycoprotein do synthesise the antigen although only some isolates are capable of undergoing phenotypic variation. The absence of this and possibly other surface markers within subpopulations of trichomonads would

appear to ensure the survival of trichomonads vulnerable to the lytic actions of antibody. Alternatively, positive phenotype organisms which may be killed by host antibody might bind to existing immunoglobulins against parasite components also present on negative phenotype parasites and act as an avoidance mechanism. Nonetheless, data presented by Alderete *et al.* (1986b) indicated that phenotypic variation generates negative phenotype trichomonads with enhanced virulence exhibited by their potential for immune evasion capabilities and rates of contact-dependent killing of cells in monolayer cultures. Thus phenotypic variation among trichomonad populations with respect to specific surface markers may be a significant aspect of biology of this complex host-parasite relationship (Alderete et al. 1986b).

Trichomonads have been demonstrated to absorb media components onto their surface (Peterson and Alderete 1982). Although it is not known to what extent this happens *in vivo* it seems likely that this would occur during infection. Although this process may have a nutritional rather than immunological function Torian *et al.* (1984) has shown the absorbed medium components to be antigenic. *Trichomonas vaginalis* has also been shown to shed antigens into the growth medium (Alderete and Garza 1985). Again it is not known whether the release of antigens occurs *in vivo* but clearly have the potential to be immunopathogenic and to divert the host immune response away from the parasites themselves. Alderete *et al.* (1991) reported the presence of serum anti-proteinase antibody among patients with trichomoniasis. Sera from all infected patients, but none from sera of normal uninfected women, possessed IgG to numerous trichomonad cysteine proteinases. However, the presence of this serum antibody disappeared after patients were cured of the *Trichomonas vaginalis* infection. This is interesting

as no host immunity is found, even after repeated infections by this parasite. The fact that patients with trichomoniasis have serum antibodies to trichomonad proteinases indicates that proteinases are expressed *in vivo* and would indicate their presence in the vagina of infected women. Evidence reported by Alderete *et al.* (1991) on vaginal washes showed the presence of proteinases and antibodies to such hydrolases in the vaginas of infected women confirming findings from mice. The findings of this study indicate that proteinases may be important to the *Trichomonas vaginalis* host relationship.

It has been shown that monoclonal antibodies raised against the parasites can apparently kill antigen-positive strains of *Trichomonas vaginalis* in a complement independent manner (Alderete & Kasmala 1986). Burgess (1986) has also described nine surface reactive anti-*Tritrichomonas foetus* monoclonal antibodies, six of which promote complement-mediated lysis and one of which acts as an opsonin for monocyte phagocytosis. Hodgson *et al.* (1990) also reported monoclonal antibodies against *Tritrichomonas foetus* which mediated complement-dependent and independent killing of this parasite and prevented adherence to bovine vaginal epithelial cells. They suggested that the antigens recognised by these monoclonal antibodies may induce protective immunity. This suggestion was also supported by the work of Corbeil *et al.* (1989) with *Tritrichomonas foetus*. Surface-reactive bovine antiserum to this parasite was found to prevent adherence to bovine squamous vaginal epithelial cells. The inhibitory antiserum was specific for several medium- to high-molecular-weight membrane antigens as detected by Western blotting. The ability of surface-reactive antibodies to prevent adherence and to agglutinate and immobilize *Tritrichomonas foetus* was concluded to indicate that they may be protective.

Secretory IgA, which is the normal antibody class to be present on the mucosal surfaces, has in fact been shown to mediate a cytotoxic mechanism. This involves the specific sensitization by IgA of bacterial cells for killing by means of antibody dependent cell mediated cytotoxicity by gut associated lymphoid tissue lymphocytes (Tagliabue *et al* 1983). It is not known, however, if trichomonads are susceptible to this form of killing. Cell mediated killing of *Trichomonas vaginalis* has been shown with macrophages and neutrophils (Martinotti *et al.* 1985 ; Rein *et al.* 1980) and trichomonads are known to activate complement by the alternative pathway resulting in lysis of the parasites (Holbrook *et al.* 1981 ; Demes *et al.* 1986).

Nothing specific is known about the host defence mechanisms against trichomonads although the almost invariable failure of *Trichomonas vaginalis* to spread from the genitourinary system may be attributed to such mechanisms. There is no evidence of acquired immunity for trichomoniasis. However, results of many studies have suggested that defence mechanisms do exist. Lytic activity towards the parasites present in unheated serum has been attributed to the existence of natural antibodies, to cross reaction with normal flora, to infection with commensal trichomonads or to genetic predisposition (Samuels & Chun-Hoon 1964), and as stated above the alternative pathway activation of complement (Holbrook *et al.* 1982 ; Demes *et al.* 1986). There is also evidence of host specific serum antibodies to trichomonads. However, they appear to be ineffective in eliminating the parasites and the protective function of such antibodies is not known (Su-Lin 1982 ; Alderete 1984 ; Ackers *et al.* 1975). Polymorphonuclear neutrophils and macrophages, which have been shown to have an important defensive role in some intracellular pathogens, have also been shown to have trichomonacidal activity. It is not clear how crucial they are against this

extracellular organism as Demes *et al.* (1985) found no correlation in the number of polymorphonuclear leucocytes in the vagina of infected patients with those of uninfected people. Stryt *et al.* (1991) reported active migration of the parasites away from neutrophil products and suggested that this phenomenon might be a means by which trichomonads avoid the microbicidal functions of host phagocytes.

1.9 Aims of the project

From the foregoing introduction it is obvious that many aspects of these parasites have yet to receive detailed attention. This lack of knowledge is becoming increasingly more significant as resistance to the successful drug metronidazole, discussed earlier, develops in many areas of the world.

A potentially important area of trichomonad research is a group of enzymes, the hydrolases, which we now know to be released by the parasites during culture. These enzymes are released in large quantities which may implicate an important role in the host-parasite relationship. Although their precise functions are not known the hydrolases almost certainly participate in the survival of the parasites during the growth of *Trichomonas vaginalis* and *Tritrichomonas foetus* in their respective hosts. Evidence for cell free factors being involved in host cell pathogenesis (section 1.7) has been presented by several authors although what form they take and how they are produced and released is not known. It is easy to envisage that these pathogenic factors could include released hydrolases although there is no good evidence as yet for this. Recent work by Alderete *et al.* (1991a) has, however, shown parasite extracellular proteinases to be antigenic, and antibodies to

these enzymes have been demonstrated in host sera and the vaginal fluid of infected patients (Alderete *et al.* 1991b)(section 1.8). It is important therefore to understand the mechanism of biosynthesis and secretion of hydrolases by trichomonads.

The project involved specific hydrolases which were already known to be released at high activities from *Tritrichomonas foetus* and *Trichomonas vaginalis*. The activities selected were NAGase and acid phosphatase for the respective parasites. Rather than with regard to any function they may have *in vivo* these enzymes were selected due to the amount released from these parasites.

The original aim of the project was to purify sufficient of one or more of these enzymes and to raise antibodies against the purified proteins which would then be used in experiments to follow biosynthesis and release of the enzyme. This could be combined with measurements of enzyme activities in the cells and the extracellular medium. However, the lack of antigenicity of the only enzyme successfully purified, NAGase 1 of *Tritrichomonas foetus*, did not allow such studies to be undertaken. The aims of the project were therefore expanded to undertake a more detailed characterization of NAGase from *Tritrichomonas foetus* and other trichomonads, and to perform a limited study of the properties of acid phosphatase.

2.0 Materials & Methods

2.1 Materials

Unless otherwise stated all chemicals used were of analar grade, or the purest available, and obtained from either BDH (Glasgow,Scotland) or Sigma Chemical Co. Ltd., (Poole, Dorset, England).

2.2 Trichomonads

2.2.1 Species and strains

Tritrichomonas foetus, clone F2, was derived from the Pfizer strain originally obtained from Dr. D.L. Linstead (Wellcome Research Laboratories, Beckenham, Kent, London.). Clone KV1, was obtained from Dr D. Lindmark, Department of Biology, Cleveland State University, Cleveland, Ohio, 44115, USA. Clone CA84-2, was obtained from Dr D. Burgess, Veterinary Molecular Biology Laboratory, Montana State University, Bozeman, Montana 59717 (Burgess 1988).

Tritrichomonas augusta, clone B2, previously identified incorrectly as *Trichomitus batrichorum* (Lockwood *et al.* 1988), was derived from an isolate obtained from the intestine of the leopard frog, *Rana pipiens* (Coombs 1976).

Trichomonas vaginalis, clone G3, cloned in 1976 has been cultivated *in vitro* for more than 15 years (Coombs 1976).

2.2.2 Culture

All trichomonads were routinely cultivated axenically in Modified Diamonds Medium (MDM) containing 10% (v/v) inactivated horse serum (recipe shown in table 5). All cultures were checked daily for contamination by microscopic observation. Contaminated cultures were discarded. Trichomonad cultures were subpassaged every 1–2 days at an approximate starting density of 10^4 organisms ml^{-1}

2.3 Harvesting

2.3.1 Growth medium

Trichomonads were routinely harvested at an approximate density of 2.5 x 10^6 cells/ml. The cells were washed twice, in the original volume of medium, with 0.25M sucrose. Cell pellets were then collected by low speed centrifugation (500g for 10 min at 4°C in a Sarstedt LC1–K bench centrifuge) and either used immediately or stored at –20°C until required. Trichomonad growth medium was harvested as follows. Cells were washed, in the original volume of serum free medium and then incubated in serum free medium for 6h. The medium was then collected by low speed centrifugation (500g for 10 min at 4°C in a Sarstedt LC1–K bench centrifuge) and filtered gently through a 0.2 μm Nalgene filter to remove any remaining cells. The absence of cells was confirmed by microscopic observation. The filtered medium was used immediately or stored at –20°C until required. No loss of hydrolase activity was apparent on storage.

Table 5. Modified Diamond's medium (Diamond 1957). Components for 1 litre final volume.

Ingredients	g
trypticase	20
yeast extract	10
maltose	5
ascorbic acid	1
KCl	1
$KHCO_3$	1
KH_2PO_4	1
K_2HPO_4	0.5
$FeSO_4$	0.1

The ingredients above were dissolved in 900 ml distilled water, the pH adjusted to 6.3-6.4, and made up to 1 litre volume. The medium was dispensed into glass bottles and autoclaved for 20 min at 15 lb in^{-2}. The sterilised 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.

Trypticase was supplied by Beckton Dickinson and Co. (Between Towns Road, Cowley, Oxford, England) ; heat–inactivated horse serum by Gibco Biocult (Paisley, Scotland) and yeast extract by Difco Laboratories (West Molesey, Surrey, England).

2.3.2 Resuspension of trichomonads

Trichomonads were grown as described in section 2.2.2 and collected, as described in section 2.3.1, during log phase growth at an approximate density 2.5 x 10^6. The cells were collected during the log phase growth to reduce the possible occurrence of cell lysis. The harvested cells were washed twice, in a volume equivalent to that of the original culture, with 0.25M sucrose before being resuspended in serum free medium. After resuspension the cells were incubated at 37°C for 6h after which they were harvested and either used immediately or stored at -20°C until required.

2.4 Subcellular fractionation of trichomonads

Pellets of trichomonads, harvested as described above, were resuspended in 0.25M sucrose before being 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 homogenate was then centrifuged at 500g for 5 min in a Sorvall RC 5B refrigerated centrifuge (SS-34 rotor). The supernatant was removed and the pellet was again homogenised before being combined with the original supernatant. This method resulted in lysis of more than 95% of the trichomonads as judged by microscopic observation. The homogenates were then separated into four fractions by differential centrifugation using the method of Steinbuchel and Muller (1986). The four fractions were defined as nuclear, large particle, small particle and non–sedimental fractions. The nuclear fraction was sedimented at 500g for 4 min in a Sorvall RC 5B refrigerated centrifuge (SS-34 rotor), resuspended and washed in 0.25M sucrose. The

supernatant from the nuclear fraction was then centrifuged to sediment the large particle fraction (*Tritrichomonas foetus* and *Trichomonas vaginalis* were centrifuged at 2 500g and 5 000g respectively for 10 min). The small particle fraction was then sedimented at 50 000g for 60 min at 4°C in an MSE 65 ultracentrifuge (10x10 rotor) and the resulting supernatant formed the non–sedimentable fraction.

2.5 Ammonium sulphate precipitation

Protein was precipitated by the addition of crystalline ammonium sulphate. The precipitated protein was collected by centrifugation 13,300g for 20 min at 4°C, using a GSA rotor, in a Sorvall FC–5B centrifuge.), redissolved, and dialysed overnight against 0.05M Tris / HCl, pH 7.5.

2.6 Enzyme assays

All enzyme assays were conducted in microtitre plates. Assays for NAGase activity used stock solutions of 10mM 4–nitrophenyl –N–acetyl–ß–D–glucosaminide, and for acid phosphatase 4–nitrophenyl phosphate (disodium salt), as the substrates. 1 ml of the substrate stock solution was mixed with 0.4 ml of 0.2M sodium acetate / acetic acid pH 5.5 and 35 µl of this mixture was added to each well together with a variable amount of sample and d.d. water to make a total volume of 50 µl. The final concentration of the substrate was 5.0 mM. The assays were carried out at 37°C. A solution of 1M sodium carbonate (150 µl) was added to stop the reaction. The production of p–nitrophenol was determined using a Biorad ELISA reader fitted with a 414 nm filter. The use of a 414 nm filter reduced

interference from the coloured trichomonad medium during the assay. A change of 1 OD was calculated to be equivalent to 1.58 µmol p-nitrophenol per ml of sample when a 15 µl sample is used. Controls for these assays contained water and sterile medium as substitutes for cell homogenates or filtered growth medium respectively. The controls were used to blank the microtitre plate reader before readings were taken.

2.7 Protein determination

Protein was routinely determined using the method described by Sedmak and Grossberg (1977). Bovine serum albumin was used as the standard and new standard curves were constructed for each fresh batch of reagent.

2.8 Purification procedures for NAGase

2.8.1 Precipitation

NAGase, from *Tritrichomonas foetus*, was precipitated by the addition of crystalline ammonium sulphate, to produce a 0-60% (w/v) fraction. The precipitation was performed as described in section 2.5 and was used as the starting material for the purification of extracellular NAGase.

2.8.2 Ion exchange chromatography

A column of DEAE-Sepharose (height 9 cm, diameter 2.5 cm) was equilibrated with 0.05M Tris / HCl, pH 7.5. The sample was loaded and the column was washed through with 50 ml of 0.05M Tris / HCl pH 7.5. NAGase

bound to the column and was eluted with a NaCl gradient at a concentration of 0.1M in a total volume of 200 ml. Any remaining protein was removed using 3M NaCl and the column was equilibrated with the starting buffer. Fractions were collected at 2.5 ml / tube and assayed for NAGase activity using the standard procedure. Fractions containing NAGase activity were pooled and concentrated under nitrogen using an Amicon Ultrafiltration Cell fitted with an XM50 membrane filter. This step was also used to dialyse the sample by flushing through 0.01M Tris / HCl pH 7.5.

2.8.3 Hydrophobic interaction chromatography

A column of phenyl Sepharose (height 13 cm, diameter 1 cm) was equilibrated with 0.5M ammonium sulphate. The sample was brought to 0.5M by the addition of the solid. The precipitate was removed by centrifugation in an Eppendorf 5415 microcentrifuge (16,000 g for 2 min) and the supernatant collected prior to loading on the column. The column was washed with 0.5M ammonium sulphate and the unabsorbed protein was eluted. The bound proteins were eluted with a decreasing linear gradient from 0.5M ammonium sulphate to 0.1M sodium acetate / acetic acid, pH 5.5 of total volume 40 ml. NAGase was then eluted by the addition of 50 ml of 5% (w/v) PEG. The column was equilibrated with starting buffer before reuse. Fractions were collected at 25 drops / tube, assayed for NAGase activity and the active fractions pooled.

2.8.4 Ligand affinity chromatography

Cyanogen bromide-activated Sepharose 4B was coupled to 2-acetamido-N-(-aminocaproyl)-2-deoxy—D-glucopyranosylamine

essentially as described by Lotan *et al.* (1973). The column was equilibrated with 0.05M phosphate buffer pH 7.0 before loading the sample. 10 ml of the starting buffer was washed through the column which removed the unabsorbed protein. Two peaks of NAGase activity were eluted. The first peak was obtained when 20 ml of 0.5M phosphate buffer was loaded and the second resulted from the addition of 20 ml of 0.05M phosphate buffer pH 7.0 containing 0.2M N–acetyl–glucosamine (Edwards *et al.* 1975). The NAGase fractions were pooled and either used immediately or stored at –20°C. The column was equilibrated with starting buffer before reuse.

2.8.5 Gel filtration chromatography

Gel filtration was achieved on an S300 column (height 100 cm, diameter 2.5cm) using the running buffer 0.05M phosphate pH 7.0 containing 0.25M NaCl. The molecular weights of the hydrolases were determined by comparing their elution volumes with those of proteins of known molecular weights. The calibration proteins used were cytochrome C (M_r 12 500), ovalbumin (M_r 45 000), bovine serum albumin (M_r 66 000), alcohol dehydrogenase (M_r 150 000) and ß-amylase (M_r 200 000). 10 mg of each protein was dissolved in 1 ml of the running buffer, loaded on to the column and eluted under identical conditions to those used for elution of the hydrolases.

2.8.6 Preparative isoelectric focusing

Preparative isoelectric focusing was performed using the Biorad Rotofor IEF Cell by the method descibed in the Biorad instruction manual (© Bio-rad Laboratories Ltd, Bio-rad House, Maylands Avenue, Hemel Hempstead,

Herts, HP2 7TD, 1989). This method allows the separation of proteins by their isoelectric point with no gel matrix involved. Assembly of the apparatus was performed as explained in section 3 of the instruction manual. The membranes, which stabilise the pH gradient, were equilibrated for 24 h in the appropriate electrolyte solution before use and then used for five Rotofor runs before being replaced. The anion exchange membranes were soaked in 0.1M NaOH and the cation exchange membranes in 0.1M H_2PO_4. The filters were also kept in these solutions between runs. The trichomonad samples were prepared, by lysis in 0.25% Triton X 100, and made up to 50 ml with d.d. H_2O and the appropriate ampholytes (2% [v / v]). Equal volumes of ampholytes 3-5 and 4-6 were used unless stated otherwise. The Rotofor run was performed at a constant power of 12W for not longer than 6 h. The run was presumed to be completed when the voltage had been constant for at least 1 h. The focusing chamber was cooled at all times by the cooling finger, which extends through the focusing chamber and the electrode assemblies. The cooling finger was kept at a temperature of 4°C using a refridgerated water bath. After the run was completed the samples were collected using a vacuum provided by a water pump. This allowed all 20 fractions to be simultaneously aspirated from the cell and delivered to the collection tubes. The pH of each fraction was then recorded and the fractions either used immediately or stored at -20°C until required.

The preparative isoelectric focusing procedure described above was used in both the purification of NAGase from *Tritrichomonas foetus*, and for some initial studies of the hydrolases (section 3.3.3) and the proteinases from trichomonads (section 3.6).

2.8.7 Analytical isoelectric focusing

Samples were analysed on 10% polyacrylamide gels by electofocusing (IEF) in the pH range of 3.5–5.2 using an LKB 2117 Multiphor System essentially as described by Winter *et al.* (1977). The pH range was created by using an equal mixture of the two ampholytes 3-5 and 4-6. Ampholytes were purchased from Biorad Laboratories Ltd. (Watford, Herts, England.). Electrofocusing was performed at a constant power of 25W for approximately 3h. Electrofocusing was complete when the final voltage and current stabilized at approximately 1030V and 28 mA respectively. After isoelectrofocusing proteins or NAGase activities were determined using the staining methods described in section 2.9.3.

To demonstrate the relationship between the electrophoretic and isoelectric forms a simple 2D electrophoresis system was devised. The procedure involved running a sample containing NAGase on an SDS PAGE mini gel, cutting out a lane of width 10 mm from the gel, incubating it in 2.5 % (v/v) Triton X100 for 30 min and then placing the gel strip on an IEF gel at right angles to the direction of the current. After focusing the NAGase bands were detected using the standard procedure (section 2.9.3).

2.9 Polyacrylamide gel electrophoresis

Gel electrophoresis was carried out in a Biorad vertical mini gel apparatus (gel size=0.75 x 82 x 80 mm) and run at 16 mA / gel. SDS-PAGE samples were prepared by mixing them with an equal volume of sample buffer (0.125 M Tris / HCl pH 6.8, 4% (w/v) SDS, 5% (w/v) 2–mercaptoethanol, 20% (v/v)

glycerol.). For all gels used for detecting enzyme activity, samples were run immediately after mixing with sample buffer and were not incubated at 100°C for 2 min before electrophoresis. Where complete denaturing was required (non-activity gels) samples were heated at 100°C for 2 min before electrophoresis. Polyacrylamide gel electrophoresis was carried out using the SDS discontinuous buffer system as described by Hames (1981). The acrylamide concentration of the separating gels was 7.5% (w/v) unless otherwise indicated. After electrophoresis the gels were stained by the procedures described in sections 2.9.1-2.9.4. Non denaturing PAGE was carried out using an identical method with the exception that neither SDS nor 2-mercaptoethanol were included in any of the buffer systems. Calculations of molecular weights of proteins after SDS-PAGE were performed by comparison to molecular weight standards run on the same gel. However, for activity stained gels prestained molecular weight standards were used which, owing to the staining procedure, run slower than the unstained standards during electrophoresis and were therefore used only as a guide and no molecular weights were calculated using the prestained standards.

2.9.1 Protein staining

Protein bands were visualised by staining in 0.05% PAGE blue 83 in 25% (v/v) isopropanol, 10% (v/v) acetic acid. Molecular weights of individual proteins were determined from their mobility compared to those of protein standards (high molecular weight standard mixture SDS–6H, Sigma Chemical Co. Ltd.). Silver stain (Sigma silver stain kit) was also used when more sensitivity was required.

2.9.2 Proteinase activity staining

Proteinases were visualised after SDS-PAGE by running samples in gels which contained 0.2% gelatin. After electrophoresis the gels were incubated for 30 min in 2.5% (v/v) Triton X100 to remove the SDS and restore proteolytic activity. The proteinase bands were developed by immersing the gels in 0.1M sodium acetate / acetic acid buffer pH 5.5 containing 1 mM DTT for 3 h at 37°C (Lockwood *et al.* 1987). The bands were then stained as described for protein staining.

2.9.3 NAGase activity staining

NAGase activity on SDS-PAGE gels was detected after electrophoresis by incubating gels in 2.5% (v/v) Triton X100 for 30 min followed by incubation at 37°C in 0.5 mM naphthol AS–BI–N–acetyl—D–glucosamide, dissolved in 5% (v/v) aqueous methoxy ethanol, made up with 0.2M sodium acetate/acetic acid buffer, pH 5.5. After an appropriate incubation time (2–5h) an equal volume of 4% (v/v) Brij 35 and Fast Garnet Salt (1 mg/ml) was added (Hayashi 1965). NAGase activity stained as pink bands ; examples illustrating this colour are given in figures 11 and 13 only.

2.9.4 Acid phosphatase activity staining

After electrophoresis the gels were incubated in 2.5% (v/v) Triton X100 for 1h and then in 0.1M sodium acetate / acetic acid buffer, pH 5.5, for 15 min. The gels were then shaken overnight at 4°C in 20 ml of the acetate buffer

containing 4 mM α–naphthyl phosphate and 25 mg of Fast Garnet Salt. Acid phosphatase activity stained as purple/black bands.

2.10 Preparation of antibodies

2.10.1 Preparation of samples

The following antigens were used for the preparation of antibodies.

(1) Purified NAGase 1 as described in section 3.4.1.

(2) Purified NAGase 1 as described in section 3.4.2.

(3) Electro-purified NAGase 1. SDS-PAGE strip gel was run and the NAGase 1 band retained. The gel strip containing the antigen was frozen at -20°C until required. The strip was defrosted and blotted on a tissue to remove any excess water. It was then washed three times for 15 min in PBS before being crushed using a glass rod in 0.5 ml of PBS. This solution was then sonicated for 5 min followed by 5 min incubation on ice. This process was repeated until no lumps of gel were left. The appropriate amount of Freund's adjuvant (0.75 ml) was added and the mixture was shaken until an emulsion was achieved for immunisation.

(4) Crude cell extract prepared as described in section 2.3 and lysed by freeze/thawing three times.

2.10.2 Immunisation and collection of serum

The above antigens (1ml containing approximately 100 mg protein) and 1.25 ml of Freund's complete adjuvant (Sigma) were then shaken into an emulsion for immunisation.

Female, New Zealand White rabbits were used to raise antisera. The injections of the rabbits were administered subcutaneously at three separate points. Three weeks later a booster injection containing the same amount of antigen and Freunds incomplete adjuvant was administered in the same manner. After a further two weeks another identical booster was administered using the same procedure.

2.10.3 Preparation of serum

The serum was prepared by allowing the blood to clot for 24 h at 4°C. The serum was then carefully decanted, aliquoted and stored until needed at –20°C.

2.11 Western blotting

Samples were separated using both non-denaturing and denaturing SDS PAGE (section 2.9). After electrophoresis the gels were incubated for 20 min at room temperature in transfer buffer (0.025M Tris, 0.19 M glycine, 20% (v/v) methanol) before being blotted onto nitrocellulose paper at a current of 0.2 amps for 1h. The nitrocellulose was then blocked for 3h at room temperature in phosphate buffered saline (PBS ; 10 mM K_2HPO_4 / KH_2PO_4 pH 7.4) containing 0.2% gelatin. At this stage the nitrocellulose was either used

immediately or stored at 4°C. For staining the nitrocellulose was incubated for 1 h, with shaking at room temperature, with the diluted serum in a fresh solution of PBS containing 0.2% gelatin and 0.1% Triton X100. Incubation with the antibody was followed by 4 x 5 min washes in PBS. Goat anti–rabbit antibody conjugated to either alkaline phosphatase or horse radish peroxidase (Sigma Chemical Co. Ltd., Poole, Dorset, England) diluted 1:1000 in PBS buffer was added for a second 1h incubation. This was followed by 3 x 5 min washes in PBS, containing 0.2% gelatin and 0.1% Triton X 100, and a further wash in PBS for at least 5 min.

For glycoprotein staining the procedure was the same as above except either Concanavalin A or peanut horse radish peroxidase conjugated lectins (10 µg/ml)(Sigma Chemical Co. Ltd., Poole, Dorset, England.) were used instead of the second conjugated antibody.

Staining of blots was performed as follows:

For peroxidase :

Diaminobenzidine tetrachloride (0.14M) in 0.05M Tris/HCl buffer, pH 7.4. A volume of 1 µl of 30% (v/v) hydrogen peroxide was added to start the reaction and when the staining was sufficient the reaction stopped by repeated washes with water.

For alkaline phosphatase :

A volume of 66 µl of 5-bromo-4-chloro-3-indolyl-phosphate (50 mg/ml) was dissolved in formamide and 132 µl of Nitro-Blue Tetrazolium (50 mg/ml), dissolved in 70% (v/v) DMSO,

was added to 20 ml of TBS (10 mM Tris/HCl pH 7.6, 150 mM NaCl.) just before use. When the staining was sufficient the reaction was stopped by repeated washes with water.

2.12 Immunoprecipitation

Samples of cell lysates (100 µl) were equilibrated with 10 mM Tris/HCl pH 7.4, 100 mM NaCl, 5 mM EDTA, 2.5% (v/v) Triton X100 (900µl) by mixing for 1h at room temperature. Pansorbin (50 µl) purchased from Calbiochem, Behring Diagnostics, La Jolla, California, USA. was added at this stage to avoid non–specific binding later, and mixed for a further 1h before centrifugation in an MSE Microcentaur Microcentrifuge at high speed for 5 min. The supernatant was then removed to a clean tube. Antiserum (1:100) was added, and after shaking for 1.5 h the tube was centrifuged as described above. The pellet was retained and washed twice in 10 mM Tris HCl pH 7.4, 100 mM NaCl, 5 mM EDTA, 0.1% SDS and then once in the same buffer containing no SDS.

2.13 Deglycosylation

A sample of pure protein was denatured by incubating at 100°C for 2 min with an equal volume of 0.2 mM sodium phosphate buffer pH 8.0, 50 mM EDTA, 0.2% SDS, 1% (v/v) 2–mercaptoethanol, 1% (v/v) Triton X100. A total of 40 units of glycopeptidase F (1 unit is the enzyme activity which hydrolases 1 nmol dansyl fetuin glycopeptide within 1 min at 37 °C at pH 7.2), obtained from Boehringer Mannheim Biochemica (Lewes, E. Sussex, England.), were then added and the mixture incubated at 37°C overnight.

The glycopeptidase F had been tested and confirmed by the manufacturer to contain no ß-galactosidase, ß-glucosidase, α and ß-mannosidase, ß-N-acetylhexosaminidase, α-L-fucosidase, sialidase or protease activity. The samples were then analysed by SDS-PAGE as described in section 2.9. The gel was stained for protein using the standard procedure (section 2.9.3).

2.14 Circular dichroism

Circular dichroism (CD) spectra were recorded at 20°C using a Jasco J-600 Spectropolarimeter. Protein was monitored between 190 and 260 nm in a quartz cuvette of pathlength 0.1 cm, in the presence and absence of 3M guanidine hydrochloride (GdnHCl), ultra pure grade. GdnHCl was supplied by Bethesda Research Laboratories (BRL), Life Technologies Inc, Gaithersburg, MD 20877, USA. The enzyme and GdnHCl were pre-incubated for 15 min at room temperature prior to the measurement of the spectrum. All measurements were made using 10 mM sodium phosphate buffer pH 7.5 and were corrected for the addition of GdnHCl. The observed ellipticity values were converted to mean residue ellipticity values using a value of 112 for the mean residue weight of an amino acid.

$$\text{Mean residue ellipticity} = \frac{112 \times \text{recorded ellipticity}}{10 \times \text{cell path length} \times \text{protein concentration}}$$

Protein concentration is in g / ml. Recorded ellipticity is measured in degrees and thus the units of the mean residual ellipticity are deg.cm^2/dmol.

3.0 Results

3.1 Characterisation of NAGase activity from *Tritrichomonas foetus* and *Trichomonas vaginalis*

3.1.1 Introduction

Many species of protozoa contain high levels of hydrolytic enzymes and in a number of instances the hydrolases are also secreted (section 1.6). Trichomonads are no exception and possess a variety of hydrolases, first described by Muller (1973). Lockwood *et al.* (1988) later reported the continual release of several hydrolases from *Trichomonas vaginalis* and *Tritrichomonas foetus* during axenic growth *in vitro*. These enzymes include NAGase, acid phosphatase, α-mannosidase, β-glucosidase, proteinases (Lockwood *et al.* 1988 ; North *et al.* 1989) and neuraminidase (Silva Filho *et al.* 1989). Lockwood *et al.* (1988) found that, as a result of this release, over 50% of the total hydrolase activity present in a culture could be extracellular. The large quantities of enzymes released suggest that the process could be of significance to the host-parasite interaction and thus merited further investigation.

In *Tritrichomonas foetus* NAGase, a hexosaminidase, has the highest activity of those hydrolases detected, and indeed it was found to be six times greater than the NAGase activity from *Trichomonas vaginalis* (Lockwood *et al.* 1988). Thus it was selected as the most suitable activity for studying enzyme release in *Tritrichomonas foetus*. This chapter describes the results of the characterisation of NAGase activity in unfractionated samples of cell lysates from *Tritrichomonas foetus* and *Trichomonas vaginalis*.

3.1.2 Hydrolase assays

All assays for NAGase were performed using the standard procedure described in section 2.6. In this section the optimisation, standardisation and initial characterisation of this enzyme activity are described. NAGase assays were either performed on total parasite lysates, prepared by incubation of cells with 0.25% Triton X100, to measure intracellular NAGase, or were performed on filtered medium to assay the total NAGase activity released by the parasites.

The results presented here are generally from single experiments but in all cases the data have been confirmed by at least two other independent determinations.

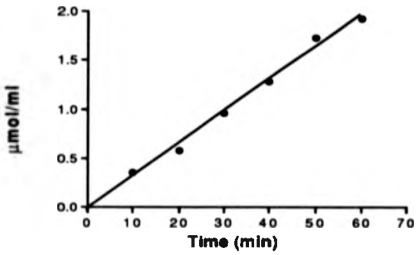
3.1.3 Time dependence of NAGase assays

In the assay for NAGase activity production of 4-nitrophenol was linear over a period of at least 60 min (figure 5). This was true using different amounts (approximate range of 10-100 µg total protein) of samples. No subsequent assay was run for more than 60 min.

3.1.4 Temperature dependence of NAGase assays

The *Tritrichomonas foetus* intracellular NAGase had an optimum temperature of between 60°C and 70°C (figure 6A). The temperature optimum for intracellular NAGase of *Trichomonas vaginalis* was 60°C (figure 6B). The rate of product formation in the non-enzyme controls was negligible at all

(A)



(B)

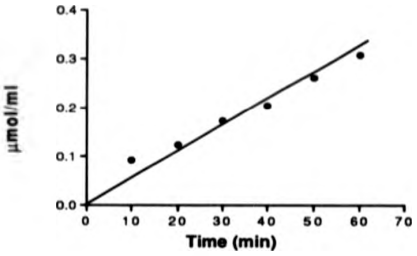
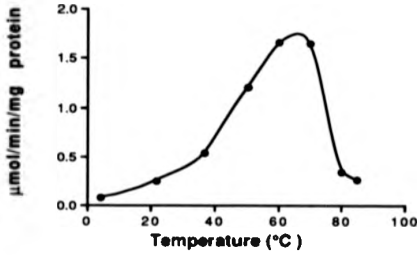


Figure 5. Time course for NAGase assay with lysates of (A) *Tritrichomonas foetus* and (B) *Trichomonas vaginalis*.

Assay performed as described in section 2.6. Amount of protein in samples was 50 µg.

(A)



(B)

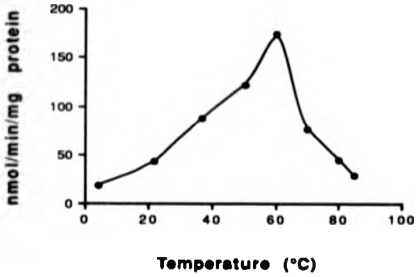


Figure 6. The temperature dependence of NAGase activity in lysates of (A) *Thtrichomonas foetus* and (B) *Trichomonas vaginalis* .
Assays were carried out at the temperatures indicated over a time period of 20 min. Amount of protein in samples was 50 µg.

temperatures. Because the enzymes were obviously stable it was appropriate to use the physiological temperature of 37°C as the standard assay temperature.

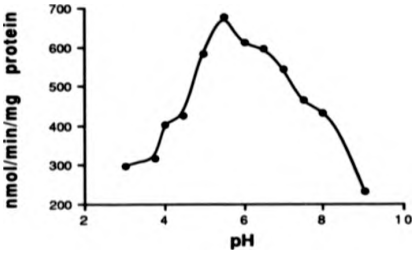
3.1.5 pH dependency of NAGase assays

The pH dependency curve for NAGase activity from *Tritrichomonas foetus* gave a distinct peak at pH 5.5 while that from *Trichomonas vaginalis* had a broad optimum from pH 4.0 to pH 6.0 (figure 7). Therefore pH 5.5 was used in all subsequent assays. The result confirmed that the NAGase activities from these species are indeed acid hydrolases although they still exhibited significant activity over a broad range of pH's.

3.1.6 Kinetics of NAGase

NAGase obeyed Michaelis Menten kinetics. Typical results are given in figure 8. The K_m's, with the substrate p-nitrophenyl-N-acetyl-ß-D-glucosaminide, for NAGase of *Tritrichomonas foetus* and *Trichomonas vaginalis* were determined. To calculate these values a substrate concentration range of 0.25 to 2.5 mM was used. The K_m and its standard error, were calculated by three independent determinations using Enzfitter version 1.05 supplied by Elsevier Soft. *Tritrichomonas foetus* intracellular NAGase activity was calculated to have a K_m of 0.95 $\pm$ 0.12 mM and the extracellular activity a K_m of 0.86 $\pm$ 0.16 mM, indicating no significant difference between the K_m's. The intracellular NAGase activity of *Trichomonas vaginalis* was similarly found to have a K_m of 0.71 $\pm$ 0.27 mM. The K_m's of the intracellular NAGases from the two species of trichomonads

(A)



(B)

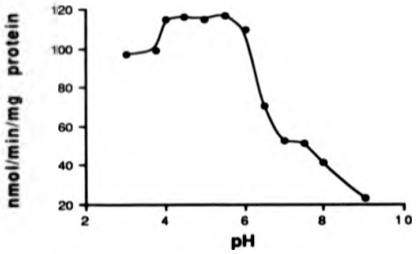


Figure 7. The pH dependence of NAGase activity in lysates of (A) *Tritrichomonas foetus* and (B) *Trichomonas vaginalis* .
Assays were carried out at the pH indicated over a time period of 30 min.
Amount of protein in samples was 50 µg.

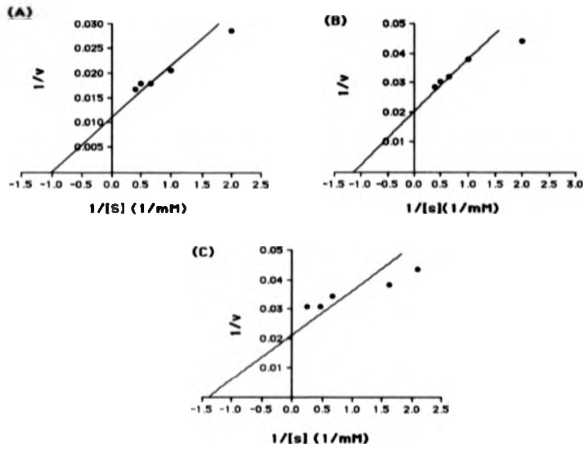


Figure 8. Double reciprocal plot for *Tritrichomonas foetus* (A) crude intracellular NAGase (B) crude extracellular NAGase, and *Trichomonas vaginalis* (C) crude intracellular NAGase. V is in units of nmol/min/ml.

were thus not significantly different. The K_m for the extracellular *Trichomonas vaginalis* NAGase activity could not be calculated owing to the low level of activity.

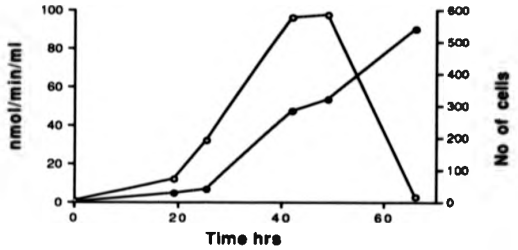
3.1.7 Release of NAGase from *Tritrichomonas foetus* and *Trichomonas vaginalis*

NAGase is released from *Tritrichomonas foetus* and *Trichomonas vaginalis* during logarithmic growth when parasites are cultivated axenically in MDM (figure 9). The activity in the medium increases during all stages of growth. Lockwood *et al.* (1988) have already shown that the appearance of extracellular activity during the active growth phase is unlikely to be due to parasite lysis as the exclusively intracellular enzymes lactate dehydrogenase, serine sulphydrase and malate dehydrogenase are not detectable in the medium during this time. A continued increase in NAGase activity was, however, also observed at the end of the stationary phase when viable cell numbers were decreasing, and it is possible that cell lysis may contribute to this later release. *Tritrichomonas foetus* released greater amounts of NAGase than *Trichomonas vaginalis* reflecting the difference in the intracellular level. This confirms results obtained by Lockwood *et al.* (1988).

3.1.8 Release of NAGase from *Tritrichomonas foetus* and *Trichomonas vaginalis* during incubation in serum free MDM

Growing trichomonads in media containing serum presents a problem when analysing released hydrolases because of the large amounts of serum-

(A)



(B)

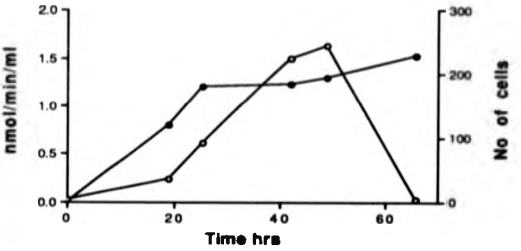


Figure 9. Release of NAGase from (A) *Trichomonas foetus* and (B) *Trichomonas vaginalis*.

Extracellular NAGase activity (nmol/min/ml) (•) and cell number ($x10^4$) (o).

Cultures were grown in complete MDM inoculated at 10^4 cells/ml.

derived proteins that are present. North *et al.* (1989) concluded that the characteristics of enzyme release after the transfer of parasites to serum free MDM were sufficiently similar to those observed in complete cultures to justify the use of this simple transfer procedure. Therefore, both species of trichomonads, harvested during the log phase of growth, were incubated for 6h in serum free MDM and the amount of hydrolase released was then determined. NAGase activity was released during incubations in serum free MDM (figure 10). There was an increase in activity over the six hours. North *et al.* (1989) have already shown that the appearance of the extracellular NAGase activities is unlikely to be due to cell lysis as the strictly intracellular enzyme, serine sulphydrase, was not detectable in the medium.

3.1.9 Summary

A preliminary characterisation of NAGase activity from *Tritrichomonas foetus* and *Trichomonas vaginalis* was performed. This enzyme was found to be an acid hydrolase with a temperature optimum from both species of around 60°C. The K_m's of NAGase from the two parasites were not found to be significantly different. The release of NAGase was confirmed although *Tritrichomonas foetus* was found to release greater amounts of the hydrolase than *Trichomonas vaginalis*.

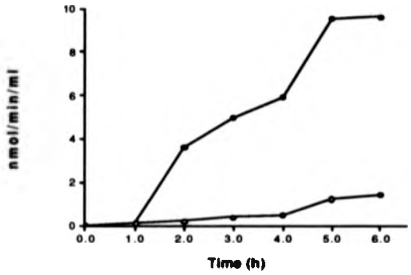


Figure 10. The release of NAGase from *Tritrichomonas foetus* (•) and *Trichomonas vaginalis* (o) during 6 h incubation in serum free MDM. NAGase activity indicated as nmol/min/ml. Cells were pregrown in complete MDM to an approximate density of 2 x 10^6 cells/ml, harvested by centrifugation, washed and resuspended in serum free MDM. A full description of this method can be found in section 2.3.2.

3.2 Analysis of NAGase by SDS-PAGE

3.2.1 Introduction

Proteins are separated by SDS-PAGE under denaturing conditions largely according to their mass. The basis of electrophoresis is that a molecule carrying a net charge will move in an electric field. Electrophoretic separations of protein mixtures are nearly always carried out in polyacrylamide gels as these are chemically inert and are readily formed. Moreover, their pore sizes can be controlled by choosing appropriate concentrations of acrylamide and methylenebisacrylamide, a cross-linking reagent. Before electrophoresis protein samples are mixed with a solution of SDS, an anionic detergent that disrupts nearly all noncovalent interactions in proteins. ß-mercaptoethanol or DTT may also be added to reduce disulphide bonds. Relatively large amounts of SDS bind to the main chains of amino acid residues of each disrupted polypeptide producing an essentially equal charge/mass ratio for all proteins. Under these conditions the mobility of most polypeptide chains is linearly proportional to the logarithm of their inverse mass and hence the molecular weight of most unknown proteins can be calculated by comparison with the mobilities of known molecular weight standards using a standard curve. Although SDS-PAGE is a good technique for analysing most proteins, it should be emphasised that some carbohydrate-rich proteins and membrane proteins have been found to migrate anomalously (Hames 1981).

Here SDS-PAGE was used to identify multiple forms of intracellular and extracellular NAGase and to make comparisons between different strains and species of trichomonad. Electrophoresis was also used to monitor the

release of NAGase from *Tritrichomonas foetus* when the parasites were grown and incubated in the presence of various inhibitors.

3.2.2 Resolution of four forms of NAGase from *Tritrichomonas foetus*

Proteins possessing NAGase activity from *Tritrichomonas foetus* were visualised using the activity stain technique described in section 2.9.3. Samples for activity gels were not incubated at 100°C before electrophoresis to avoid loss of enzyme activity. Four major forms of NAGase were found in lysates of *Tritrichomonas foetus* prepared during axenic growth in MDM containing 10% (w/v) horse serum. The apparent molecular weights were calculated to be 54 000, 89 000, 100 000 and 158 000 (figure 11). These four major forms of NAGase activity are refered to as NAGases 1-4 as defined in table 6. A less active NAGase band was sometimes detected in both extracellular and intracellular samples which had an apparent M_r in the range between 80 000 and 90 000. This form of NAGase was, however, not always detected probably due to its activity being below the limit of detection by this method. The specific activity of NAGase from cell lysates of this organism was calculated to be 740 ± 112.2 nmol/min/mg cell protein (table 7). It was not possible to use the method to analyse NAGase in extracellular medium from growing cells because of the large excess of serum proteins present which interfered with SDS-PAGE with no separation of proteins occuring.

The same four major forms of NAGase, with apparently identical molecular weights, were also found to be released from this organism after transfer of

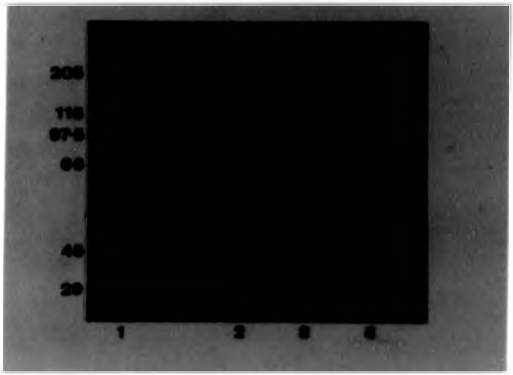


Figure 11. Activity staining of NAGase on SDS-PAGE.

Lane (1) Molecular weight markers (2) *Tritrichomonas foetus* crude cell lysate (3) *Tritrichomonas foetus* crude medium preparation (4) *Trichomonas vaginalis* crude cell lysate. 50 nmol / min NAGase activity loaded to each lane.

Table 6. Properties of the four major NAGase activities of *Tritrichomonas foetus* , clone F2.

NAGase form	Apparent M_r from SDS-PAGE activity gels	Apparent pI from IEF
1	54 000	4.5
2	89 000	4.7
3	100 000	4.9
4	158 000	4.6

The apparent molecular weights of the four major NAGase forms were calculated from a standard graph constructed using known molecular weight standards which were run on the same gel as the trichomonad samples. SDS and ß-mercaptoethanol were present as reducing agents but the samples were not incubated at 100°C before electrophoresis. The pI's of the NAGase activities were determined by isoelectric focusing, again using standards with known isoelectric points. The correspondence between M_r and pI values was deduced from analysis using a simple two dimensional eletrophoresis system, described in section 2.8.7, and Rotofor isoelectric focusing, described in section 2.8.6.

Table 7. Intracellular NAGase activity in trichomonads.

species	specific activity (nmol/min/mg cell protein)
Tritrichomonas foetus, F2	740 ± 112.2
KV1	690 ± 56.8
CA84-2	186
Tritrichomonas augusta	322 ± 42.1
Trichomonas vaginalis	97 ± 10.3

Standard deviations calculated from three separate determinations although this was not possible for *Tritrichomonas foetus* strain CA84-2 owing to lack of time.

the parasites to serum free medium (figure 11). During incubation total NAGase activity in the extracellular medium increased continuously over the period. This was demonstrated both by activity assays and SDS-PAGE. The release of the enzymes was also investigated by sampling the medium every hour, concentrating by ammonium sulphate precipitation, separating by SDS-PAGE and staining for NAGase activity (figure 12). The relative levels of the four forms of NAGase were similar throughout the incubation period. The different mobilities of the enzymes in different samples are unlikely to reflect variations in M_rs but are almost certainly caused by the differing amounts of concentrated medium components present in the samples. This is supported by the observations that the molecular weights of the NAGase forms can be seen to be approximately the same after 6h as after 1h. The apparent differences seen after 2 and 3 h are probably artifactual owing to the greater concentration factor needed in the first hours of the experiment.

Four intracellular enzymes with NAGase activity were also observed after analytical isoelectric focusing of *Tritrichomonas foetus* lysates and crude medium preparations (figure 13). Using a simple two dimensional electrophoresis system (section 2.8.7) it was shown that NAGase 4 (apparent M_r 158 000) had a pI of 4.6 and NAGase 1 (apparent M_r 54 000) a pI of 4.5. The two other forms, could not be separated using this method. However, the relationships of these forms to their isoelectric points were subsequently determined using preparative isoelectric focusing, described later in section 3.3.2. The pI values of 4.7 and 4.9, corresponded to NAGase 2 and 3 respectively as defined in table 6. The additional band seen in the cell lysate with a pI of 4.5 is probably the 85 000 M_r form although this has not been confirmed.

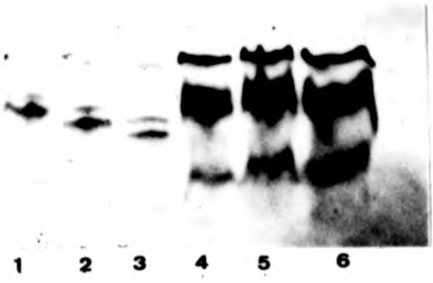


Figure 12. Analysis by SDS-PAGE of the release of NAGase activity during 6h incubation in serum free MDM. Lane numbers refer to the time in hours that samples were collected after initial cell resuspension. Cells were inoculated at an approximate density of 2 x 10^6 cells/ml. These samples represent equal volumes of medium.

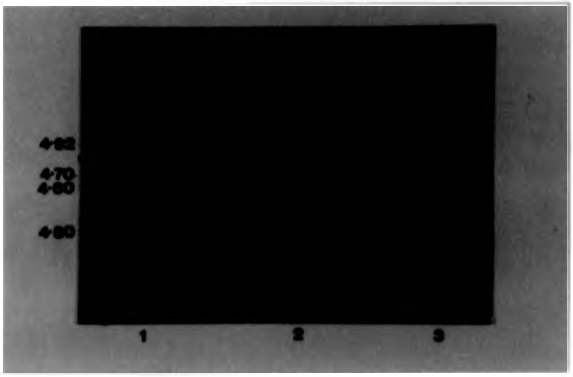


Figure 13. Isoelectric focusing gel of the NAGase activities from *Tritrichomonas foetus*, clone F2.

Lane (1) *Tritrichomonas foetus* cell lysate. (2) *Tritrichomonas foetus* crude medium preparation. (3) a mix of the cell lysate and crude medium preparation shown in lane (1) and (2). approximately 50 nmol / min NAGase activity loaded to each lane.

Trichomonads have high levels of cysteine proteinase activity which could have altered the size during sample preparation and analysis, but the addition of the proteinase inhibitors leupeptin and E-64 to samples before electrophoresis did not affect the NAGase activity patterns (section 3.2.4).

The NAGase activities of additional strains of *Tritrichomonas foetus* and other species of trichomonad were investigated to examine any species specific or strain specific enzyme forms. *Tritrichomonas foetus* strain, KV1, was investigated using the SDS-PAGE staining and the preparative isoelectric focussing procedures described in section 2.8.6. This strain had been originally cloned from the same isolate as strain F2 and had an intracellular specific activity of 690 ± 56.8 nmol/min/mg cell protein for NAGase (table 7). Five major forms of intracellular and extracellular NAGase were identified with apparent M_rs of 54 000, 85 000, 89 000, 104 000 and 158 000 (figure 14). Four of these forms have near identical apparent molecular weights to those described for *Tritrichomonas foetus* F2. A NAGase activity of similar size to the apparent 85 000 M_r NAGase form of strain KV1 was also occasionally seen in strain F2 at much lower activity (data not shown here). It is probable that the NAGase forms in these two strains represent identical or near identical proteins expressed at different relative levels.

The NAGase activity from another clone of *Tritrichomonas foetus*, CA84-2, was also investigated. This trichomonad was recently isolated in California (Burgess 1988) and is completely independent of the strains F2 and KV1. Figure 15 compares the NAGase activities of F2 and CA84-2. Four major forms of NAGase activity were again found in strain CA84-2. However there

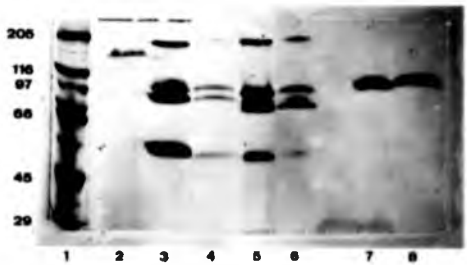


Figure 14. Activity staining of NAGase on SDS-PAGE.

Lane (1) Molecular weight standards (2) *Trichomonas vaginalis* cell lysate (3) *Tritrichomonas foetus*, clone F2, cell lysate (4) *Tritrichomonas foetus*, clone F2, crude resuspension medium preparation (5) *Tritrichomonas foetus*, clone KV1, cell lysate (6) *Tritrichomonas foetus*, clone KV1, crude resuspension medium preparation (7) *Tritrichomonas augusta* cell lysate (8) *Tritrichomonas augusta* crude resuspension medium preparation. Approximately 50 nmol / min NAGase activity loaded to each lane.

Figure 15. Activity staining of NAGase on SDS-PAGE.

Lane (1) *Tritrichomonas foetus*, clone F2, cell lysate (2) *Tritrichomonas foetus*, clone CA84-2, cell lysate. Approximately 50 nmol/min NAGase activity loaded to each lane.

was a difference in that the relative intensities of staining for NAGase 1 and NAGase 3 in CA84-2 were the opposite of that in F2. This strain was found to have a intracellular specific activity of 186 nmol/min/mg cell protein for NAGase (table 7). This is slightly lower value than that found for strains F2 and KV1 although it was only possible to perform one assay for this strain and so no definite conclusions can be made. The results did confirm that the general NAGase activity banding pattern is a common feature of *Tritrichomonas foetus*.

Trichomonas vaginalis cells, which have a specific activity of 97 ± 10.3 nmol/min/mg cell protein for NAGase (table 7), have much lower levels of activity than *Tritrichomonas foetus* and also release less of this enzyme into the medium (figure 9). Only one intracellular NAGase enzyme was detected by SDS-PAGE and this has an apparent M_r of 138 000 (figure 14). Unfortunately, owing to the relatively low sensitivity of the staining method no extracellular enzyme(s) has been detected after either SDS-PAGE or isoelectric focusing, although extracellular NAGase activity can be detected using 4-nitrophenyl-N-acetyl-ß-glucosamide as a substrate in a microtitre plate assay (data not shown).

The NAGase activities of *Tritrichomonas augusta*, a non-pathogenic intestinal trichomonad, were also investigated. One major form with an apparent M_r of 107 000 was detected in cell lysates and an identical form was apparent in extracellular samples (figure 14). NAGase activity from cell lysates of this organism was found to have a specific activity of 322 ± 42.1 nmol/min/mg cell protein (table 7).

3.2.3 Determination of molecular weights by gel filtration

Gel filtration is commonly used to determine the molecular weights of proteins in their native forms. In the case of gel filtration chromatography the proteins are separated on the basis of their relative ability to enter pores in the beads of the matrix. Generally smaller proteins are able to enter the pores and hence have a greater available volume to pass through, resulting in greater column retention times compared to larger molecules which pass directly through the spaces between the beads of the matrix.

The native molecular weights of the NAGase of *Tritrichomonas foetus*, strain F2, *Trichomonas vaginalis* and *Tritrichomonas augusta* were determined by gel filtration chromatography using a Sephacryl 300 column as described in section 2.8.5. These results are shown in figure 16A. Surprisingly, the intracellular NAGase activity from *Tritrichomonas foetus* eluted as a single peak which corresponded to an M_r of 80 000 (figure 16B). The determination was repeated several times and the same result obtained even when samples were run in the presence of 1M NaCl. The fractions of the activity peak from the gel filtration column were analysed by SDS-PAGE. All four forms of NAGase were found in every fraction and appeared to co-elute from the gel filtration column (data not shown). Gel filtration was repeated using a Sephadex G-150 gel matrix (section 2.8.5) and again one peak of NAGase activity was found. The extracellular NAGase activity of *Tritrichomonas foetus*, F2 was also found to elute from the Sephacryl 300 column in a single peak coinciding with an approximate M_r of 80 000. The single peak of NAGase activity is discussed in section 4.3.

Trichomonas vaginalis and *Tritrichomonas augusta* NAGase activities were

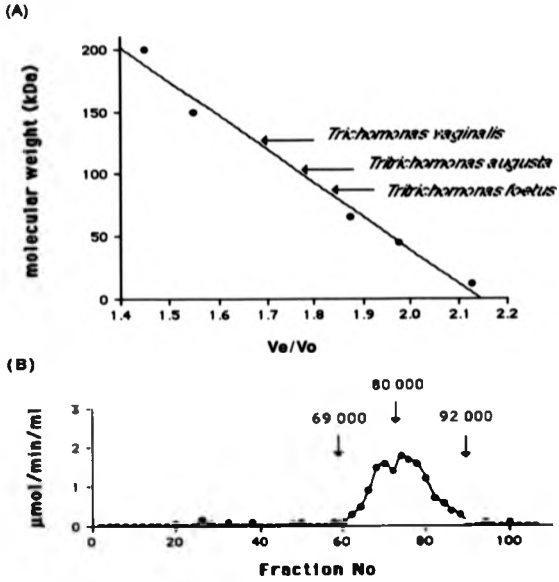


Figure 16. Gel Filtration Chromatography using a Sephacryl 300 column. Details of the column are described in section 2.8.5. (A) Positions of the NAGase activity peaks of the three species of trichomonads are indicated with arrows. Elution volume of the protein / Void volume of the blue dextran standard is indicated as Ve/Vo. (B) Profile of NAGase activity from *Tritrichomonas foetus* cell lysate. Arrows indicate M_r positions of the NAGase activity peak. Approximately 1mg protein loaded to each column run and NAGase activity assays were performed as described in section 2.6.

eluted in fractions corresponding to apparent M_rs of 130 000 and 100 000 respectively. Thus the molecular weights detemined by gel filtration for the NAGase of these species corresponded closely to those found by SDS-PAGE (section 3.2.2).

3.2.4 The subcellular distribution of the four forms of NAGase

There is evidence that the lysosomal population of trichomonads may be heterogeneous. To investigate the question of whether NAGase forms are differentialy distributed among the compartments, subcellular fractions of *Tritrichomonas foetus* were obtained using the method described in section 2.4.

Because of high proteinase activity in lysosomes, leupeptin, a cysteine proteinase inhibitor, was included in one set of samples to suppress the endogenous proteinase activity and allow examination of the extent of proteolysis in the fractions (North 1989). Some samples were also incubated at 100°C for 2 min before electrophoresis to inhibit any proteolysis (lanes 1-6 of figure 17). Comparison of the two coomassie blue stained gels (figure 17) shows that proteolysis did occur, especially in the large particle fraction, and was inhibited in the leupeptin-containing samples. The differing extents of proteolysis suggested that most proteinases were contained in the large particle fraction in agreement with the known location of the cysteine proteinases (Lockwood *et al.* 1988).

The highest NAGase activity was contained in the large particle fraction (figures 18). All four forms were present in all the fractions. NAGase 4 was detected in all the fractions although very faintly in the nuclear and small

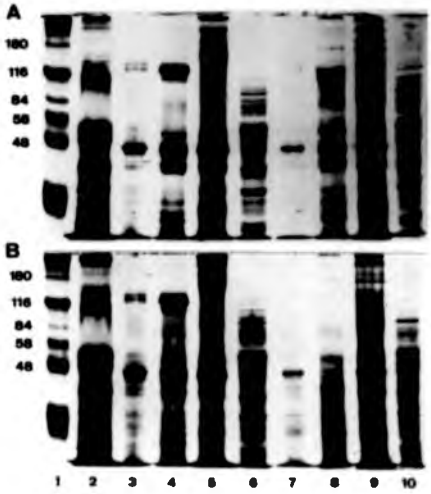


Figure 17. Protein analysis of the subcellular fractions of *Tritrichomonas foetus*.

Lane (1) Molecular weight standards (2) cell lysate, 50 µg protein loaded (3,7) nuclear fraction, 5 µg protein loaded (4,8) large particle fraction, 15 µg protein loaded (5,9) small particle fraction, 50 µg protein loaded (6,10) non-sedimental fraction, 7 µg protein loaded

Samples run in lanes 1-6 of both gels were incubated at 100°C for 2 min before electrophoresis. Samples run in lanes 7-10 were not incubated at 100°C for 2 min before electrophoresis. All samples in gel A contained leupeptin (10µM) which was added at the same time as the sample buffer before heating.

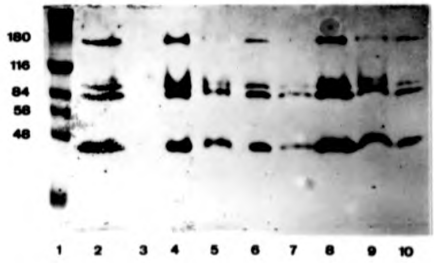


Figure 18. SDS-PAGE analysis of the NAGase activity of subcellular fractions of *Tritrichomonas foetus* .

Lane (1) Molecular weight standards (2) cell lysate (3,7) nuclear fraction (4,8) large particle fraction (5,9) small particle fraction (6,10) non-sedimented fraction.

Approximately 25 µg protein loaded to each lane. Samples in lanes 7-10 were incubated with leupeptin before and during electrophoresis to inhibit proteinase activity. Samples run in the other lanes were not incubated with a proteinase inhibitor.

particle fractions and cannot be seen from figure 18. The relative activities of the four major forms in each fraction were the same indicating that there was no differential subcellular localisation of the different forms. Interestingly, although the presence of leupeptin inhibited the otherwise extensive proteolysis in the large particle fraction, all four major forms of NAGase activities were still observed in the leupeptin-treated samples, suggesting it was unlikely that any of the NAGase forms was generated by proteolysis of another of the major forms. The enzymes appear to be resistant to proteinases released during cellular disruption.

3.2.5 The effect of various agents on *Tritrichomonas foetus* NAGase activity forms

As an initial investigation into the processes which may be involved in the biosynthesis and release of the different forms of NAGase from *Tritrichomonas foetus*, the parasites were grown and incubated in the presence of various agents which might interfere with these processes. Table 8 indicates the effects of the agents tested while table 9 gives the concentrations used and at which stage the agents were tested i.e. whether during growth or after resuspension of the parasites.

Parasites grown in serum containing MDM were inoculated at 5×10^4 cells/ml. The growth of the cells was monitored by microscopic observation at regular intervals. Cells were collected by centrifugation as described in section 2.3.1 and spent media filtered through 0.2 µm nitro-cellulose Nalgene filters to remove any remaining cells. NAGase activity in the cells and the spent medium was analysed. For experiments involving

Table 8. Agents tested in the investigation of NAGase release from *Tritrichomonas foetus*.

Agent	inhibition	Reference
Ethanol	solvent for cycloheximide and monensin	
Cycloheximide	inhibits protein synthesis, by preventing peptidyl transferase activity	Stryer (1988) p 759
Monensin	ionophore which disrupts golgi function	Stryer (1988) p 990
Tunicamycin	blocks N-linked glycosylation by inhibiting the addition of Glc NAc to dolichol phosphate , the first step in the formation of the core oligosaccharide	Stryer (1988) p 778
NH_4Cl	elevation of the lysosomal pH	Seglen (1983)
Glc NAc	substrate analogue	
Sucrose	changes the osmotic potential of the medium	
Hydroxynorvaline	inhibitor of N-linked glycosylation as it is an analogue of threonine and replaces this amino acid in the tripeptide required for glycosylation.	Docherty & Aronson (1985)

E64 *	irreversible inhibitor of cysteine proteinases, acts by binding to the active site	North (1989) North *et al.* (1990a)
Leupeptin *	inhibitor of serine and some cysteine proteinases, acts by binding to the active site	North *et al.* (1990a)
Z-Phe-Arg CHN_2 * Z-Phe-Phe CHN_2 *	irreversible cysteine proteinase inhibitors which react with the cysteine active site	North *et al.* (1990)

* These proteinase inhibitors have been shown to be effective against the proteinases of *Tritrichomonas foetus*.

Table 9. Analysis of NAGase release from *Tritrichomonas foetus* , clone F2, in the presence of various agents.

agent	concentration	reference for concentration used	growing cells	resuspended cells
Ethanol	0.1%		•	
Cycloheximide	1.7 mM	North *et al.* (1989)	•	
Monensin	2 µM	Tapper & Sundler (1990)	•	
Tunicamycin	2.4 - 24 µM	North *et al.* (1989)	•	•
NH_4Cl	15 mM	Seglen (1983)		•
Glc NAc	0.1M			•
Sucrose	0.1M			•
Hydroxynorvaline	10 mM	Docherty & Aronson (1985)		•
E64	0.28 mM	North *et al.* (1990)		•
Leupeptin	0.4 mM	"		•

Z-Phe-Ala CHN_2	10μM	North *et al.* (1990)	*
Z-Phe-Phe CHN_2	20 μM	North *et al.* (1990)	*

* indicates that cells were grown in complete MDM, including the agent, or resuspended for a 6h period in serum free MDM, including the agent. The agents were added at the concentrations indicated above.

resuspended parasites, cells were collected during log phase growth as described in section 2.3.2, then resuspended in serum free media at a density of approximately 5 x 10^6 cells/ml and incubated for a further 6 h.

Of the reagents tested for their effect during growth neither tunicamycin (1.22 μM), deoxyglucose (10 mM) nor monensin (2μM) had any measurable effect on growth (figure 19). Higher concentrations of tunicamycin (2.44 μM to 24.4 μM) did inhibit the growth of *Tritrichomonas foetus* cells in a concentration dependent manner (figure 20). Cycloheximide (1.7 mM) also had an inhibitory effect, reducing the growth rate by two-fold (figure 19).

Deoxyglucose, monensin, cycloheximide and tunicamycin at all the concentrations tested had no effect on the level of NAGase activity either inside or outside the cell. SDS-PAGE analysis revealed that deoxyglucose, monensin and cycloheximide had no effect on the pattern of NAGase activities in the cells. As explained in section 3.2.2 it was not possible to analyse the extracellular growth medium. With tunicamycin, however, an extra band of intracellular NAGase activity, with an apparent M_r of 48 000, was evident. The relative activity of NAGase 3 was always lower in experiments (figure 21) but a direct relationship between this extra band and NAGase 3 or one of the other 'normal' NAGase forms has not been demonstrated. It seems likely, however, to have been a non-glycosylated form of one of them.

When cells were resuspended for 6 h in serum free medium with NH_4Cl (15 mM), GlcNAc (0.1 M), sucrose (0.1 M), hydroxynorvaline (10mM), E-64 (0.28 mM), Z-Phe-Ala-CHN_2 (10 μM) or tunicamycin at concentrations up to 24.4

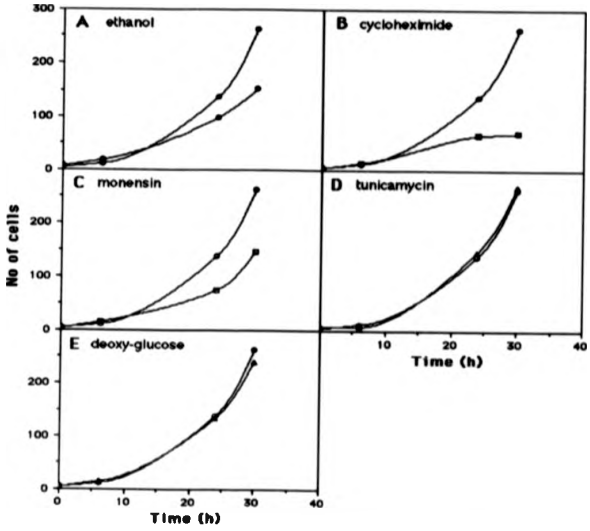


Figure 19. Analysis of the growth of *Trichomonas foetus* in the presence of various agents.

In all the graphs the control cells are indicated for comparison (•). Graph (A) ethanol 0.1% (B) cycloheximide 1.7 mM (C) monensin 2 μM (D) tunicamycin 2.44 μM (E) deoxy-glucose 10 mM.

Number of cells x 10^4.

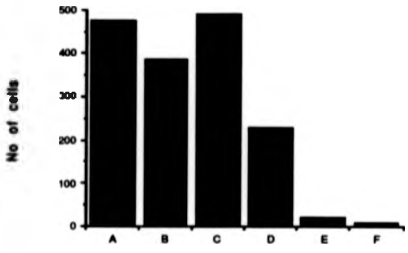


Figure 20. Inhibition of the growth of *Tritrichomonas foetus* after 55 h of growth in the presence of various concentrations of tunicamycin.
Column (A) control (B) methanol 0.1% (C) tunicamycin 2.4 μM (D) tunicamycin 9.6 μM (E) tunicamycin 19.2 μM (F) tunicamycin 24 μM.
Number of cells x 10^4. Cells inoculated at 10^4 cells/ml.

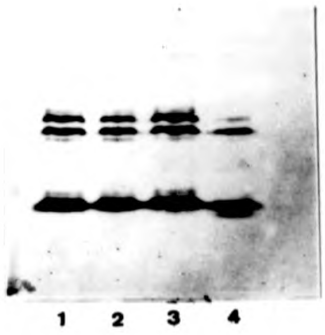


Figure 21. NAGase activity from *Tritrichomonas foetus* in the presence of tunicamycin.

The cells were grown for 50 h before being harvested in the usual method (section 2.3.1). Approximately 30 nmol/min NAGase activity loaded to each lane.

Lane (1) cell lysate (2) lysate of cells grown in the presence of 1% (v/v) methanol (3) lysate of cells grown in the presence of 2.4 μM tunicamycin (4) lysate of cells grown in the presence of 9.6 μM tunicamycin.

μM there was no effect on the amount of NAGase activity in the cells or released by them. Furthermore SDS-PAGE analysis showed identical patterns of NAGase staining for control cells and those incubated with the various reagents. As an example the activity gels for tunicamycin-treated cells are shown (figure 22). There is no evidence for an extra '48 000 M_r' band apparent after long-term treatment of growing cells. This highlights the difficulties of finding reagents which affect NAGase activities and patterns in short term experiments. Although NAGase 1 has been shown to be a glycoprotein it was not altered in either the growth or resuspension experiments described in this section.

There were, however, two reagents which had some effect on NAGase after cells were resuspended in serum free medium. Incubation with either of the cysteine proteinase inhibitors leupeptin (0.4 mM) or Z-Phe-Phe-CHN_2 (20μM) resulted in a change in the NAGase staining pattern for intracellular samples (figure 23). Extra bands of NAGase activity were present at positions corresponding to apparent M_r values in the range from 70 000 to 60 000 with one additional band with an apparent M_r of 45 000. In the particular example shown the fifth NAGase band ('85 000 M_r'), which could only sometimes be detected in *Tritrichomonas foetus* F2 samples, is readily apparent at high levels in both the control and inhibitor-treated cells. Surprisingly it was also present in the medium of cells incubated with Z-Phe-Phe-CHN_2. The results suggest that there are additional active forms of NAGase which in the absence of inhibitors are rapidly converted by proteolysis to other active or inactive forms. The four major forms, however, have been shown to be stable to proteolysis (section 3.2.4). Neither inhibitor affected the net level of NAGase activity in the cells or the medium when measured by assays (section 2.6).

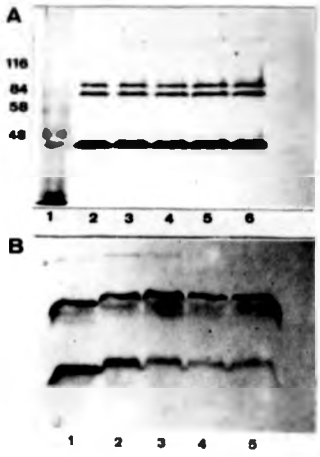


Figure 22. SDS-PAGE analysis of NAGase activity after 6 h resuspension of *Tritrichomonas foetus* in the presence of tunicamycin.

Gel A : Cell lysates of harvested cells (section 2.3.1). Lane (1) Molecular Weight Markers (2) control (3) methanol 1% (v/v) (4) 4.8 µM tunicamycin (5) 12 µM tunicamycin (6) 24 µM tunicamycin.

Gel B : Samples of resuspension medium which were filtered through 0.2 µm nitrocellulose filters and concentrated by ammonium sulphate precipitation (section 2.5). Lane (1) control (2) methanol 1% (v/v) (3) 4.8 µM tunicamycin (4) 12 µM tunicamycin (5) 24 µM tunicamycin.

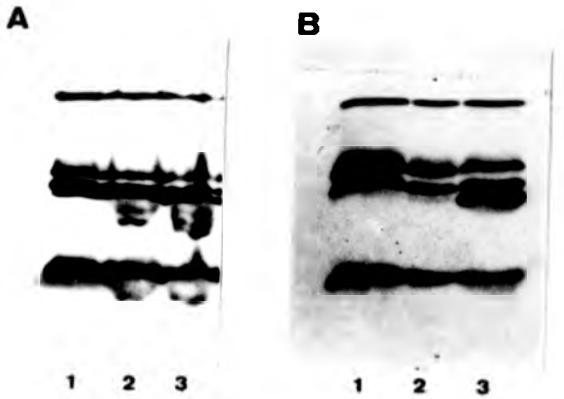


Figure 23. SDS-PAGE analysis of NAGase activity after 6 h incubation of *Tritrichomonas foetus* in the presence of cysteine proteinase inhibitors.
Samples of gel A are cell lysates of harvested cells (section 2.3.1) while samples of gel B are prepared by concentration of the incubation medium using ammonium sulphate precipitation (section 2.5).
Lanes (1) control (2) 0.4 mM leupeptin (3) 20 μM Z-Phe-Phe CHN_2

There was a clear difference between the effects of Z-Phe-Phe CHN_2 and Z-Phe-Ala CHN_2, the latter having no effect on the NAGase activity pattern. This may, however, have been due to the lower concentrations of Z-Phe-Ala CHN_2 used. Z-Phe-Ala CHN_2 appears to be a better inhibitor of cysteine proteinases in cell lysates although it is more significant that Z-Phe-Phe CHN_2 is a more effective inhibitor of cell growth (North *et al.* 1990). This may reflect differences between the ability of the two inhibitors to enter the cells which could also have been responsible for the difference seen in the experiments described here.

Only limited information is available from these types of experiment, and although longer incubation times might have yielded more data on the effects of the various agents tested it was not appropriate to increase the resuspension periods owing to the eventual degeneration and lysis of the parasites leading to false results. Moreover only active forms of NAGase can be measured and so any biosynthetic precursers are not detected and therefore a complete picture of NAGase biosynthesis cannot be gained. These limitations might be overcome if antibodies to one or more of these NAGase forms were available. The purification of NAGase, which would then be used to raise specific antibody, was therefore a requirement for further studies.

3.2.6 Summary

SDS-PAGE analysis has characterized and demonstrated the existence of four major NAGase forms present both intracellularly and extracellularly in all *Tritrichomonas foetus* strains and one form in each of *Trichomonas vaginalis* and *Tritrichomonas augusta*.

Growth of *Tritrichomonas foetus* in the presence of tunicamycin, an inhibitor of N-linked glycosylation, generated an extra band of NAGase activity and a reduced NAGase 3 activity on SDS-PAGE. The extra band probably represents a deglycosylated form. Resuspension of this parasite in the presence of the cysteine proteinase inhibitors leupeptin and Z-Phe-Phe CHN_2 also generated a number of extra bands possibly owing to interference in biosynthetic processing. This will be discussed later.

3.3 Separation of NAGases and the purification of NAGase 1 from *Tritrichomonas foetus*

3.3.1 Introduction

The most widely used methods for the purification of specific proteins from crude samples are those of ion-exchange, gel filtration, hydrophobic interaction and affinity chromatography.

Ion-exchange chromatography relies on the ability of a particular protein to bind to a charged matrix at a specific pH. Proteins with no net charge or the same charge as the the gel matrix will pass straight through the column, while oppositely charged proteins bind. Bound proteins are eluted either by changing the pH and hence the net charge of the protein or by the application of a salt gradient to displace the protein by ion exchange.

Hydrophobic interaction chromatography is another commonly used purification procedure. This technique relies on the ability of hydrophobic portions of a protein to associate with a non-polar matrix, not primarily because they have a high affinity for each other, but because water bonds strongly to itself, driving the hydrophobic interaction. The bound proteins are then eluted from the column using an ammonium sulphate gradient which acts to change the properties of the elution buffer. Different proteins are eluted at different concentrations depending on their hydrophobicity.

Specific affinity chromatography has more recently been developed and is a very powerful protein purification technique. This technique takes advantage

of the high affinity of many proteins for specific chemical compounds, these often being the proteins natural substrates, ligands and cofactors; or analogues of these. The specific chemical groups are covalently attached to a matrix such as cyanogen bromide activated Sepharose and it is often possible to bind a specific protein of interest from a very crude mixture. This protein can then be eluted by washing with a buffer containing the free substrate, ligand, cofactor or analogues of these.

It is clear from foregoing experiments that there is more than one NAGase form in *Tritrichomonas foetus* and so purification is needed not only to separate NAGase from other proteins but ideally also to purify a single enzyme form. The purification of an enzyme is a crucial stage in any detailed investigation to characterise its activity and structure and as a first step in the raising of antisera. This leads to information about any critical roles it may play in biological systems.

3.3.2 Preparative isoelectric focusing using the Rotofor Cell

The Rotofor cell is a preparative, free solution, isoelectric focusing apparatus that provides a new procedure for protein characterisation and purification. Separation of proteins by isoelectric focusing is based on the fact that all proteins have a pH dependent net charge. This net charge is determined by the amino acid sequence of the protein, the carbohydrate composition and the pH of the environment. When a protein is electrophoresed through an established pH gradient, it will migrate until it reaches the pH at which the net charge on the protein is zero; at that point it is said to be focused.

Ampholytes which are small, charged molecules are used to establish the pH

gradient. When voltage is applied to a system of ampholytes and proteins, all the components migrate to their respective pI's. Ampholytes rapidly establish the pH gradient and maintain it for long periods, allowing the slower moving proteins to focus in that gradient. Ion exchange membranes are used in the Rotofor cell to separate the sample from the electrolyte while allowing current to pass through and set up the pH gradient. Cation and anion exchange membranes are used. The cation exchanger is negatively charged and will repel negatively charged ions, preventing them from contaminating the electrolyte. The anion exchanger works in the opposite way, its positive charge repelling positive ions. Using the ion exchange membranes gives a concentration gradient of the ions at the respective ends of the sample chamber. The highest concentration of negative ions will be next to the cation exchanger and the highest concentration of positive ions will be next to the anion exchanger.

The cell incorporates a cylindrical focusing chamber with an internal cooling finger. Separation of proteins occurs within the annulus formed by the inside wall of the chamber and the cooling finger. Rotation at 1 rpm around the focusing axis stabilizes the solution against convective and gravitational disturbances. A series of 19 parallel monofilament polyester membranes divide the focusing chamber into 20 discrete compartments each holding one fraction. After focusing the solution in each compartment is rapidly collected, without mixing. This method is especially advantageous when activity needs to be maintained as the proteins are in solution in their native conformation. After the initial fractionation, proteins can be further purified by refractioning over a narrower pH gradient and a higher voltage which leads to a better separation of the protein. Biorad have reported that the preparative fractionation of 51 ml of lyophilized cell culture supernatant containing

approximately 2.4g of protein has been successfully performed.

The Rotofor cell was used primarily to separate the four forms of NAGase activity found in *Tritrichomonas foetus* (section 3.2.2), identify their respective isoelectric points and act as a major step in the purification of NAGase (section 3.4.4).

3.3.3 Analysis of trichomonad proteins by Rotofor isoelectric focusing

Preparative isoelectric focusing was performed using the Rotofor cell as outlined above and described in detail in section 2.8.6. Because of the capacity of the Rotofor to deal with large amounts of protein, effective separation of proteins can be achieved from very crude samples. Figure 24 is a coomassie stained gel showing the separation of the proteins from *Tritrichomonas foetus* crude cell lysates by preparative isoelectric focusing. Different proteins can be seen to have focused at their isoelectric points along the pH gradient created during the Rotofor run. For example, a major protein with an approximate Mr of 70 000 has focused between pH 4.5 and 4.7 and another with an apparent Mr of 50 000 at a pH of 5.9.

3.3.4 Analysis of NAGase from *Tritrichomonas foetus* strains and other trichomonad species by Rotofor isoelectric focusing

NAGase activities from *Tritrichomonas foetus* (F2 and KV1), *Trichomonas vaginalis* and *Tritrichomonas augusta* were initially characterised and compared using preparative isoelectric focusing.

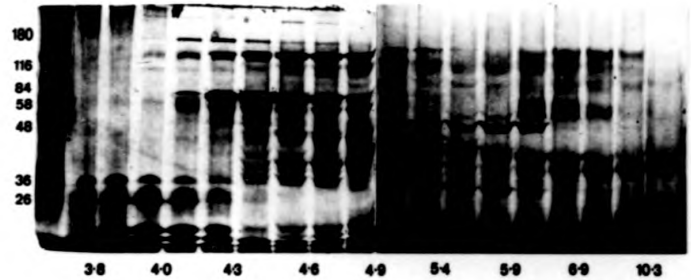


Figure 24. Two dimensional separation of proteins from *Tritrichomonas foetus*, clone F2, cell lysates by preparative isoelectric focusing (the Rotofor IEF cell) and SDS-PAGE. The molecular weight standards are indicated in the first lane and the pH of the fractions is indicated along the bottom of the gels. Approximately 300 mg of protein loaded to the Rotofor.

The NAGase activities of the two strains of *Tritrichomonas foetus*, F2 and KV1, have been shown by SDS-PAGE to be identical with respect to the number of enzyme forms and to their corresponding molecular weights (section 3.2.2). By preparative isoelectric focusing (figure 25) it was found that NAGase activity from these strains also have similar pI's. From these initial studies it can be concluded that NAGase activities from both F2 and KV1 are similar. All four NAGase activities were focused in a pH range between 4.5-5.5 which confirms findings from the analytical isoelectric focusing results described in section 3.2.2.

The NAGase activity in *Tritrichomonas augusta* focused at a slightly higher pH of 5.5 (figure 25). This figure also shows that the *Trichomonas vaginalis* NAGase activity focused at pH 4.5.

After preparative isoelectric focusing the yield of NAGase activity from *Tritrichomonas foetus* and *Tritrichomonas augusta* was about 40% of the total activity applied. For *Trichomonas vaginalis* the yield was much lower at 15% (figure 25). Such yields are not impressive and losses may have been due to a number of factors. Precipitates were formed during the Rotofor runs of trichomonad material in fractions corresponding to pH's in the range 3.0-7.0. These fractions contained the NAGase activities and the precipitation could result in a percentage of the NAGase activity being lost. These precipitates did not redissolve on dialysis of fractions containing them against buffers of higher pH (7.5-10.0). The proteins may also be subjected to inactivation under the pH conditions involved during the procedure. Another possible source of activity loss could be caused by the occurence of proteolysis. It has, however, been shown that proteinases are unlikely to have degraded NAGase (section 3.2.4) and for this reason and the high cost at this scale

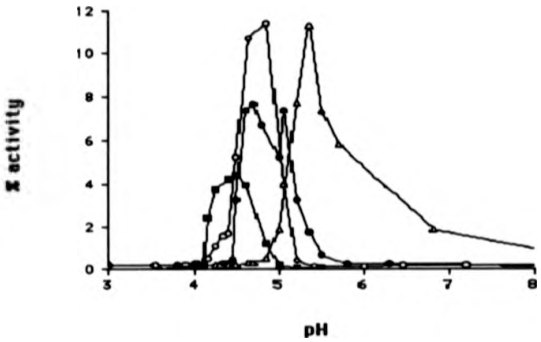


Figure 25. Analysis of NAGase activity by preparative isoelectric focusing of *Tritrichomonas foetus*, F2 and KV1, *Tritrichomonas augusta* and *Trichomonas vaginalis*.

Tritrichomonas foetus F2 (•), NAGase activity loaded 19 µmol/min, yield of activity 45.7%. *Tritrichomonas foetus* KV1 (o), NAGase activity loaded 27 µmol/min, yield of activity 38.1%. *Tritrichomonas augusta* (Δ), NAGase activity loaded 14.4 µmol/min, yield of activity 39.1%. *Trichomonas vaginalis* (▪), NAGase activity loaded 3.3 µmol/min, yield of activity 15.7%.

Preparative isoelectric focusing was performed, using the Rotofor IEF cell, separately on each of the four trichomonad species. The harvested fractions were then assayed for NAGase activity using the usual assay described in section 2.6 and the pH of each fraction identified.

proteinase inhibitors were not included during the Rotofor runs.

3.3.5 Complete separation of the four major forms of NAGase from *Tritrichomonas foetus*

Three Rotofor runs were used to separate and partially purify NAGase activities (table 10). The original samples used were crude lysates of *Tritrichomonas foetus*. Typically each of the original samples contained approximately 300 mg of protein. This sample was run in the Rotofor Cell, the harvested fractions assayed for NAGase activity, and the active fractions then pooled. The pooled fractions were then collected and divided into two samples which were re-run consecutively without the addition of more ampholytes. This resulted in the further separation of the activities owing to the increase in the spread of the narrower pH range across the twenty fractions. The initial pH gradient, ranging from pH 2.0-11.0 in the first run, changed to a significantly narrower spread of pH, e.g. 4.0-6.0, during the second run. After the second set of Rotofor runs the fractions containing NAGase activity were again split, as shown in table 10, and then concentrated using an Amicon XM50 filter. Table 11 shows the specific activities and percentage yields of NAGase activity for this procedure. Analysis of the concentrated samples was also performed using SDS-PAGE (figure 26) after which NAGase 4 activity was not detected. Almost complete separation of the remaining three forms was, however, achieved. The identification of forms 2 and 3 (table 6) was important in showing that NAGase 3 (M_r 100 000) focused at a higher pH than NAGase 2.

As separation of the enzyme forms was possible and single bands were seen on activity gels the results from SDS-PAGE, analytical isoelectric

Table 10. The partial purification and separation of NAGase from *Tritrichomonas foetus* by preparative isoelectric focusing. The pH of the pooled samples are indicated. More details can be found in table 11.

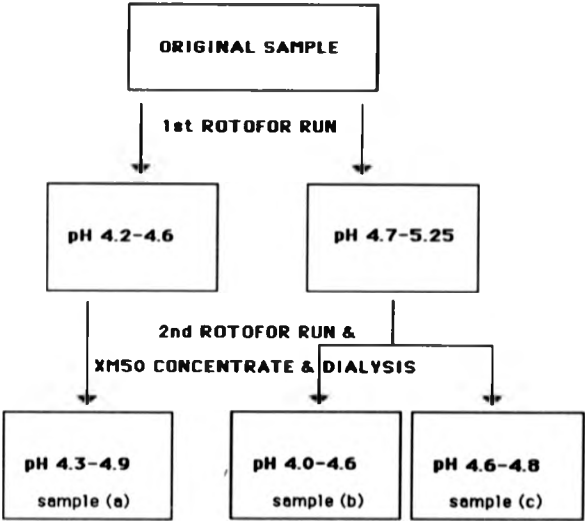


Table 11. The partial purification and separation of NAGases from *Thitrichomonas foetus* by the Rotofor IEF cell.

sample	volume ml	total protein mg	total activity nmol/min	specific activity nmol/min/mg protein	% yield
cell lysate	24.0	321.0	99 000	317	100.00
run 1	19.0	22.0	4 700	215	5.00
run 2	1.5	6.0	2 880	484	3.00
sample (a)	0.5	2.0	950	475	0.96
sample (b)	0.5	2.1	980	478	0.99
sample (c)	0.5	1.9	950	500	0.96

Run 1 and run 2 and samples a, b and c refer to the stages described in table 10.

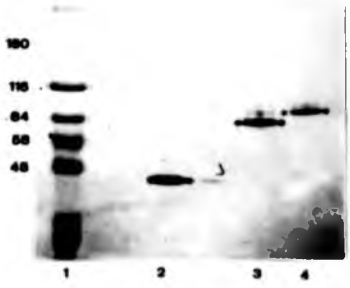


Figure 26. The separation of the NAGase activities by SDS-PAGE from extracellular samples of *Tritrichomonas foetus* by the Rotofor IEF cell.
Lane (1) Molecular weight standards (2) sample a (3) sample b (4) sample c.
See tables 10 and 11 for information on samples a, b and c.

focusing and now preparative isoelectric focusing have shown that four major forms of NAGase activity exist in *Tritrichomonas foetus*. Therefore these results confirmed that the four forms of NAGase are not generated as a result of using SDS-PAGE. The Rotofor cell was used in the purification of NAGase 1 described in section 3.3.7.

3.3.6 Purification of NAGase enzymes of *Tritrichomonas foetus* from resuspension medium

The purification of NAGase from the resuspension medium of *Tritrichomonas foetus* was attempted to try and take advantage of the release of this enzyme. The process should act to separate this enzyme from the thousands of non-released intracellular proteins present when lysates are the starting material.

Log phase growth medium was used as the starting material for purification. The original growth medium contained NAGase at a specific activity of 1.3 µmol/min/mg protein which increased to 4.12 units with a yield of 88% after precipitation in the 0-60% ammonium sulphate fraction (table 12).

After dialysis all this material was loaded onto a DEAE cellulose column. A DEAE anion exchange column was chosen as all four major forms of NAGase had an isoelectric point below pH 7.0. They would therefore be negatively charged at pH 7.0 and hence bind to the DEAE column. A gradient of ammonium sulphate was then used to elute the proteins bound to the column. The NAGase activity eluted as a single peak at about 0.25 M ammonium sulphate (figure 27). The pooled peak fractions from the DEAE cellulose column were then loaded onto a phenyl-Sepharose column and,

Table 12. The partial purification of extracellular NAGase from *Thtrichomonas foetus* .

	volume (ml)	total protein (mg)	total activity (µmol / min)	specific activity µmol/min/mg protein	% yield
1	660	23.0	29.8	1.30	100.0
2	15	6.4	26.2	4.12	88.0
3	4	2.1	25.7	12.24	86.2
4	18	0.8	9.4	12.00	32.0
5	6	0.3	1.0 (3.0)	3.30 (9.90)	3.4 (10.2)

The stages of purification are numbered as follows : (1) Original filtered growth medium. (2) 0 - 60 % fraction of an ammonium sulphate precipitation of the growth medium. (3) Ion exchange chromatography. (4) Hydrophobic interaction chromatography. (5) affinity chromatography. The numbers in the brackets represent approximate values that have been corrected for inhibition by N-acetyl-glucosamine. It has been extracted from figure 30 that the N-acetyl-glucosamine present in the samples inhibited NAGase activity by approximately three fold after affinity chromatography.

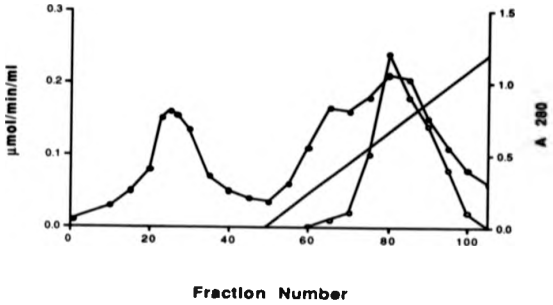


Figure 27. Ion exchange chromatography with DEAE Sepharose. Extracellular NAGase activity (µmol/min/ml) distribution (o) and protein measured at A280 (•) is shown. The salt gradient (0-0.5M) is represented by the diagonal line. The sample loaded was prepared by ammonium sulphate precipitation and dialysis as described in section 2.5.

after reduction of the salt concentration, the NAGase activity again eluted as a single peak containing 32% of the original activity (figure 28). This represented a significant loss of NAGase activity and did not result in any increase in the specific activity (table 12).

Affinity chromatography using an 2-acetamido-N-(-aminocaproyl)-2-deoxy-D-glucopyranosylamine ligand column (section 2.8.4), resulted in the resolution of the NAGase eluted from the phenyl-Sepharose column into three peaks (figure 29). The first NAGase peak was the material which passed straight through the column. The second peak probably eluted as a result of the increase in molarity of the buffer and the third and largest peak appeared after elution with N-acetylglucosamine.

The afore procedure resulted in the partial purification of an NAGase with a M_r of 54 000. The complete purification is summarised in table 12. The yield of activity was high until after the hydrophobic interaction chromatography step. The loss of activity at this stage could have been the result of NAGase remaining bound to the column since the specific activity did not fall significantly. However, the fraction eluted with N-acetylglucosamine gave a lower specific activity despite displaying the greatest purity as judged by SDS-PAGE (data not shown). It should be noted that the NAGase fractions from the affinity column contained up to 0.2M N-acetyl-glucosamine. The effect of this maximum concentration on the NAGase activity was an approximate three fold inhibition (figure 30). Owing to the small amounts of purified NAGase available, no attempt was made to remove the N-acetylglucosamine by dialysis.

The net result of this purification was a NAGase of reasonable purity (no

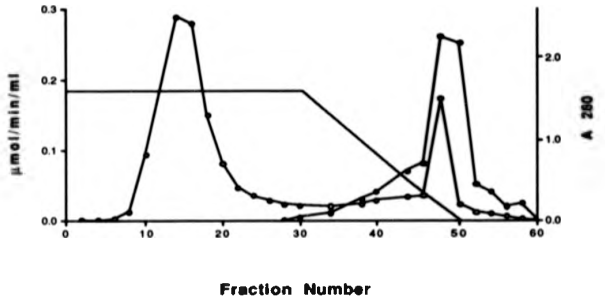


Figure 28. Hydrophobic interaction chromatography with Phenyl Sepharose. Extracellular NAGase activity (µmol/min/ml) distribution (•) and protein measured as A280 (o) is shown. The ammonium sulphate concentration (0-0.25M) is represented by the solid line. The sample loaded was prepared as described in section 3.3.6.

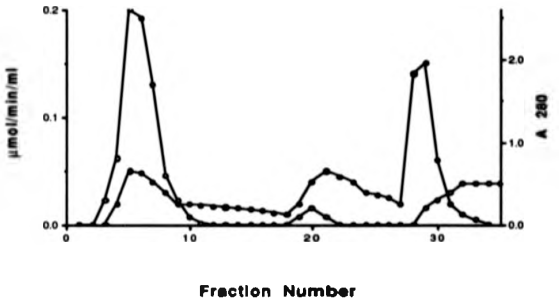


Figure 29. Affinity chromatography on Sepharose 4B with 2-acetamido-N-(-aminocaproyl)-2-deoxy-ß-D-glucopyranosylamine. Extracellular NAGase activity (µmol/min/ml) distribution (•) and protein measured as A280 (o) is shown. The sample loaded was prepared as described in section 3.3.6. The NAGase activity was measured in the presence of N-acetylglucosamine and the later fractions must be underestimated.

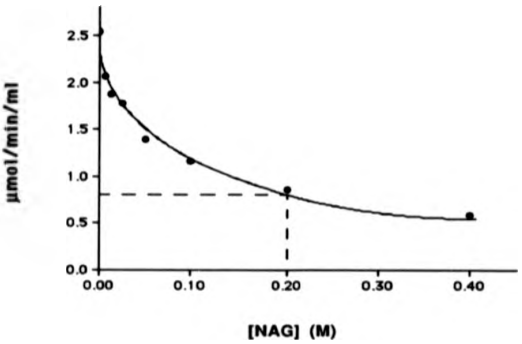


Figure 30. Inhibition of the microtitre plate NAGase activity assay by N-acetyl-glucosamine.

N-acetyl-glucosamine at the concentration indicated was added to *Trichomonas foetus* cell lysate before being assayed for NAGase activity by the standard procedure (section 2.6). NAGase activity was found to be inhibited three fold by 0.2 M N-acetyl-glucosamine.

PAGE data shown) but of disappointingly low specific activity and yield. This purification was not regarded as sufficiently successful for the preparation of pure antigen. Alternative purification steps to replace steps such as phenyl-Sepharose chromatography were considered but henceforth it was decided to concentrate on the purification of the intracellular enzymes as discussed in section 3.3.7.

3.3.7 Purification of Intracellular NAGase.

Extracellular and intracellular NAGase have been shown to be identical by SDS-PAGE (section 3.2.2) and so it was decided to purify one or all forms of NAGase from cell lysates.

Preparative isoelectric focusing using the Rotofor system (section 3.4.2) was used for the first stage of purification. A *Tritrichomonas foetus* cell lysate derived from approximately 2×10^9 cells was made up to 45 ml with d.d. H_2O and ampholytes as described in section 2.8.6. At the end of the Rotofor run the fractions were collected and assayed for NAGase activity. The active fractions were pooled and concentrated, using an Amicon XM50 filter. This concentrated sample was then applied to the affinity column and run as described in section 2.8.4. The results are summarised in table 13. As judged by SDS-PAGE (figure 31) the resulting NAGase 1 was pure when stained with coomassie blue, however, using silver stain two high M_r protein bands were observed (data not shown). Nevertheless the purity of this preparation was considerably higher than that derived using the previous method (section 3.4.6) and was used for further experiments.

Table 13. Purification of NAGase 1 from cell lysates of *Tritrichomonas foetus*.

	volume (ml)	total protein (mg)	total activity (µmol / min)	specific activity µmol/min/mg protein	% yield
1	45.0	216.0	169.2	0.78	100.0
2	13.0	10.4	51.3	4.90	30.1
3	2.5	14.2	45.2	3.20	26.7
4	2.5	1.1	3.0 (9.0)	2.70 (8.1)	1.8 (5.4)

The stages of purification are numbered as follows : (1) Preparative isoelectric focusing of *Tritrichomonas foetus* lysate diluted with d.d. H_2O and ampholytes (section 2.8.6). (2) Harvested fractions containing NAGase activity from the Rotofor run. (3) Concentration of the active fractions using an Amicon XM50 filter. (4) affinity chromatography as described in section 2.8.4. The numbers in the brackets represent approximate values that have been corrected for inhibition by N-acetyl-glucosamine. It has been extracted from figure 30 that the N-acetyl-glucosamine present in the samples inhibited NAGase activity by approximately three fold after affinity chromatography.

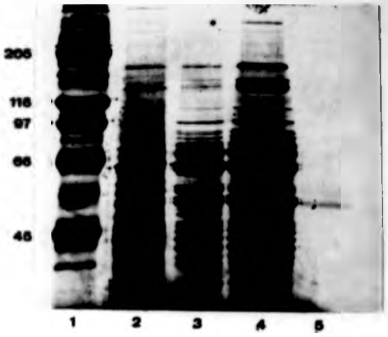


Figure 31. Purification of NAGase 1 from *Trichomonas foetus*.
Lane (1) Molecular weight standards (2) cell lysate (3) harvested fractions from preparative isoelectric focusing containing NAGase activity (4) concentrated fractions from preparative isoelectric focusing (5) fraction containing NAGase 1 after affinity chromatography. The stages of purification are described in more detail in section 3.3.7. Proteins stained with coomassie blue stain.

3.3.8 Summary

Traditional two dimensional electrophoresis has been used to study the proteins and antigens of *Trichomonas vaginalis* (Alderete *et al.* 1986). Using the Rotofor followed by SDS-PAGE two dimensional electrophoresis was also acheived. In some ways the Rotofor is superior to IEF gels for the first stage of a two dimensional separation gel as it provides material, either for further purification, or for traditional SDS-PAGE gels allowing much simpler application of specific detection techniques, such as immunoblotting or radiolabel detection. The Rotofor Cell also eliminates the problems associated with determining spots and streaks on traditional two dimensional gels as compared to the easily interpreted, precisely known, M_rs of bands on SDS-PAGE. Separation of NAGase activities from all four species of Trichomonads showed that the three pathogenic species, namely *Tritrichomonas foetus* , strains KV1 and F2 and *Trichomonas vaginalis* all had activities focussing at pH 4.5-5.0, with the two strains of *Tritrichomonas foetus* having identical pIs. The non-pathogenic form, *Tritrichomonas augusta* was distinct in having a NAGase activity focussing at pH 5.5-6.0, perhaps reflecting a difference in the parasites environment, lifestyle and possibly enzyme function.

Two schemes were designed to purify NAGase from *Tritrichomonas foetus* . The first purification procedure for extracellular NAGase found problems with the loss of NAGase activity when the affinity column was used in the last stage. There may be a number of reasons for this. Firstly the low specific activities of NAGase from the affinity column are partly the result of inhibition by high concentrations of N-acetyl-glucosamine in the elution buffer. It is probable that the actual specific activities are approximately three fold higher

than those measured and 'corrected values' are indicated in brackets in the relevant tables. It is unlikely that any NAGase remained bound to the column since N-acetylglucosamine is thought to be a highly effective elutant, although this cannot be ruled out entirely.

The second purification scheme was developed using cell lysates as starting material since previous work had indicated the intracellular and extracellular forms to be identical. This was more successful although the same problems with the affinity column were found. This scheme, however, resulted in a protein with NAGase activity, as judged by SDS-PAGE, with an apparent M, of 54 000 (NAGase 1) which was used in further experiments. Initial separation of the NAGases by preparative isoelectric focusing followed by purification using the affinity column may be a successful procedure for the isolation of the other three NAGase forms which could form the basis of future projects.

3.4 Antibody Production

3.4.1 Introduction

The principal function of the immune system is to protect animals from infectious organisms and their toxic products. When an individual first encounters an antigen the cells of the immune system recognize the antigen and produce an immune response. The immune reaction can take the form of cell mediated immunity or, involve the production of antibodies directed towards the antigen, or both.

Raising antisera to purified proteins is a process which has allowed the development of many techniques which are invaluable to investigate the biosynthesis, mechanism of release and function of proteins. In such techniques antisera are used in pulse chase experiments which are used to follow the biosynthesis of a specific protein, identify any precursors and determine the intracellular paths of the enzymes release. Antisera can also be used to identify the presence of enzymes *in vivo* which may identify their possible functions in host-parasite relationships.

Three separate attempts were made to raise polyclonal antisera against purified NAGase. On all occasions female New Zealand white rabbits were used and the injections were administered subcutaneously. Non-immune sera were always collected from the rabbit ear before antigen injection commenced.

Antibodies were needed against NAGase 1 to:

1. Identify any cross reactivity between the four main forms of NAGase by Western Blotting. If cross reactivity occured antibodies would be used to identify the multiple forms and determine whether these all relate to active forms or whether inactive forms exist within and are released by the organisms.

2. Identify any cross reaction between NAGases in different strains and species of trichomonads.

3. Determine any cross reactivity between NAGase 1 and other hydrolases of trichomonads.

4. Determine by analysis of immunoprecipitates of radiolabelled trichomonad proteins, derived from different subcellular fractions, the mechanism and rate of biosynthesis and release and identification of NAGase precursors.

5. Be used as potential inhibitors in cytotoxic studies against host cell lines to identify any nutritional function(s) of the hydrolase.

3.4.2 Antisera against extracellular NAGase

The NAGase used was purified from resuspension medium using the procedure described in section 3.3.6. Rabbits were injected with 100 μg of native protein antigen, which is within the suggested dose range (Harlow &

Lane 1988). The protein antigen was mixed with 1.25 ml of Freund's complete adjuvant and injected into three separate sites along the back of the rabbit. After a period of three weeks a booster injection which contained 100 µg of protein and 1.25 ml of Freund's incomplete adjuvent was administered. A test bleed (1 ml from the ear) was taken two weeks later and followed by two more identical boosters and three more test bleeds at suitable intervals. The rabbit was then totally bled (approximately 200ml) and the serum stored in 1 ml aliquots at -70 °C until required. Pre-immune sera was collected.

In western blots of total *Tritrichomonas foetus* crude lysates and growth media a number of proteins were recognised by sera from a test bleed after the second booster. However, only two main bands were recognised both of which had apparent M_rs greater than 180 000 (also seen after the third booster in figure 32). Nothing was recognised in samples of the purified extracellular NAGase used as the antigen. A band with an M_r of approximately 40 000 to 50 000 kDa was recognised in the *Tritrichomonas foetus* cell lysate by serum taken after the third booster but was not seen in any of the other samples including the pure enzyme (figure 32). Also under the totally denaturing conditions used here NAGase 1 would have an apparent M_r of 70 000 (section 3.5.6). No bands were recognised in the *Trichomonas vaginalis* cell lysate. It is probable that the weakly cross-reacting bands recognised in trichomonad samples represented contaminant protein present in the original NAGase preparation or one which shared common antigen groups like carbohydrates.

Using this antiserum western blot analysis of the subcellular fractions of *Tritrichomonas foetus* was performed (figure 33). The bands recognised by

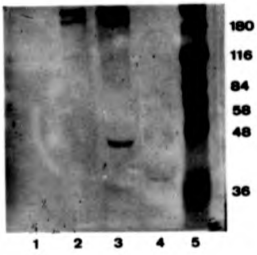


Figure 32. Western blot analysis of antiserum raised against purified extracellular NAGase 1 from *Tritrichomonas foetus* (section 3.4.2).

Lane (1) Purified extracellular NAGase, 2 µg protein (section 3.3.6) (2) *Tritrichomonas foetus* crude medium, 50 µg protein (3) *Tritrichomonas foetus* cell lysate, 50 µg protein (4) *Trichomonas vaginalis* cell lysate, 50 µg protein (5) Molecular Weight Standards.

Titre of antiserum 1:200.

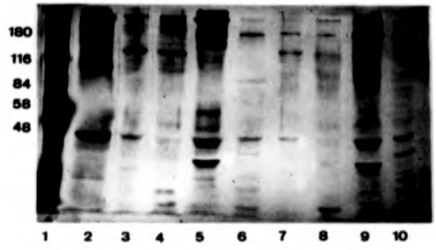


Figure 33. Western blot analysis of subcellular fractions of *Tritrichomonas foetus*.

Lane (1) Molecular weight standards (2) cell lysate (3,7) nuclear fraction (4,8) large particle fraction (5,9) small particle fraction (6,10) non-sedimented fraction. Samples of lanes 7-10 were incubated with leupeptin (10 μM) before and during electrophoresis to inhibit proteinase activity.

Approximately 25 μg protein loaded in each lane. Blot screened with antibody raised against purified extracellular NAGase after the last bleed (section 3.4.2).

Titre of antiserum 1:200.

this antiserum were compared, using the subcellular fractions of *Tritrichomonas foetus* analysed for NAGase activity and described earlier (figure 18). It was found that most of the antigens recognised were in the small particle fraction and not the large particle fraction where most NAGase activity is localised. This was taken to confirm that the anitserum did not recognise NAGase.

3.4.3 Antisera against intracellular NAGase 1

NAGase 1 was purified as described in section 3.4.7. Antiserum was prepared exactly as described in section 2.10 using 100 µg of native purified protein mixed with 1.25 mg of the appropriate Freund's adjuvant.

After the final booster injection no bands were recognised with immune serum that did not also cross-react with pre-immune serum and in all cases cross-reactivity was weak (figure 34). The immune sera did not recognise the purified enzyme (data not shown). For this reason, and the cross reaction of the non-immune sera, the antisera was concluded to have no use.

In an attempt to eliminate contaminants NAGase 1 was prepared as described in section 3.4.7 and was run on strip SDS-PAGE. The position of NAGase 1 was determined both by staining a small section of gel for NAGase activity and by staining with coomassie blue and then comparing the position with that of purified NAGase 1 (figure 35). The appropriate protein band was then cut out, denatured, and prepared for injection into the rabbit as described in section 2.10.1. The other bands seen above the main protein band in this figure are believed to be NAGase 1 which has been denatured to differing extents by the preparation and during SDS-PAGE

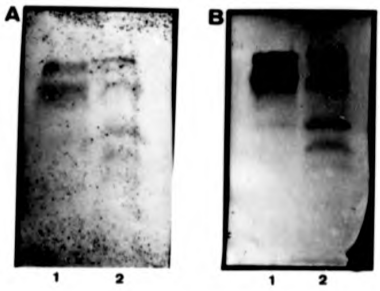


Figure 34. Western blot analysis using pre-immune serum or immune serum against native NAGase 1.

Lanes (1) *Tritrichomonas foetus* cell lysate (2) *Tritrichomonas foetus* crude medium preparation. Approximately 50 µg protein loaded to each lane.

Gel A incubated with pre-immune sera and Gel B incubated with immune sera as described in section 2.11.

Titre of antisera 1:100

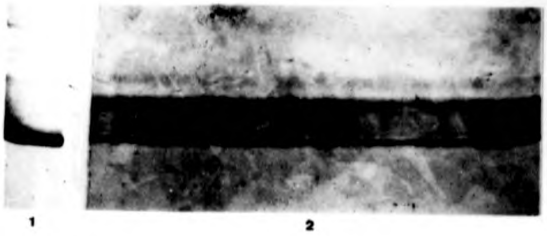


Figure 35. Strip SDS-PAGE of NAGase 1 used for antigen preparation.
Lane (1) purified NAGase 1, approximately 2 µg protein loaded. Stained for NAGase activity as described in section 2.9.3. (2) coomassie blue stain of NAGase 1, approximately 40 µg protein loaded.
NAGase 1 was purified as described section 3.3.7. The dark stained protein band which corresponded to the NAGase activity was cut out and prepared for injection into a rabbit by the method described in section 2.10.1.

(section 3.5.6). The rabbit was bled after the second booster, two months after the first injection, as the treatment appeared to be having a somewhat detrimental effect on the animal used.

Immunoblots and immunoprecipitates of prepared samples showed no recognition at all using this antisera and it was concluded that this preparation had not been antigenic in this rabbit.

3.4.4 Antiserum against *Tritrichomonas foetus* whole cell lysate

Because of the difficulties in raising antibodies to the purified enzyme, with native or denatured preparations, antiserum to whole cell lysates was raised to determine whether or not NAGase is an antigen of *Tritrichomonas foetus*, and to determine whether purified NAGase 1 is recognised by such antiserum.

Tritrichomonas foetus cell lysate (0.4 mg) was prepared with 1.25 ml of complete Freund's adjuvant and injected in the usual manner into a rabbit. Three weeks later this injection was repeated using incomplete adjuvant and the rabbit bled a week later. Immunoblots showed the presence of a number of antigenic bands in the *Tritrichomonas foetus* cell lysate and the filtered growth medium. However, purified NAGase 1 was not recognised on a blot. Some major antigenic bands of this parasite were, however, identified and shown in figure 36. Most recognition was found to intracellular and extracellular proteins with apparent M_rs greater than 116 000 although a doublet was seen in the cell lysate with apparent M_rs of 40 000 to 42 000.

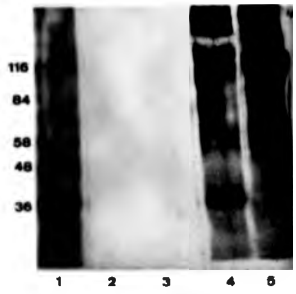


Figure 36. Western blot analysis of antiserum against crude *Tritrichomonas foetus* cell lysate.

Lane (1) Molecular weight standards (2) non-denatured purified NAGase 1 (section3.3.7), 2 µg protein (3) denatured purified NAGase 1, 2 µg protein (4) *Tritrichomonas foetus* cell lysate, 50 µg protein (5) *Tritrichomonas foetus* crude medium extract, 50 µg protein.

Titre of antiserum 1:200

3.4.5 Summary

Four separate attempts to raise antisera against NAGase from *Tritrichomonas foetus* were performed and in no circumstance was there any indication that NAGase was recognised in the prepared samples. Using *Tritrichomonas foetus* total cell lysates it was possible to demonstrate that antibodies could be raised to proteins in rabbits, but NAGase 1 appears to be non-antigenic. Unfortunately neither resources nor time allowed the use of other species to attempt production of antibodies but this would be an important consideration for future work in this area.

3.5 Characterisation of NAGase 1 from *Tritrichomonas foetus*

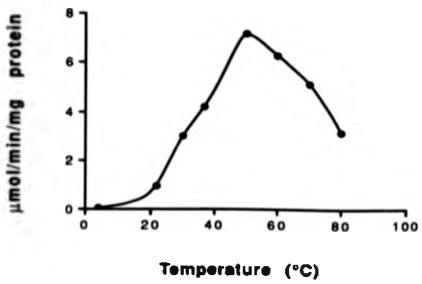
3.5.1 Introduction

During its purification it was noticed that NAGase 1 exhibited certain unusual characteristics, especially on SDS-PAGE, and it was therefore thought important to investigate more closely some of the properties of this enzyme. A variety of techniques are available to study protein structure and in this section the results of SDS-PAGE analysis, endoglycosylase treatment and circular dichroism spectrophotometry of purified NAGase 1 are presented. SDS-PAGE, discussed previously (section 3.2.1), can give information on the size and number of polypeptides present in a protein sample and can be used to distinguish, for example, catalytic and non-catalytic subunits. Treatment of a protein with endoglycosylases will release any covalently linked carbohydrate moieties and consequently increase its mobility on SDS-PAGE, hence generating information on the size, and by the use of specific glycosylases, type of carbohydrate/protein linkage.

3.5.2 Temperature dependency of NAGase 1 activity assay

Using p-nitrophenyl-N-acetyl-ß-D-glucosaminide as the substrate the temperature dependency of purified NAGase 1 was determined. This is shown in figure 37A. Under the assay conditions the pure enzyme had a temperature optimum of 45°C. This was lower than seen in section 3.1.4 where the NAGase activity in crude extracts had an optimum of between 60 and 70°C. This higher temperature optimum seen with the NAGase crude

(A)



(B)

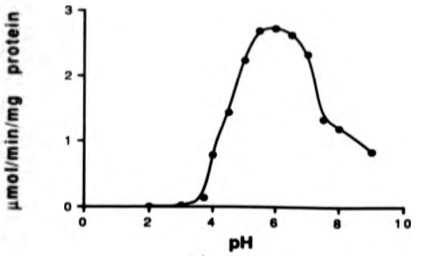


Figure 37 The (A) temperature and (B) pH dependency of NAGase 1 activity of *Trichomonas foetus* .

Assays were carried out at the temperatures and pH values indicated over a time period of 30 min. Amount of protein in samples was 5µg.

samples may be due to either the enzyme activities being stabilised by the high concentrations of other proteins present, or, each NAGase form may have a different temperature dependency and it is the accumulation of these which gives a value of between 60°C and 70°C.

3.5.3 pH dependency of NAGase 1 activity assay

The pH optimum of NAGase 1 was between 5.5 and 6.0 (figure 37B). This was similar to that found for NAGase crude activity in cell fractions described in section 3.1.5.

3.5.4 The molecular weight of NAGase 1 by SDS-PAGE

The apparent M_r of NAGase 1 was found to be 54 000 by SDS-PAGE analysis using activity gels, as shown in figure 38. A coomassie blue stained gel of pure NAGase 1 is shown, with the same sample stained for NAGase activity to confirm that the protein band compared with the NAGase activity. Analysis of NAGase 1 by gel filtration chromatography was performed on a Sephacryl S300 column as described in section 2.8.5. NAGase 1, like the NAGase activity in crude lysates (section 3.2.3), was eluted from the gel filtration column in a volume corresponding to an apparent M_r of 80 000 (figure 39).

3.5.5 Denaturation of NAGase 1 and analysis on SDS PAGE

NAGase 1 run under the conditions used for activity staining gave a single coomassie blue stained band with an apparent M_r of 54 000 (figure 38). When NAGase 1 was incubated for 2 min at 100°C prior to electrophoresis,

Figure 38. SDS-PAGE analysis of NAGase1.

Lane (1) Molecular Weight Standards (2) Coomassie blue stain of NAGase 1 (3) NAGase 1 stained for activity using the method described in section 2.9.3. 2 µg protein was loaded to each lane. Both samples were run on the same gel and split after electrophoresis for the separate staining procedures. The samples contained SDS and ß-mercaptoethanol and were not incubated at 100°C before electrophoresis.

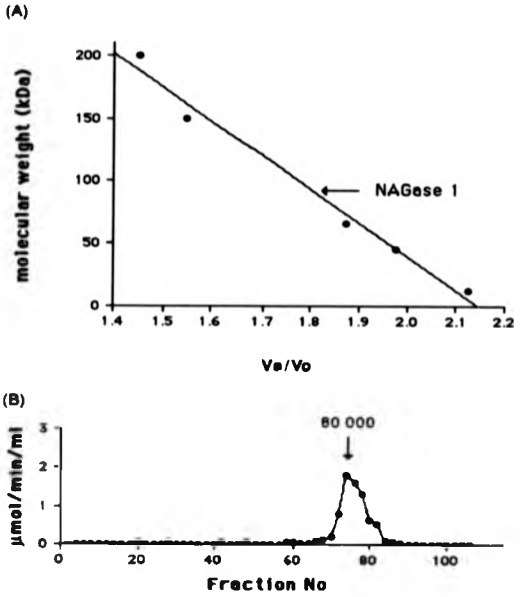


Figure 39. Gel Filtration Chromatography of purified NAGase 1 using a Sephacryl 300 column. Details of the column are described in section 2.8.5. (A) The position of the NAGase 1 activity peak is indicated with an arrow. Elution volume of the protein/void volume of the blue dextran standard is indicated as Ve/Vo. (B) Profile of NAGase 1 activity. Arrow indicates M_r position of the NAGase 1 activity peak. Approximately 25 µg protein loaded to each column run and NAGase activity assays were performed as described in section 2.6.

using the standard conditions employed for SDS-PAGE, the mobility of the enzyme was found to change. Figure 40 shows a coomassie blue stained gel demonstrating that total denaturation resulted in a decrease in mobility of the enzyme. After denaturation, NAGase 1 stained as a doublet with an apparent M_r of 68 000 and 70 000. There was also an increase in the intensity of staining which also occured when silver stain was used instead of coomassie blue stain. In figure 40 there are other coomassie stained bands which may represent forms of NAGase that have been denatured simply by electrophoresis without incubation at 100 °C. The same shift in mobility was also apparent if the acrylamide concentration of the gel was 12.5% or when using urea PAGE (Anderson *et al.* 1983)(figure 41).

To analyse this change in mobility further, the temperature, and dependence of the shift on the components of the sample buffer, were investigated.

The results above have shown that at a temperature between room temperature and 100 °C a shift in electrophoretic mobility of NAGase 1 was produced. To determine at what temperature this transition occured NAGase 1 was incubated at different temperatures for 5 min in the presence of complete electrophoresis sample buffer, that is with SDS and β-mercaptoethanol. NAGase 1 started to lose activity, as judged by staining, at 37°C. The transition to inactive enzyme was complete after a 5 min incubation at 50°C (figure 42, gel A). This loss of activity correlated with the decrease in mobility and at 50°C the transformation was in fact complete within 2 min (figure 42, gel B).

When NAGase 1 was incubated at 50°C before mixing with electrophoresis buffer it was stable and remained fully active after 15 min, and on

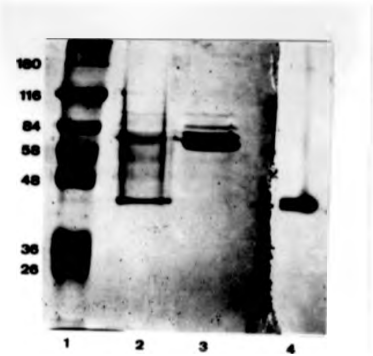


Figure 40. Effect of incubation at 100°C for 2 min before SDS-PAGE of NAGase 1

Lane (1) Molecular weight standards (2) Coomassie blue stain of NAGase 1 (3) Coomassie stain of denatured NAGase 1 which had been incubated with sample buffer at 100°C for 2 min before electrophoresis (4) NAGase 1 stained for activity using the procedure described in section 2.9.3.

Approximately 2 µg protein loaded to each lane. Sample mixed with sample buffer, half incubated at 100°C for 2 min, and then all samples run in the appropriate lanes on the same gel. The gel was then split after electrophoresis for the separate staining procedures.

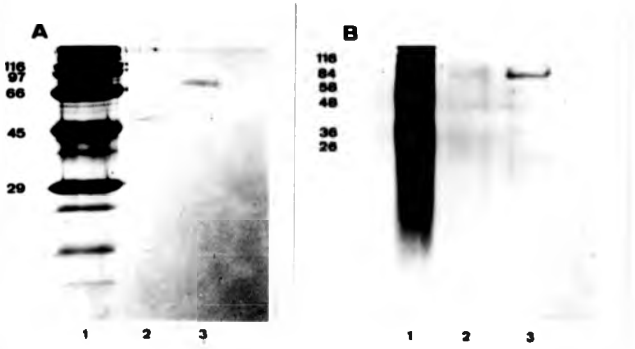


Figure 41. Effects of electrophoresis conditions on the decrease in mobility of denatured NAGase 1.

Lane (1) Molecular Weight Standards (2) NAGase 1 (3) NAGase 1 incubated with sample buffer at 100°C for 2 min before electrophoresis.

Gel A - 12.5% SDS-PAGE.

Gel B - urea PAGE (Anderson *et al.* 1983). The native NAGase is not visible on this photograph but was detectable on the original gel.

Approximately 2 µg protein loaded to each lane. Both gels are stained for protein using coomassie blue stain.

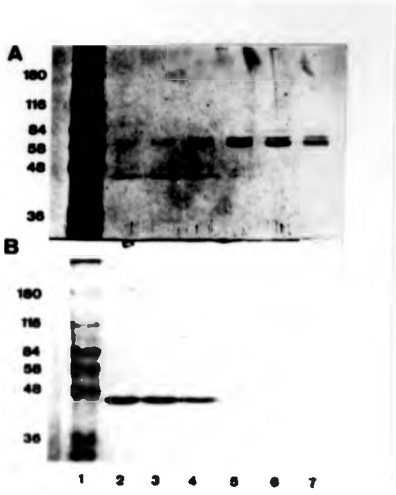


Figure 42. Effect of temperature on the mobility of NAGase 1 during SDS-PAGE.

Lanes 2-7 contained samples of NAGase 1 which were incubated at the temperatures indicated for 5 min in the presence of SDS-PAGE sample buffer prior to electrophoresis. Approximately 2 µg protein was loaded on to each lane. Lane (1) Molecular Weight Standards (2) 4°C (3) 25°C (4) 37°C (5) 50°C (6) 60°C (7) 70°C.

Gel A was stained for protein with coomassie stain. Gel B was stained for NAGase activity. Both gels were loaded and run at the same time.

subsequent SDS-PAGE analysis it had a mobility equivalent to an apparent M_r of 54 000. When NAGase 1 was incubated at 100°C with or without SDS and ß-mercaptoethanol the change in electrophoretic mobility occured (data not shown). Purified NAGase 1 was therefore incubated at 50°C for 5 min with different components of electrophoresis sample buffer to determine what component(s) was necessary for the change in mobility to occur. After incubation the samples were analysed by SDS-PAGE (figure 43). The shift in mobility was found to occur only if both SDS and ß-mercaptoethanol were present.

A likely explanation for the changes in mobility of NAGase 1 during SDS-PAGE may relate to a large number of disulphide bonds present in the enzyme. When incubated in the presence of SDS and ß-mercaptoethanol these bonds would be dissociated which may change the strucure of the protein causing it to run slower on SDS-PAGE. Alternatively, proteins which contain large carbohydrate moieties have been shown to run in an abnormal manner during SDS-PAGE (Hames 1991).

3.5.6 NAGase 1 is a glycoprotein

Many lysosomal enzymes are glycoproteins. To determine whether NAGase 1 was a glycoprotein it was incubated with the enzyme Glycopeptidase F which removes any N-linked carbohydrate moieties (section 2.13). This enzyme cleaves the N-glycan linkage between asparagine and the carbohydrate chain which increases the mobility of the deglycosylated protein during SDS-PAGE. Using SDS-PAGE analysis it was found that the native form of NAGase 1 did not deglycosylate under the same conditions as the ovalbumin control, even when the N-glycopeptidase F concentration was

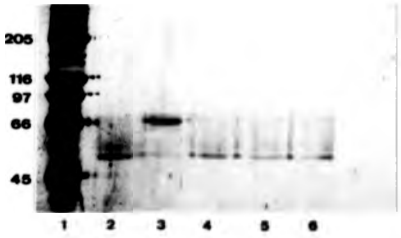


Figure 43. Effect of pre-incubation of NAGase 1 for 5 min at 50°C with different components of SDS-PAGE sample buffer.

Lane (1) Molecular Weight Standards (2) NAGase 1 incubated with no sample buffer (3) NAGase 1 incubated with complete SDS-PAGE sample buffer (4) NAGase 1 incubated with sample buffer containing no SDS (5) NAGase 1 incubated with Sample buffer containing no ß-mercaptoethanol (6) NAGase 1 incubated with sample buffer containing no glycerol.

Approximately 2 µg protein loaded to each lane. Gel stained for protein with coomassie stain.

increased by a factor of ten. However, when NAGase 1 was incubated at 100°C for 2 min, before incubation with N-glycopeptidase F, its mobility was increased on SDS-PAGE by the equivalent of approximately 2000 Da (figure 44), that is from apparent M_rs of 70 000 and 68 000 to apparent M_rs of 68 000 and 66 000. This suggested that NAGase 1 was a glycoprotein.

Information of the glycoprotein content of *Tritrichomonas foetus* was achieved using two peroxidase conjugated lectins : concanavalin A and peanut lectin. Crude preparations and samples partially purified using preparative isoelectric focusing of *Tritrichomonas foetus* were run on SDS-PAGE, transferred to nitrocellulose and stained for glycoproteins as explained in section 2.11. Figure 45 shows a coomassie blue stained gel (gel A) and blots of the same gels stained for glycoproteins with the two lectins (gels B & C).

Although the native form of NAGase 1 did not appear to be recognised by either of the lectins (figure 45, gels B & C, lanes 7), the concanavalin A lectin did recognise the denatured enzyme (figure 45, gel B, lane 8) which can therefore be concluded to contain high mannose type carbohydrate. NAGase 1 was not recognised by the peanut lectin which suggests that this enzyme does not contain O-linked carbohydrate.

One other protein in the partially purified NAGase 1 preparation, with an apparent M_r of 50 000, was strongly recognised by concanavalin A in both denatured and non-denatured samples (figure 45, gel B). This perhaps suggests that the carbohydrate moiety is fully exposed to the lectin staining on the native and denatured form of this protein. In non-denatured samples two other major proteins, with apparent M_rs of 40 000 and 50 000, were recognised by peanut lectin in the partially purified sample and a third in the

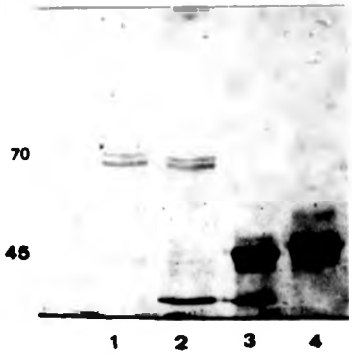


Figure 44. Deglycosylation of NAGase 1.

Lane (1) NAGase 1 (2) NAGase 1 incubated with N-glycopeptidase F (3) ovalbumin control (4) ovalbumin incubated with N-glycopeptidase F.

Deglycosylation performed as described in section 2.13. Control samples run in lanes 1 and 3 were incubated in the same manner as samples 2 and 4 except with no N-glycopeptidase F present. The apparent molecular weights indicated are predicted from other electrophoresis runs.

NAGase 1 was denatured before incubation with N-glycopeptidase F and all samples were denatured by incubation at 100°C for 2 min before electrophoresis.

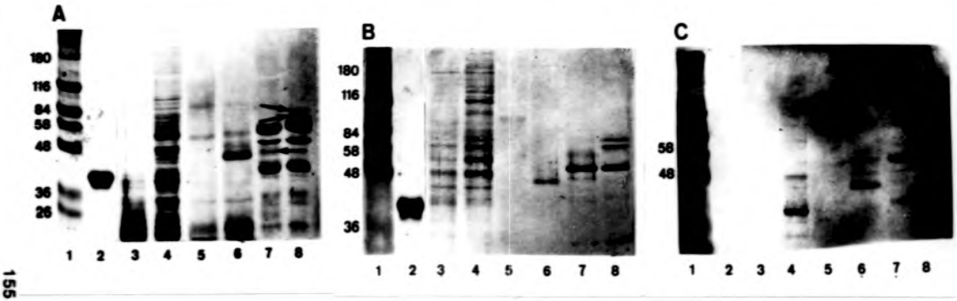


Figure 45. Lectin binding staining of *Tritrichomonas foetus* proteins.

Lane (1) Molecular Weight Markers (2) Ovalbumin, 2 µg protein (3) *Tritrichomonas foetus* lysate, 10 µg protein (4) cell lysate incubated at 100°C for 2 min before electrophoresis, 10 µg protein (5) spent growth medium, 10 µg protein (6) spent growth medium incubated with sample buffer at 100°C for 2 min before electrophoresis, 10 µg protein (7) preparative IEF purified (section 2.8.6) NAGase 1, 5 µg protein (8) preparative IEF purified (section 2.8.6) NAGase 1 incubated with sample buffer at 100°C for 2 min before electrophoresis, 5 µg protein. Gel A - coomassie blue stained. Gel B - concanavalin A staining. Gel C - peanut lectin staining. Staining procedures with the lectins were performed as described in section 2.11.

denatured sample with an apparent M_r of 48 000 (figure 45, gel C). In the *Tritrichomonas foetus* cell lysate many glycoproteins are recognised and distinct differences between recognition of the two lectin stains can be seen. For example a major glycoprotein recognised by peanut lectin was seen at an approximate M_r of 30 000 (lane 4) and in the extracellular samples three glycoproteins were recognised by peanut lectin while only one was seen by concanavalin A staining (figure 45, gels B & C, lanes 6).

In gel A there is extensive proteolysis apparent in lanes 3 and 5. The same samples when totally denatured before electrophoresis (lanes 4 and 6) show no proteolysis. In general the lectins recognised more proteins in samples which had been denatured by incubation at 100°C. It is not clear whether this was due to the lack of proteolysis in these samples or if denaturing the proteins allowed better recognition by the lectin. The inclusion of a proteinase inhibitor such as leupeptin during sample preparation would answer this question.

3.5.7 Sequencing of NAGase 1

To determine the structure of NAGase 1, sequencing using the automated Edman Degradation reaction was attempted, by the Biochemistry Department of Aberdeen University. However, the electrophoresis stages, to transfer the sample to nitrocellulose, prior to sequencing, were not successful and could not be repeated owing to the lack of purified material. This would, however, be a priority for future work in this field.

3.5.8 Circular dichroism analysis of NAGase 1

Certain types of protein secondary structure, particularly alpha helix, differentially absorb light of a particular polarity. The measurement of this differential absorption of light is known as circular dichroism. Circular dichroism can give information about the amount and, to some extent, type of secondary structure present in a particular polypeptide. Such studies might shed some light on the unusual behaviour of NAGase 1 on SDS-PAGE. Circular dichroism analysis was performed as described in section 2.14.

Figure 46 shows the trace produced from the circular dichroism analysis of purified NAGase 1. The blue line is the trace for the native NAGase. A computer analysis of this trace predicted that the enzyme contained very little alpha-helix, a limited amount of ß-sheet and was mostly a "random coil" structure. The red line is the trace for NAGase 1 which had been incubated at 100°C for 2 min; conditions which were known to change the electrophoretic mobility (figure 40). It shows an identical structure to the native NAGase with no unfolding. Unfolding was achieved when the enzyme had been incubated with 3M guanidine hydrochloride (the green line). Unfortunately this type of analysis cannot be performed in the presence of SDS or 2-mercaptoethanol as these compounds absorb polar light. However, the CD data suggested that the unusual migration of the detnatured protein was not due to the breakdown of the secondary structure but possibly due to aggregation which must be taken into consideration. Another possibility is that in the native form the enzyme contains large amounts of very tightly coiled random structures, or perhaps large amounts of disulphide bonds. In either of these cases denaturation of the enzyme might produce a much looser and larger structure which would appear to have a

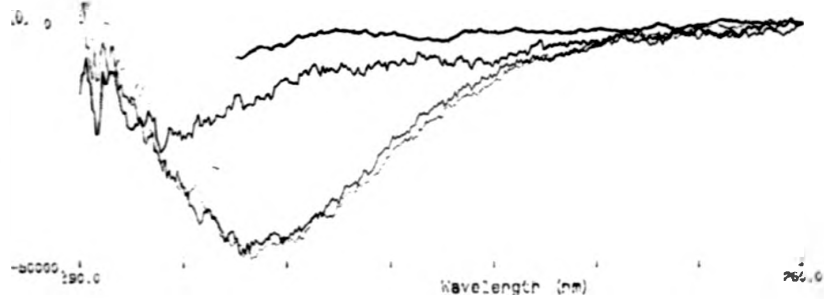


Figure 46. Circular dichroism analysis of NAGase 1. Blue line trace of untreated NAGase 1, red line trace of treated NAGase 1, green line trace of treated NAGase 1 incubated with 3 M Guanidine Hydrochloride, black line trace of treated NAGase 1 incubated with 3 M Guanidine Hydrochloride and then dialysed to remove the Guanidine hydrochloride. Treatment of NAGase 1 involved the enzyme being incubated at 100°C for 2 min. CD analysis performed as described in section 2.14.

larger molecular weight on SDS-PAGE. The unfolding and exposure of more protein domains may also explain the increased affinity of NAGase 1 for coomassie and silver stain when denatured.

3.5.9 Summary

Although purified NAGase 1 was found to have an apparent M_r of 54 000 after SDS-PAGE, when it was completely denatured prior to electrophoresis, there was a decrease in mobility, and NAGase 1 gave a doublet of apparent M_rs of 68 000 and 70 000. These are believed to be the true molecular weights and agree with the gel filtration data. This decrease in mobility was found to occur at 50°C in the presence of sample buffer which correlated with the loss of NAGase activity. At 50°C both SDS and mercaptoethanol had to be present in order for this apparent increase in M_r to occur.

Using N-glycopeptidase F and a concanavalin A lectin, NAGase 1 was characterised as an N-linked glycoprotein and some initial sudies of this enzyme were attempted using circular dichroism analysis.

3.6 Characterisation of acid phosphatase activity

3.6.1 Introduction

Acid phosphatase is a major hydrolase activity released from *Trichomonas vaginalis* during axenic growth *in vitro*. *Trichomonas vaginalis* has been shown to contain and release approximately twice as much of this enzyme as *Tritrichomonas foetus* (Lockwood *et al.* 1988). The physiological function for this extracellular enzyme is not yet known. Initial experiments were performed to assess the suitability of acid phosphatase for the study of hydrolase biosynthesis and release from trichomonads. Studies on the acid phosphatase activities from the trichomonad species are described in this chapter.

3.6.2 Phosphatase assays

Assays for phosphatase used the substrate 4-nitrophenyl phosphate (disodium salt) and were performed by the standard procedure described in section 2.6. The following results were obtained from single experiments but in all cases the data have been confirmed by at least two other independent determinations. Controls for these assays contained water or sterile medium. The controls were used to blank the microtitre plate reader before the assays were read.

3.6.3 Time dependence of phosphatase activity

In the assay for phosphatase activity the production of 4-nitrophenol was

linear at least up to 60 min (figure 47). This result remained true for different amounts (approximate range of 10 - 50 µg total protein) of samples. No subsequent assay was run for more than 60 min.

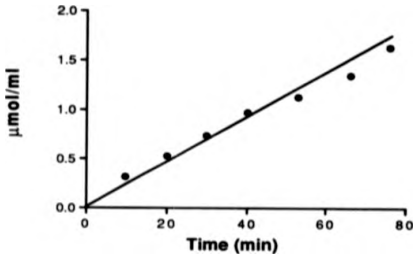
3.6.4 pH dependence of phosphatase activity

The pH optima for the acid phosphatase from the two species were similar to those for NAGase in that the enzyme from *Tritrichomonas foetus* exhibited a sharp optimum of pH 5.0 while the enzyme from *Trichomonas vaginalis* had a much broader optimum of pH 4-5 (figure 48). However unlike NAGase activity (figure 7) the acid phosphatase activity from both species showed a very sharp drop in activity between pH 5.5 and 6.0. When the samples assayed at pH 6.5 and above were dialysed against 0.1 M acetic acid/sodium acetate buffer pH 5.5 reactivation of the enzyme occurred. Therefore acid phosphatase was not irreversibly inactivated.

3.6.5 Temperature dependence of acid phosphatase activity assay

The temperature optimum for the acid phosphatase activities of both *Tritrichomonas foetus* and *Trichomonas vaginalis* was 60°C. At 80°C, however, the acid phosphatase activity of *Tritrichomonas foetus* still retained 40-50% of the optimum activity (figure 49)(at this temperature NAGase activity was only 10% of the optimum, figure 6). The rate of product formation in the non-enzyme controls was negligible at all temperatures.

(A)



(B)

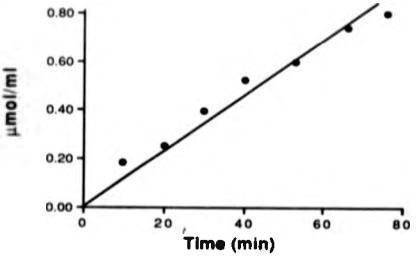
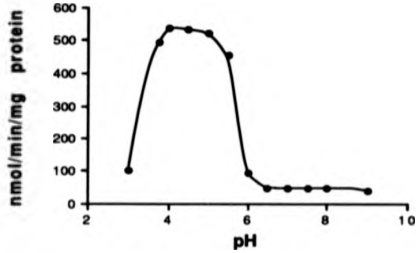


Figure 47. Time course for phosphatase assay with lysates of (A) *Trichomonas vaginalis* (B) *Tritrichomonas foetus*.
Assays performed as described in section 2.6. Amount of protein in samples was 30 µg.

(A)



(B)

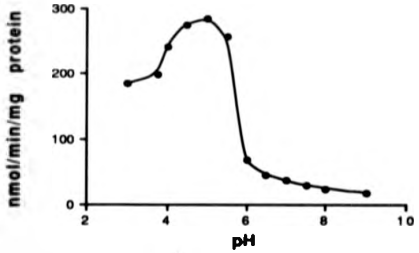
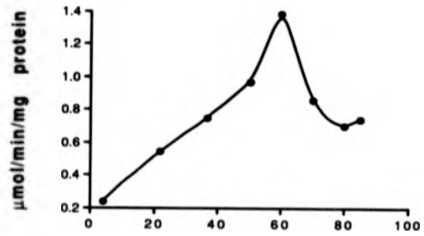


Figure 48. The pH dependency of acid phosphatase activity in lysates of (A) *Trichomonas vaginalis* and (B) *Tritrichomonas foetus*.
Assays were carried out at the pH indicated over a time period of 30 min. Amount of protein in samples was 30 µg.

(A)



(B)

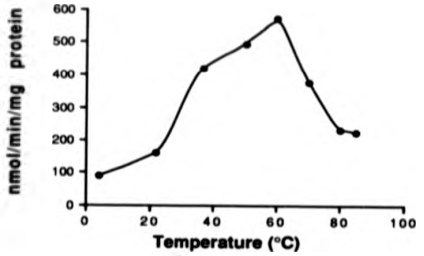


Figure 49. The temperature dependency of phosphatase activity from lysates of (A) *Trichomonas vaginalis* and (B) *Tritrichomonas foetus*.
Assays were carried out at the temperatures indicated over a time period of 30 min. Amount of protein in samples was 30 µg.

3.6.6 Kinetics of acid phosphatase

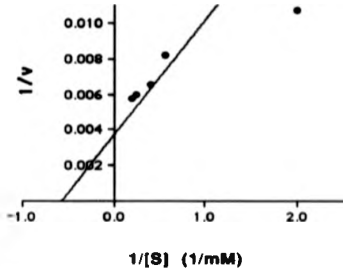
The K_m for the total intracellular acid phosphatase in lysates of *Trichomonas vaginalis* and *Tritrichomonas foetus* was determined for the substrate 4-nitrophenyl phosphate (disodium salt) and obeyed Michaelis Menten kinetics (figure 50). The K_m's and their standard errors were calculated using Enzfitter version 1.05 supplied by Elselvier Soft from a substrate concentration range of 0.25 to 5.0 mM. *Trichomonas vaginalis* acid phosphatase was calculated to have a K_m of 1.67 $\pm$ 0.15 while acid phosphatase from *Tritrichomonas foetus* was found to have a K_m of 1.36 $\pm$ 0.25.

3.6.7 Release of acid phosphatase from *Tritrichomonas foetus* and *Trichomonas vaginalis*

Acid phosphatase was released from both parasites during logarithmic axenic growth in MDM (figure 51). Enzyme activity in the medium increased during all stages of growth. An increase was also observed at the end of the stationary phase during the decline in the number of live parasitic cells. *Trichomonas vaginalis* secreted greater amounts of acid phosphatase than *Tritrichomonas foetus*. This confirmed observations made by Lockwood *et al.* (1988).

Acid phosphatase was also released by both organisms during short term incubations in serum-free MDM. There was an increase in activity over the 6h which was not thought to be due to lysis of the cells (figure 52). North *et al.* (1989) have shown, using the same procedure, that serine sulphydrase, a strictly intracellular enzyme, was not detectable in the medium. As a proportion of the total enzyme activity *Trichomonas vaginalis* released

(A)



(B)

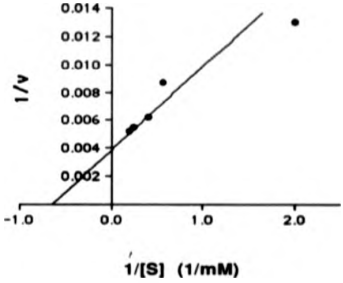
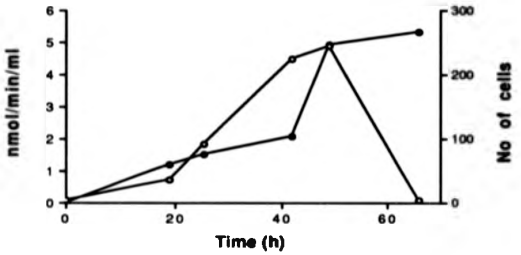


Figure 50. Double reciprocal plot for crude acid phosphatase from (A) *Trichomonas vaginalis* cell lysates (B) *Tritrichomonas foetus* cell lysates. V is in units of nmol/min/ml.

(A)



(B)

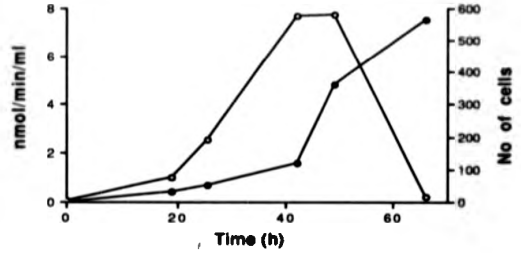


Figure 51. Release of acid phosphatase from (A) *Trichomonas vaginalis* and (B) *Tritrichomonas foetus*.

Enzyme activity (nmol/min/ml) (•) and cell number ($x10^4$) (o).

Cultures were grown in complete MDM and inoculated at 4×10^4 cells/ml.

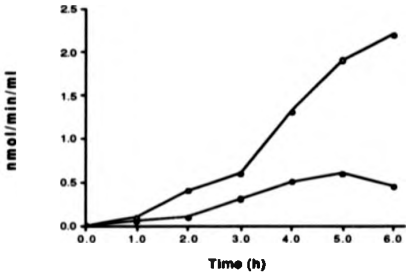


Figure 52. The release of acid phosphatase from *Trichomonas vaginalis* (o) and *Tritrichomonas foetus* (•) during 6 h incubation in serum free MDM. Acid phosphatase activity in nmol/min/ml. Cells were pregrown in complete MDM to an approximate density of 2 x 10^6 cells / ml, harvested by centrifugation, washed and resuspended in serum free MDM. A full description of this method can be found in section 2.3.2.

greater amounts of acid phosphatase than *Tritrichomonas foetus* did. As with NAGase (section 3.1.8), there were no differences in the characteristics of the released acid phosphatase from these organisms while growing in complete medium and after resuspension into serum free media.

3.6.8 Resolution of acid phosphatases by SDS-PAGE

SDS-PAGE analysis was used to analyse the acid phosphatase activities of *Trichomonas vaginalis*, *Tritrichomonas foetus* (F2 and KV1) and *Tritrichomonas augusta*. Gels were stained for acid phosphatase activity (section 2.9.4) using α-naphthyl phosphate as the substrate. One major form of acid phosphatase was resolved with an apparent M_r of 126 000 in *Tritrichomonas foetus* and *Tritrichomonas augusta* but not in *Trichomonas vaginalis* in which no activity could be detected using this method (data not shown). *Tritrichomonas foetus* and *Tritrichomonas augusta* enzyme activity was detected in both intracellular and extracellular samples.

The subcellular fractions of *Tritrichomonas foetus*, strain F2, were also analysed. Acid phosphatase activity was present in all fractions but was predominant in the small particle fraction (figure 53).

Non-denaturing PAGE (section 2.9) was used in an attempt to detect acid phosphatases which were not visualised using partially denaturing SDS-PAGE. Gels were run with no SDS or ß-mercaptoethanol present at any stage of the process. Intracellular and extracellular acid phosphatase activity from *Trichomonas vaginalis* stained as a doublet which had apparent M_rs of

Figure 53. Activity staining of acid phosphatase on SDS-PAGE in *Trichomonas foetus* subcellular fractions.

Lane (1) cell lysate (2) nuclear fraction (3) large particle fraction (4) small particle fraction (5) non-sedimental fraction (6) crude medium preparation. Approximately 50 µg protein loaded to each lane.

The apparent molecular weight indicated is that predicted from other electrophoresis runs.

160 000 and 150 000 (figure 54). Acid phosphatase activity bands were also visualised for the other trichomonad species. Acid phosphatase activity in *Tritrichomonas foetus*, strains F2 and KV1 corresponded to a single band with an approximate M_r of 126 000 while the enzyme from *Tritrichomonas augusta* had a slightly higher apparent M_r of 130 000. Interestingly the high molecular weight 'streaky' stained acid phosphatase activity was only observed in the intracellular samples perhaps suggesting the presence of more than one enzyme form or active precursors.

3.6.9 Determination of molecular weight by gel filtration chromatography

Gel filtration chromatography was performed using a standardised Sephacryl S300 column (section 2.8.5). *Trichomonas vaginalis* acid phosphatase activity was eluted from the gel filtration column corresponding to an apparent M_r of 160 000 (figure 55). All the other acid phosphatase activities were found to have an apparent M_r of 130 000 by this method. These results were consistent with the apparent molecular weight values found for the enzymes by SDS-PAGE.

3.6.10 Summary

Release of acid phosphatase was confirmed for *Trichomonas vaginalis*, *Tritrichomonas foetus* and *Tritrichomonas augusta*. These activities were further characterised by SDS-PAGE. *Trichomonas vaginalis* was found to contain and release two forms of this enzyme while for the other species of trichomonads only one form of acid phosphatase was detected. As with NAGase (section 3.2.2), all intracellular and extracellular enzyme activities

Figure 54. Activity staining of acid phosphatase after non-denaturing PAGE. Lane (1) *Tritrichomonas foetus*, clone F2, crude cell lysate (2) *Tritrichomonas foetus*, clone F2, crude medium preparation (3) *Tritrichomonas foetus*, clone KV1, crude cell lysate (4) *Tritrichomonas foetus*, clone KV1, crude medium preparation (5) *Trichomonas vaginalis* crude cell lysate (6) *Trichomonas vaginalis* crude medium preparation (7) *Tritrichomonas augusta* crude cell lysate (8) *Tritrichomonas augusta* crude medium preparation. Approximately 20 µmol / min acid phosphatase activity loaded to each lane. Crude cell lysates were prepared by lysing cell pellets with 0.25% Triton X100 and the crude medium preparations were collected as described in section 2.3.2.

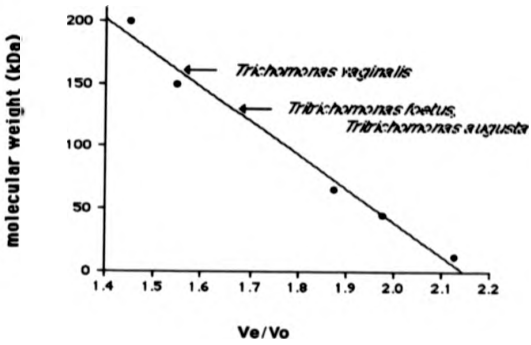


Figure 55. Gel Filtration Chromatography using a Sephacryl 300 column. Details of the column and Molecular Weight Standards are described in section 2.8.5. Positions of the acid phosphatase activity peaks of the three species of trichomonads are indicated with arrows. Elution volume of the protein / Void volume of the blue dextran standard is indicated as Ve/Vo.

were thought to be identical.

3.7 Use of Rotofor isoelectric focusing in a preliminary investigation of the proteinases of trichomonads

3.7.1 Introduction

Trichomonads contain and release multiple cysteine proteinases (Lockwood *et al.* 1987). Lockwood *et al.* (1987), using gelatin-SDS-PAGE, have identified at least eleven different proteinases of *Trichomonas vaginalis* of which the most active proteinases have M_rs between 38 000 and 96 000. However, an even greater complexity has now been demonstrated using traditional two dimensional-gelatin-SDS-PAGE which has resolved as many as 23 distinct proteinases (Neale & Alderete 1990). These proteinases had isoelectric points in the range from 5.7 to 7.0. Lockwood *et al.* (1985; 1986) has also described the purification of two intracellular proteinases from this trichomonad with M_rs of 18 000 and 64 000 and isoelectric points of 5.2 and 5.9 respectively. The most active cysteine proteinases of *Tritrichomonas foetus* have M_rs between 20 000 and 40 000 distinguishing them from the apparently larger proteinases of *Trichomonas vaginalis* (North 1991)(figure 56). A detailed review of the molecular weights and properties of the proteinases of trichomonads can be found in North (1991). There has been no information published on the isoelectric points of proteinases from *Tritrichomonas foetus* or *Tritrichomonas augusta*.

An evaluation of the use of preparative isoelectric focusing as the first step in the separation and purification of the proteinases of these organisms was performed. The proteinases of trichomonads have been characterised in greater detail than the other hydrolases of these organisms. The applicability

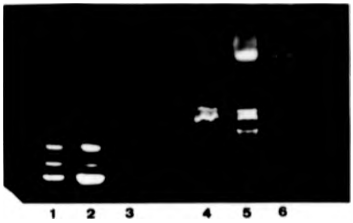


Figure 56. Gelatin SDS-PAGE of the cysteine proteinases of *Thtrichomonas foetus* and *Trichomonas vaginalis*.

Lane (1) 0-40% (2) 40-60% (3) 60-80% ammonium sulphate precipitations of *Thtrichomonas foetus* cell lysate. Lane (4) 0-40% (5) 40-60% (6) 60-80% ammonium sulphate precipitations of *Trichomonas vaginalis* cell lysate. Amount of protein loaded to each lane 10μg. Ammonium sulphate precipitations performed as described in section 2.5.

of the Rotofor and analysis of results was also obtained by the investigations into the proteinases.

3.7.2 Analysis of proteinases by preparative isoelectric focusing

Intracellular and extracellular samples of *Tritrichomonas foetus* which were separated by preparative isoelectric focusing using the Rotofor cell (section 2.9.2), were analysed by gelatin SDS-PAGE. The intracellular (figure 57) and extracellular (figure 58) proteinases of this trichomonad demonstrated different profiles after electrophoresis. At least five bands with pI's between pH 3.4 and pH 5.0 can be seen in intracellular fractions, whereas the majority of bands from the extracellular sample focused in the fractions of pH 4.5 to pH 6.0. This is interesting and worth further investigation as studies performed on the other hydrolases have all indicated that the intracellular and extracellular forms are identical.

The intracellular proteinases from *Tritrichomonas foetus* strain KV1 were also analysed using this technique (figure 59). As with strain F2 the majority of the proteinase activities focused in a pH range of 4.2-4.8. There were, however, some intrastrain differences. A higher M_r proteinase activity of KV1, which focused sharply at pH 5.0, was not found in F2,. The extracellular activities of strain KV1 were not analysed.

Tritrichomonas augusta showed a slightly different profile in which the majority of the proteinase forms focussed at a pH of between 4.6-5.5 which is higher than found for *Tritrichomonas foetus* (figure 60).

The profile for *Trichomonas vaginalis* showed proteinases focussed up to pH

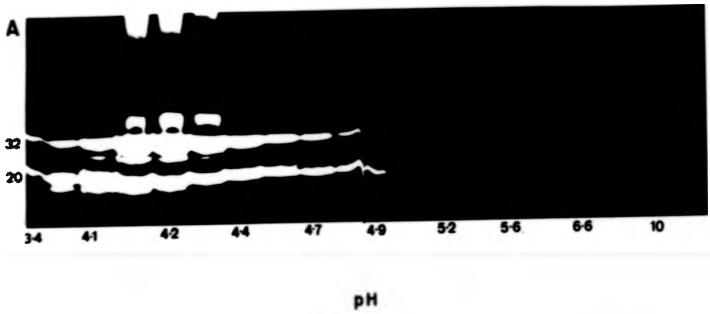


Figure 57. Analysis of proteinase activities by gelatin SDS-PAGE of isoelectrically focused fractions from *Tritrichomonas foetus* F2 cell lysates. The pH of the fractions and the approximate molecular weights are indicated. The molecular weights of the proteinases were identified by comparison with previously reported proteinase patterns (North *et al.* 1990).

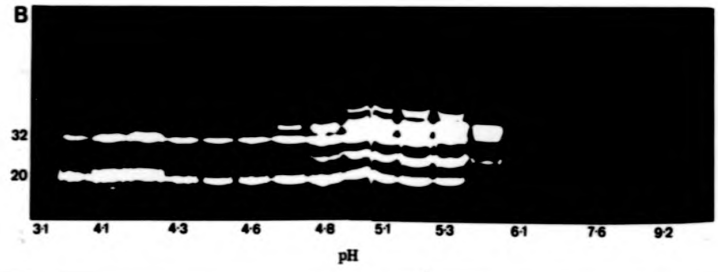


Figure 58. Analysis of proteinase activities by gelatin SDS-PAGE of isoelectrically focused fractions from *Tritrichomonas foetus* F2 crude medium preparation. The pH of the fractions and the approximate molecular weights are indicated. The molecular weights of the proteinases were identified by comparison with previously reported proteinase patterns (North *et al.* 1990).

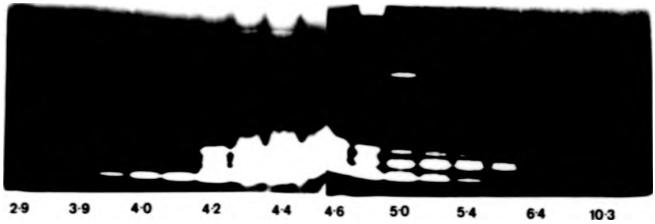


Figure 59. Analysis of proteinase activities by SDS-PAGE of isoelectrically focused fractions from *Tritrichomonas foetus* KV1 cell lysates. The pH of the fractions are indicated.

Figure 60. Analysis of the proteinase activities by gelatin SDS-PAGE of isoelectrically focused fractions from *Tritrichomonas augusta* cell lysates. The pH of the fractions and approximate molecular weights are indicated. The molecular weights range from M_rs 32 000 to 60 000 (Lockwood *et al.* 1987).

8.5 and above and with most of the proteinase activity at a higher molecular weight than the *Tritrichomonas* species (figure 61). Higher molecular weight activities are consistent with data published by Lockwood *et al.* (1987). Interestingly, there is a distinct loss of proteinase activity in two intracellular *Trichomonas vaginalis* fractions at pH 4.6-5.0, which may be the result of a co-focussing proteinase inhibitor. Surprisingly, the lowest molecular weight proteinase activity in *Trichomonas vaginalis* was not inhibited in these fractions. Although some focusing has occurred in this species the high levels of activity make it hard to distinguish individual enzymes. More work is therefore needed here with lower levels of activity in the starting preparations.

3.7.3 Summary

Interestingly some proteinase bands of the same molecular weight have focussed over very broad pH ranges. Examples of this are seen with the lowest molecular weight form of extracellular *Tritrichomonas foetus* which apparently focussed between pH 3.0 and pH 6.0 and also the lowest activity seen for *Trichomonas vaginalis* focused between pH 4.2 and pH 5.5.

This work has provided useful information on the pI's of individual enzymes and revealed some interesting differences between the intracellular and extracellular proteinases of *Tritrichomonas foetus*, strain F2, and between the enzymes of the two strains F2 and KV1 of this species. Further separation of various proteinase forms from all species of trichomonads could of course be achieved by focussing particular fractions over narrower pH ranges as discussed in section 3.3.5 and may be a starting point for future work in this area.

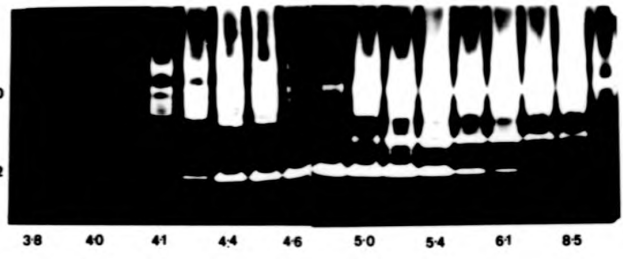


Figure 61. Analysis of the proteinase activities by gelatin SDS-PAGE of isoelectrically focused fractions from *Trichomonas vaginalis* cell lysates. The pH of the fractions and approximate molecular weights are indicated. The molecular weights of the proteinases were identified by comparison with previously reported proteinase patterns (North *et al.* 1990).

4.0 Discussion

4.1 Initial comments

Prior to this project being undertaken very little was known about the release of proteins from trichomonads. The phenomenon had certainly been shown to occur and to involve a number of different hydrolytic enzymes, and the extent to which the enzymes were released had been measured. However, neither the mechanism involved, nor the physiological significance of the process were understood.

The initial aims of this work were to attempt to investigate the mechanism of protein release by trichomonads. At least two different strategies might have been adopted. Firstly, the process could have been examined by looking at the whole range of proteins released, for example distinguishing the proteins synthesised and released by trichomonads by autoradiography after labelling cultures with ^{35}S methionine. This procedure would have the advantage of indicating a number of proteins whose fate could have been followed, thus gaining an understanding of their secretion kinetics. A disadvantage of this approach may be that the activities of individual proteins involved may not have been known. Secondly, the study could have focussed on individual proteins or groups of proteins of known activity, in this case selecting lysosomal hydrolases which had already been shown to be released. This approach would have the advantage that measurement of the activity could be used to monitor the release process, and something of the physiological significance of the process might be surmised. However, studies of individual proteins may have the disadvantage in that those chosen may not be representative or have the desired properties.

For this project it was decided to adopt the second approach, focussing on NAGase, the hydrolase released at the highest activity by *Tritrichomonas foetus* and also acid phosphatase, the hydrolase released at the highest activity by *Trichomonas vaginalis*.

A detailed understanding of the release process requires information on the properties of intracellular and extracellular forms of each enzyme to determine features which can influence the release process in order to pinpoint key steps of the biosynthesis of the proteins concerned. It was decided that it would be most useful to purify these enzymes and use the purified proteins to raise antibodies for use as probes for detecting intermediates in biosynthesis, and thus determine how proteins get outside the cell. The purified enzyme could also be analysed to provide information on its properties.

Neither of the selected enzymes turned out to be ideal. Although purification of one of the forms of NAGase was achieved it did not have the degree of antigenicity required to raise specific antibodies. Acid phosphatase proved surprisingly difficult to purify because it was unstable. Nevertheless this work has provided some further information on the release process and on the properties of some of the trichomonad hydrolases, most notably NAGase.

4.2 The release of hydrolases

NAGase and acid phosphatase were identified as typical lysosomal hydrolases. NAGase, which is often used as a lysosomal marker protein (Kivilvoma *et al.* 1990), had a pH optimum of 5.5. The enzyme also had the expected subcellular distribution as it was mostly associated with a large

particle fraction, which has similarities to eukaryotic lysosomal fractions. Acid phosphatase also has a low pH optimum (pH 5.5) but as had already been shown was present in a small particle fraction and had a different distribution from that found for NAGase. Thus the acid hydrolases from trichomonads are not uniformly distributed. Any explanation for a phenomenon common to NAGase and acid phosphatase must take this difference into account.

In *Tritrichomonas foetus*, NAGase was present in multiple forms which could be separated by SDS-PAGE and IEF. On using SDS-PAGE, the NAGase activity from intracellular and extracellular samples of *Tritrichomonas foetus* resolved into four main forms (figure 11). The same four forms were also separated by IEF (figure 13). From comparisons of these results it was concluded that the intracellular and extracellular forms of *Tritrichomonas foetus* NAGase are probably identical. All four forms of *Tritrichomonas foetus* NAGase were similarly released during the 6h resuspension in serum free medium. The apparently coordinated release of the different NAGase forms, and indeed of other types of hydrolase by these parasites, may suggest that they are all contained within a single subcellular compartment, presumably a lysosome or lysosome-like particle, which is directly involved in the release process. There is no evidence either for differential distribution of the four major forms of NAGase in this organism or, from work described here and in studies performed by North *et al.* (1989), that the process of release is regulated. Once NAGase is in the lysosomes it may be inevitable that the enzyme will be released as a consequence of exocytosis, directly from the lysosomes themselves. The data are difficult to reconcile with a separate secretory pathway. North *et al.* (1989) showed that the release process was not immediately inhibited by protein synthesis inhibitors, indicating that enzyme molecules are not released shortly after they are synthesised. These

conclusions were supported by studies showing a lack of effects of some inhibitors undertaken here (section 3.2.5). Thus the release of hydrolases from trichomonads appears to result from continuous (constitutive) loss of lysosomal contents.

Hydrolases, found in lysosomes of the cells of higher animals, are not generally released except during cell malfunction or death. For example, fibroblasts and hepatocytes in culture release acid hydrolases to the extracellular medium, only if the synthesis of a specific recognition marker is impaired in the cells. This marker, mannose-6-phosphate, which is covalently attached to the enzymes, is used for receptor mediated segregation of enzymes into the lysosomal compartments. If the receptor or the marker are lacking, the hydrolases fail to enter the lysosomal compartment, and are released, through the default secretory pathway, together with molecules belonging to the constitutive secretory pathway of the cells (von Figura & Hasilik 1986).

Many protozoa, however, release large amounts of hydrolases during normal growth in culture. Bates & Dwyer (1987) have demonstrated that acid phosphatase is a major secreted protein of the protozoan parasite *Leishmania donovani* although there are also two other forms of acid phosphatase which are not released by this organism. Release of other hydrolases from *Leishmania* have not been studied. A broad range of lysosomal enzymes is secreted from the ciliate *Tetrahymena pyriformis* during growth and starvation, including NAGase, acid phosphatase, ß-glucosidase, α-glucosidase, α-galactosidase, ß-galactosidase and α-mannosidase (Banno *et al.* 1987). The parasitic amoeba *Entamoeba histolytica* secretes

acid phosphatase, NAGase, α-glucosidase and amylase (Muller *et al.* 1988) and some studies have also suggested that proteolytic enzymes of this parasite are released (Keene *et al.* 1986). *Acanthamoeba*, another parasitic amoeba, releases the lysosomal hydrolases hexosaminidase and acid phosphatase continuously into the culture medium (Hohman & Bowers 1984). Olomu *et al.* (1986) have demonstrated that *Naegleria fowleri* contains acid phosphatase, hexosaminidase, β-glucosidase, β-galactosidase, β-fucosidase, α-mannosidase, arylsulfatase and β-glucuronidase. Although they have not yet investigated the release of these enzymes it was concluded to be highly probable that this process occurred. Therefore hydrolase release from trichomonads is not an atypical activity for protozoans. With the exception of *Leishmania donovani*, the other protozoa mentioned above release a spectrum of hydrolases as was found in the studies of *Tritrichomonas foetus* and *Trichomonas vaginalis*. Differences between trichomonads and *Leishmania*, and the lack of reports on the release of hydrolases from other flagellates suggest, however, that trichomonads are unique among this group of protozoa, with respect to the range of hydrolases released.

In the resuspension experiments described in section 2.3.2, for *Tritrichomonas foetus* the rate of NAGase release was determined as approximately 5-10% of the initial cellular activity per hour. This rate can be compared with values reported for two other protozoa, *Acanthamoeba* and *Tetrahymena pyriformis*. Similarly to trichomonads, *Acanthamoeba* releases lysosomal hydrolases continuously into the culture medium. The released enzymes can be separated into groups on the basis of their secretion kinetics. One group, which includes hexosaminidase, is secreted at

approximately 15% of the cellular activity per hour, and for the other, which contains acid phosphatase, the rate is approximately 5% of the cellular activity per hour (Hohman & Bowers 1984). The lysosomal enzymes secreted from *Tetrahymena pyriformis* during growth and starvation also fall into distinct groups based upon differences in their secretion kinetics (Banno *et al.* 1987). The first group contains acid phosphatase, ß-glucosidase and α-galactosidase, which are secreted at a rate of 4% of the initial cellular activity released per hour. The second group of enzymes which includes α-glucosidase, α-mannosidase and ß-galactosidase exhibit secretion at a rate of approximately 11-15% per hour. The third group containing hexosaminidase has the highest rate of secretion at 22% of the initial cellular activity per hour. Hexosaminidase showed a continuous increase in overall activity (intracellular and extracellular) during starvation of this organism. After 4h of starvation the intracellular hexosaminidase activity, expressed as a percentage of the initial intracellular activity, was 84% while the equivalent of 88% of the initial activity had been released during this period. The release is accompanied by continued synthesis and maintenance of high intracellular levels of hexosaminidase activity. This resembles the findings for *Tritrichomonas foetus*. In sharp contrast, after the same time period the intracellular level of acid phosphatase was 73% of the initial value while the released activity corresponds to only 16% of the initial cellular activity. There is no evidence reported that *Tetrahymena pyriformis* has lysosomal subpopulations and so it would appear that enzymes apparently in the same location can be released at different rates. However, studies of *Acanthamoeba* have provided an explanation. It has intracellular hydrolases which show a differential pH-dependent binding to membrane in the secondary lysosomes where they are almost exclusively restricted.

Membrane appears to be returned to the cell surface, after endocytosis, via small vesicles that fragment from secondary lysosomes. This vesicle shuttle appears to be the source of the secreted hydrolases. Hohman & Bowers (1984) suggest that the inner membrane and content of these vesicles undergo a marked pH shift when, following fragmentation from lysosomes, they fuse with the plasma membrane. This rapid pH shift and the differential pH-dependent membrane binding of hydrolases account for the hydrolase secretion kinetics.

As can be seen from above the rate of release of NAGase from *Tritrichomonas foetus* is slightly lower than that found for *Acanthamoeba* and *Tetrahymena* but is certainly of the same order of magnitude. Hence all of these protozoa constitutively release hydrolases at similar rates and a similar process may be involved.

A number of studies on a wide range of eukaryotic organisms have shown that lysosomal enzymes are synthesized on membrane bound ribosomes, transferred cotranslationally into the lumen of the endoplasmic reticulum and then transported through the Golgi complex, where they are likely to be subjected to processing, before they finally reach the lysosome (Stryer 1988). In *Tetrahymena thermophila* for example, hexosaminidase is known to be synthesised as a precursor polypeptide of M_r 79 000 which is converted to an M_r 59 000 polypeptide within 10 min, and this is further processed into at least three major mature active forms of M_rs 58 000 - 54 000 within 20 min (Hunseler *et al.* 1988). These forms are almost quantitatively secreted into the culture medium within 1-2 h after synthesis. The M_r 59 000 polypeptide is also partially processed into smaller polypeptides with M_rs 48 000-46 000 and M_rs less than 20 000, which are also secreted. The conversion of the M_r

79 000 into the M_r 59 000 polypeptide was concluded to be due to proteolytic processing. Further conversion into the M_r 58 000-54 000 forms involves carbohydrate processing, while the forms of M_r 48 000-46 000 and those with M_rs of less than 20 000 result from additional proteolytic cleavages of the M_r 58 000 - 54 000 polypeptides. Hexosaminidase from *Tetrahymena thermophila* is processed rapidly as only 2h after synthesis almost all mature forms of this enzyme are present in the culture medium. It is not reported in this work if any of the precursors are active.

It was possible that in *Tritrichomonas foetus* the four major active forms of NAGase might also be derived from a common precursor which underwent rapid processing before incorporation in lysosomes and release to the medium. However, there is no evidence for processing and interconversion of the active forms which were all apparently stable after cell lysis. Although it is possible that interconversion could only take place under conditions pertaining in the cell, all four forms must have been sufficiently stable to accumulate intracellularly in easily detectable amounts. Treatment with proteinase inhibitors failed to change the pattern of the four major enzyme bands (section 3.2.4). Had proteolytic processing, involving lysosomal cysteine proteinases, been involved, a change in the relative levels would have been expected. The presence of a fifth NAGase (figure 23) was variable and strain dependent (section 3.2.2) and it is possible this form might result from proteolysis either within whole cells or as a result of sample preparation.

As no difference was found between extracellular and intracellular forms of NAGase it is probable that no specific additional signals are necessary to target NAGase to the outside. Comparisons of the intracellular and extracellular enzymes produced under a variety of conditions show that the

only NAGase forms which were found in the cells, but not released into the medium, were those responsible for the additional bands found after incubation of cells with cysteine proteinase inhibitors (figure 23). There are three possible reasons why the 'abnormal' forms of NAGase activity are not released. It is possible that even in the presence of inhibitors these forms detected outside were short lived and never accumulated in the lysosomes in sufficient amounts to be released in detectable quantities. The short time period of the experiment may also have prevented them from accumulating outside in sufficient quantities, and if the incubation could have been extended then these forms could have been detected in the extracellular medium. Another possibility was that the structure of the hydrolases had been changed so they could no longer participate in the secretion process.

In addition to proteolytic processing the extent of glycosylation might be a factor in the production of multiple enzyme forms. In *Tetrahymena thermophila*, Endo F digestion of the secreted hexosaminidase polypeptides (M_r 58 000 and M_r 56 000) yields a single product with an apparent M_r of 52 000, demonstrating that these two forms differ only in their asparagine linked carbohydrates (Hunseler *et al.* 1988). It is possible, since NAGase 1 was also shown to be a glycoprotein (figure 44), that the NAGase forms only differ in the extent of their glycosylation. However, it is not possible to deglycosylate NAGase 1 under conditions where activity is retained and since it is not known if NAGases 2-4 are glycosylated no conclusions can be made. Tunicamycin treatment produced a probable non-glycosylated active form (apparent M_r 48 000), although it is impossible to relate it to NAGases 1-4 (section 3.2.2). The NAGase 3 activity band was, however, diminished in this sample (figure 21). The result showed that for at least one NAGase, glycosylation was apparently not needed for activity. Perhaps not surprisingly

tunicamycin only started to have effects on the NAGase activity pattern at concentrations at which growth was inhibited. Even long periods of exposure failed to have a dramatic effect on the pattern of NAGase suggesting poor uptake of tunicamycin into the trichomonad cells. However, it was not possible to determine whether the apparent 48 000 M_r non-glycosylated band (figure 21) would increase in intensity at the expense of any others over a longer period. It is not yet known whether the apparently non-glycosylated band, seen when the cells were grown in the presence of tunicamycin, was released as it was only observed in growing cultures in which the extracellular activity could not be examined. It would have been interesting to know whether a protein lacking its normal sugar content could be released.

Most of the inhibitors of other processes which might have been involved in secretion or other exocytotic processes (tables 8 & 9) that were tested, had no dramatic effect on NAGase activity or the NAGase activity pattern, seen on SDS-PAGE, allowing few positive conclusions to be drawn. At this stage there is no evidence for the involvement of Golgi function directly in release. The reliance on activity gels rather than totally denaturing SDS-PAGE also restricted the conclusions which could have been drawn as only active forms can be detected, making subtle changes in the amount of protein difficult to follow.

A number of questions remain unresolved, paricularly are there biosynthetic precursors to the four main forms of NAGase and what are the pathways of biosynthesis and secretion? To study these further, specific antibodies to the individual NAGase forms would be required to perform pulse chase experiments. It was therefore disappointing that purified NAGase 1 failed to elicit any immune response in rabbits.

4.3 NAGase structure

NAGase from *Tritrichomonas foetus* was chosen as a model enzyme to study the processes involved in the release of hydrolases from trichomonads. The NAGase activity profiles of all three strains of *Tritrichomonas foetus* studied were very similar, each containing the same four major forms (figures 14 and 15). There may be a difference between *Tritrichomonas foetus* strains F2 and KV1 in that a fifth NAGase band was always seen in strain KV1 samples after SDS-PAGE, but only occasionally and very faintly in strain F2. However, the strains of *Tritrichomonas foetus* F2, KV1 and CA84-2 can be concluded to have essentially similar NAGase activity profiles.

The four forms of NAGase may well be products of separate genes. What is interesting is that *Tritrichomonas foetus*, the only species to have multiple forms of NAGase, also exhibited the highest level of NAGase activity. In the absence of any evidence indicating a single NAGase precursor processed to form the four major forms it could be speculated that both the multiple forms and this higher activity have resulted because of duplication of genes which are each still expressed at normal levels. The origin of the fifth form of NAGase which appeared more consistantly in KV1 (figure 14) than in F2 may be a precursor or product of one of the other forms (discussed in section 4.2).

NAGase 1, the form purified, exhibited some unusual characteristics when analysed by electrophoresis. Samples of NAGase 1 not incubated at 100°C ran on SDS-PAGE with a mobility corresponding to an apparent M_r of 54 000 (figure 38). However, incubating samples at 100°C caused a dramatic change in mobility and the protein produced a doublet which corresponded

to apparent M_r's of around 70 000 (figure 40). At the same time NAGase 1 took up coomassie blue stain to a much greater extent. The change in mobility correlated with a loss of activity and indicated that totally denatured NAGase 1 could not be renatured to an active state after electrophoresis.

Similar anomalous behaviour has been reported for the major cysteine proteinase (cruzipain) from *Trypanosoma cruzi* (Martinez & Cazzulo 1992). The M_r of cruzipain is 36 300 as calculated from its sequence yet the apparent M_r of the enzyme, as determined by SDS-PAGE, has been reported in a range of values from 40 000 - 60 000. Martinez & Cazzulo (1992) found that the purified enzyme had a mobility corresponding to an apparent M_r ranging from 33 000 - 51 000 depending on the extent of reduction and/or incubation at 100°C of the sample. They also concluded that the reasons for various aspects of this anomalous behaviour were likely to be N-glycosylation and the presence of several disulphide bridges. These possibilities had also been considered for NAGase 1.

From the results obtained it seems most likely that the unusual behaviour of NAGase 1, when analysed by SDS-PAGE, is due to the extent to which the disulphide bonds are broken under different conditions of sample preparation. NAGases 1,2 and 3 may be similar enzymes which differ by the number of disulphide bridges and the ease which such bonds can be reduced.

Although it is not possible to define the M_r of NAGases 2 and 3, as they were not purified, it seems likely that these ran, under non-denaturing conditions, more according to their actual molecular size since the apparent M_rs of these enzymes from SDS-PAGE activity gels agrees more closely with the size

indicated for NAGase activity by gel filtration (figure 16). The single activity peak obtained after gel filtration could have contained NAGases 1, 2 and 3. A separation between NAGase 2 and 3 would probably not be detected considering the breadth of the activity peak (figure 16B) and if NAGase 1 ran according to its apparent molecular weight after total denaturation then it would also be expected to be eluted in the same peak as NAGases 2 and 3. The gel filtration data and mobility on denaturing gels of NAGase 1 suggested that this enzyme is monomeric. It seems likely that NAGases 2 and 3 may be enzymes of a similar size.

Very little is known about NAGase 4 which is in general of lower activity. The discrepancy in the apparent molecular weights of the NAGases from *Tritrichomonas foetus* after SDS-PAGE and gel filtration can be explained by assuming that the lowest activity form, NAGase 4, may have eluted from the S 300 column at the expected place but could not be detected due to its low activity. Interestingly, Edwards *et al.* (1975) purified a hexosaminidase 800-fold from extracts of *Tritrichomonas foetus*. This enzyme had an apparent M_r of 150 000, determined by gel filtration, which is more similar to the apparent M_r of NAGase 4 (158 000) than the rest of the enzyme forms. Using SDS-PAGE two other forms were supposedly detected, although no data was presented and no information was given of their apparent M_rs or whether they were active. These additional forms could, however, represent any of NAGases 1, 2 or 3.

The presence of large carbohydrate groups on proteins has also been shown to cause unusual behaviour on SDS-PAGE. This is probably because SDS only binds to the protein part of the molecule and the reduced net charge lowers the polypeptide mobility during electrophoresis (Hames

1981). NAGase1 does have an N-linked polysaccharide moiety, although it is not especially large (figure 44). The removal of the N-linked carbohydrate group resulted in a reduction in the apparent M_r of 2 000. The presence of extensive O-linked carbohydrate was not specifically investigated and therefore cannot be ruled out, although peanut lectin which detects galactose, which is more likely to be present in O-linked carbohydrate moieties, failed to stain NAGase 1 (figure 45). The presence of O-linked carbohydrate could have been responsible for anomalous behaviour on SDS-PAGE, but it is unlikely that the faster mobility would have been attributable to the relatively small N-linked carbohydrate moiety which was present. This is supported by the fact that the deglycosylated NAGase 1 actually ran faster than the intact enzyme (figure 40). It is interesting to note that the N-linked polysaccharide could only be removed from the denatured form of NAGase 1 suggesting it is held in a position which was not readily accessible by N-glycopeptidase F in the native protein.

As can be seen from table 14 the apparent molecular weights predicted for NAGases 1-4 fall within the very wide range of molecular weights found for NAGase from other sources. There is not enough information, however, to conclude whether the trichomonad enzymes are most similar to those of the groups of other organisms represented in table 14. Most studies have concentrated on the mammalian lysosomal NAGases, particularly those from humans. These enzymes exist as dimers made from combinations of two similar monomers, each monomer being derived from a single prepropolypeptide which is proteolytically processed en route to and within the lysosome to give three separate polypeptides linked by disulphide bonds (Hubbes *et al.* 1989). These mammalian enzymes are thus different from the

Table 14 Comparison of apparent M_rs of some NAGases and NAHases purified from different sources.

Species	Enzyme		Apparent M_r		Reference
Trichomonas foetus, F2	NAGase	1	54 000		figure 11
		2	89 000		"
		3	100 000		"
		4	158 000		"
Trichomonas vaginalis	NAGase		138 000		figure 14
Trichomonas augusta	NAGase		107 000		"
Tetrahymena thermophila	NAHase		55 000	monomer	Tiedtke & Rasmussen 1983
Dictyostelium discoideum	NAHase		130 -136 000	dimer 65-68 000	Graham *et al.* 1988
Trichinella spiralis	NAHase		100 000	monomer	Rhoads 1988
Penicillium oxalicum	NAGase		143 000	monomer	Yamamoto *et al.* 1985
Manduca sexta pupal hemolymph	NAGases		61 000 64 000	monomer dimer 54 000 & 10 000	Koga *et al.* 1983
Octopus vulgaris	NAGase		120 000	Tetramer (2 β subunits of 34 000 2 α subunits of 27 000)	Ceccarini *et al.* 1983

Mouse testes	NAGase		178 000	monomer	Gupta & Kapur 1981
Boar epididymis	NAGase	A	510 000	subunits of 67 000	Parkes *et al.* 1984
		B	510 000	subunits of 67 000, 29 000 and 26 000	
Hen egg white	NAHase		68 000	monomer	Ogawa *et al.* 1983
lysosomal fraction of hen oviduct	NAHase		53 000	monomer	"
Human	NAHase		100 000	Two subunits of 50 000	Hubbes *et al.* 1989

trichomonad NAGases which appear to be monomers.

The K_m determined for crude NAGase activity for p-nitrophenyl-N-acetyl-ß-D-glucosaminide in both *Tritrichomonas foetus* and *Trichomonas vaginalis* are in the same order as NAGase activities for the same substrate from a variety of organisms (table15). It was not possible to obtain the actual values for NAGase 1 owing to the inhibition by N-acetyl-glucosamine which was present during all activity assays of purified NAGase 1.

An attempt to sequence the N-terminus of NAGase 1 was unfortunately unsuccessful. However, with more time this could be repeated. A protein sequence might also allow the development of an oligonucleotide probe to screen pre-existing DNA libraries of *Tritrichomonas foetus* and *Trichomonas vaginalis* and allow the comparison of sequences of NAGases from other sources. Subsequent sequencing of NAGase cDNA or genes would provide more information on the structure of these enzymes.

4.4 Acid phosphatase

Acid phosphatase is a major released hydrolase from *Trichomonas vaginalis* which initially suggested its possible use as a model enzyme in this study. It was, however, found to be very unstable during purification and characterisation by procedures such as ion exchange or gel filtration chromatography, analysis by SDS-PAGE and preparative isoelectric focusing. After all column runs no activity could be detected in the collected fractions and the instability of this enzyme during SDS-PAGE is discussed in sections 3.6.8. The reason for this is not yet known as the instability has prevented further analysis and hence would make it difficult to purify this

TABLE 15. Comparison of the K_m of NAGases from various sources.

Species	K_m (mM)	References
Tritrichomonas foetus		
Crude intracellular	0.95 ± 0.12	figure 8A
Crude extracellular	0.86 ± 0.16	figure 8B
Purified intracellular	0.25	Edwards *et al.* (1975)
Trichomonas vaginalis		
Crude intracellular	0.71 ± 0.27	figure 8C
Triticum aestivum (wheat leaf)		
Purified intracellular	0.29	Barber & Ride (1988)
Tetrahymena thermophila		
Purified extracellular	0.49	Tiedtke (1983)
Dictyostelium discoideum		
Purified extracellular	1.5	Every & Ashworth (1973)
Mouse testes		
Purified intracellular	0.24	Gupta & Kapur (1981)

enzyme to homogeneity. It was therefore concluded that acid phosphatase was not a suitable enzyme to study these processes and investigate the possible roles of trichomonad secreted hydrolases.

Tritrichomonas foetus, strains F2 and KV1, and *Tritrichomonas batrichorum* all had one major intracellular form and one extracellular form of acid phosphatase (figure 54). *Trichomonas vaginalis,* however, had two intracellular and extracellular forms with higher apparent M_rs of around 160 000 (figure 54). This agrees with investigations by Gradus & Mathews (1985). To determine biochemical markers which might be useful for the characterisation of various taxons of trichomonads they compared the acid phosphatases from ten species and strains of these parasites. The investigation found that each strain contained one or two isoenzymes of acid phosphatase. Interestingly, the *Tritrichomonas foetus* strain (BP-4) studied by Gradus & Mathews (1985) only had one form of acid phosphatase while two out of four strains of *Trichomonas vaginalis* had two isoenzymes which is typical of the results presented in this thesis (figure 54). The other two *Trichomonas vaginalis* strains, however, contained one form of acid phosphatase. The presence of multiple forms of this hydrolase in other organisms is not unusual, for example the parasite *Leishmania donovani* contains three forms of acid phosphatase (Remaley *et al.* 1985) and *Eimeria vermiformis* has two forms which can be separated by gel filtration (Hosek *et al.* 1988).

The intracellular acid phosphatases of the trichomonads appeared as high molecular weight forms which gave an unresolved smear on SDS-PAGE, perhaps as a result of the presence of multiple processed forms. The extracellular enzyme forms appear as apparently lower M_r forms in discrete

bands on SDS-PAGE. There may be differences between the extracellular and intracellular forms of acid phosphatase, perhaps indicating a difference in the synthesis and, or processing of this enzyme compared to NAGase. It should be noted, however, as discussed in section 4.2, that acid phosphatase is not in the same subcellular compartment as NAGase (Lockwood *et al.* 1988). The high molecular weight smear was seen for all the trichomonad strains (figure 54). Interestingly, the acid phosphatase of *Leishmania donovani* is also detected as heterodisperse bands on SDS-PAGE. Mrsa *et al* (1985) purified acid phosphatase from yeast and found evidence for two enzyme forms. When subjected to electrophoresis only very diffuse bands of acid phosphatase could be detected, as is seen with *Leishmania*, and in the studies with *Trichomonas vaginalis*. The mature acid phosphatase of *Leishmania donovani* contains N-linked oligosaccharides and work with tunicamycin and monensin indicated that Golgi processing, probably glycosylation, is responsible for the heterodispersity of the mature leishmanial enzyme observed in SDS-PAGE. Work with these inhibitors involving the acid phosphatases of trichomonads may be of interest in future research.

4.5 Immunological studies

Studies, using specific antibodies, are central in investigating the mechanism of release of these hydrolases, and an understanding of these processes are essential to completely appreciate the roles which these enzymes play intracellularly and when released.

Attempts to raise antibodies to NAGase 1 in rabbits were unsuccesful (section 3.5). This result although surprising may be explained. NAGase is a

major enzyme released by *Tritrichomonas foetus in vitro*. If the process occurs *in vivo*, and there is evidence to suggest that it does (see below), NAGase would be expected to be fully exposed to the host's defence mechanisms. If large quantities of hydrolases, including NAGase, are released *in vivo* they may have evolved a structure which is not easily recognised by the immune system. This would ensure that they could carry out their function and perhaps enhance parasite survival despite the immune system. Many trichomonad proteins are known to be recognised by the hosts immune system although little is still known about the nature of the antigenic constituents of trichomonads and their immunogenic potential in the human host. There has also been very little work carried out on the antigenicity of specific trichomonad enzymes. Proteinase release probably occurs *in vivo*. Initial studies have been performed which showed the presence of proteinases in mice vaginal fluid after infection with *Trichomonas vaginalis* (Lockwood *et al.* 1987). Anti-proteinase antibodies were raised in rabbits (Lockwood 1987) and recently Alderete *et al.* (1991) described the presence of antibodies to trichomonad proteinases in the sera of *Trichomonas vaginalis*-infected patients.

Many trichomonad proteins have been shown to be antigenic. Mathews *et al.* (1987) have investigated *Trichomonas vaginalis* using differential centrifugation to yield defined subcellular fractions. It was reported that the principal antibodies in the serum of infected women were directed towards cytosolic components, not against membrane components, although all subcellular fractions contained multiple antigenic moieties. There have been no studies performed, however, on released material. Alderete *et al.* (1986c) have evaluated the protein composition of *Trichomonas vaginalis* isolates using 2D SDS-PAGE. At least 200 intrinsically labelled, and about 30 major

extrinsically labelled, proteins of molecular weights less than 120 000 were resolved. Immunoblotting of such 2D gels demonstrated the presence of a number of highly immunogenic proteins. An interesting finding was that some high molecular weight proteins, though synthesized by all isolates, were not externalised on the surfaces of some parasites. This suggests that the presence or absence of certain high molecular weight proteins on the surface of trichomonad strains may be the principal mediators of antigenic heterogeneity.

Less information is available on antigenic determinants of *Tritrichomonas foetus* although Burgess *et al.* (1986 ; 1988) identified a major surface antigen of apparent M_r 150 000. Similarly, Hodgson *et al.* (1990) have shown antigens of apparent M_rs 155 000 and between 45 000 and 75 000 which are probably also surface antigens as they were shown to mediate complement-dependant killing and prevent adherence of *Tritrichomonas foetus* to bovine vaginal epithelial cells.

Western blot analysis of antiserum raised against crude *Tritrichomonas foetus* cell lysate (figure 36) showed dominant high molecular weight antigens from both the cell lysate and crude medium extract as well as a doublet with approximate M_rs of 36 000 and 38 000 from the cell lysate. Although this does not link to any information specifically on NAGase it does confirm that some proteins from *Tritrichomonas foetus* were antigenic.

One of the most important objectives of future work must be to develop a probe for studying the synthesis, processing and release of NAGase. Although NAGase 1 proved to be non-antigenic in rabbits in these studies, it is possible that the use of another species to raise antibody may be

successful. Other avenues would be either to devise new purification procedures for the other forms of NAGase which may be more antigenic, or to choose another enzyme to investigate the biosynthesis and release of hydrolases by trichomonads.

4.6 Does released NAGase have a specific role ?

It is still too early to answer this question but there are pointers in the literature which can be considered.

Tetrahymena hydrolase secretion is apparently essential for growth. A unique mutant, MS-1, caused by a single recessive mutation has been produced which is constitutively blocked in the release of all lysosomal enzymes tested to date, under both nutrient and non-nutrient conditions (Hunseler *et al.* 1987). MS-1, however, possesses, bound within the cell, the same amount of active lysosomal enzymes as the wild type. This may indicate that the product of the mutated *sec* allele is needed for either transport to secretory lysosomes, or for insertion of lysosomal enzymes, into the correct domains of the cell membrane. Using this mutant Tiedtke & Rasmussen (1988) demonstrated that secreted acid hydrolases are essential for the survival, growth and multiplication of *Tetrahymena*.

The various species of trichomonad studied here inhabit different environments *in vivo* yet all secrete NAGase activity in culture. Significantly *Tritrichomonas augusta*, a non-pathogenic species, which inhabits the digestive tract of its host rather than the urogenital tract, nevertheless releases hydrolases including high levels of NAGase. This perhaps suggests that NAGase may not be specifically responsible for the pathogenicity of

human and bovine trichomoniasis. However, whilst not being a process specifically related to pathogenicity it is possible that the release of hydrolases, in a different enviroment to that inhabited by *Tritrichomonas augusta*, may have a role in the pathogenicity of trichomoniasis.

Hydrolases from other species have been shown to have cytotoxic effects on mammalian cells and are implicated in pathogenesis. Ravdin *et al.* (1988) found killing of host cells by *Entamoeba* occurs in three sequential events; adherence, extracellular cytolysis and lastly phagocytosis. It was observed that NAGase from this organism played a role in the breakdown of phagocytized erythrocytes and in the cleavage of glycoprotein bonds between adherent mucosal epithelial cells. However, a relationship between vesicle exocytosis and parasite cytolytic activity has not yet been resolved (Ravdin & Guerrant 1981).

There is additional evidence that hydrolases released from other protozoa may be involved in pathogenic host-parasite interactions. Chang (1978) has identified a cytopathic substance in culture filtrates from *Naegleria*. Although it was not isolated or characterised, the lytic cytopathic agent and the lysosomal hydrolases are perhaps one and the same substance. The acid phosphatase of this organism was studied to determine if it is capable of inhibiting the production of microbicidal oxygen metabolities by phagocytic cells as is the case for *Leishmania donovanii* (Saha *et al* 1985). Olomu *et al.* (1986), in similar experiments with *Naegleria* found no evidence to support this hypothesis.

In trichomonads, Savoia & Martinotti (1989) have reported cytotoxic activity of supernatants of *Trichomonas vaginalis* obtained from culture or after contact

and cultivation with trypsinized human amnion epithelial cells. Acid phosphatase, NAGase and, to a lesser degree alkaline phosphatase, esterase, ß-glucosidase and α-mannosidase were found to be present in the medium and the amount of acid phosphatase and NAGase released was found to be quantitatively related to the cytotoxicity of the parasite. Studies by Crampen *et al.* (1979) have indicated that the neuraminidases of *Tritrichomonas foetus* are able to break down molecular structures of the wombs of cows, indicated by measured cleavage rates. Both these studies implicate the involvement of hydrolases in the pathogenicity linked with trichomoniasis.

In general, therefore, the release of the hydrolases appears to be a major activity of the protozoans discussed here although a function has not yet been defined. Most studies have addressed the possibility that hydrolases are released in order to degrade host macromolecules to provide nutrients or to facilitate penetration and establishment of the parasite, either by breaking down tissue barriers or by immobilising host defence mechanisms. Thus hydrolase release may be closely linked to the pathogenesis of disease.

4.7 Concluding remarks

In general a variety of hydrolytic enzymes released from a range of parasitic protozoans have been shown to degrade host macromolecules, contribute to cytotoxicity and neutralize host cell defences, presenting a strong case for a significant role for these enzymes in pathogenicity.

There are many implications in this field of study for the development of new

antiparasitic drugs and to gain a greater knowledge and understanding of host/parasite relationships. However, with trichomonads and indeed other parasitic protozoa, knowledge of this area is scant. Very little has been documented about the biosynthesis and mechanisms of secretion of these hydrolases and only speculation about their functions *in vivo* can be made. It would, therefore, be of interest to study these processes. In addition, more *in vivo* studies must be done to determine if acid hydolases are secreted, and, if they are essential to the survival of the parasites, what are their precise functions. Obviously such experiments require antibodies to the secreted hydrolases and this would require further protein purifications. Given the worldwide suffering and economic losses caused by these organisms, it is important to continue research into the fundamentals of the growth of these organisms and host colonisation. Secreted hydrolases appear to be central to such research.

5.0 References

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