BIOCHEMISTRY OF SULPHUR-CONTAINING AMINO ACIDS IN TRICHOMONADS

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SUMMARY

S-Adenosylhomocysteine (SAH) hydrolase has been detected in crude homogenates of Trichomonas vaginalis, Tritrichomonas foetus and Trichomitus batrachorum at activities of 14, 1.2 and 3.3 nmol/min/mg protein, respectively. The enzyme from Trichomonas vaginalis was found to be soluble with pH optimum of 8.0 and apparent K_ms for adenosine and homocysteine of 100 μM and 155 μM, respectively. Ara A was shown to inhibit the T. vaginalis enzyme but only at relatively high concentrations (I_{50}, 100 μM), whereas sinefungin and 2'-deoxyadenosine had only small inhibitory effects. EDTA (I_{50}, 6 mM) and various divalent cations also inhibited the enzyme from T. vaginalis.

Homocysteine desulphurase (EC 4.4.1.2) and serine sulphydrase (EC 4.2.1.22) activities in various lines of T. vaginalis, both metronidazole-resistant and -sensitive, and other trichomonad species were assessed. Trichomonas vaginalis contained the highest homocysteine desulphurase and serine sulphydrase activities of all the species. Although the levels of the enzyme activity in T. vaginalis isolates differed, no correlation between the activities and sensitivity to metronidazole was apparent. Trichomonas vaginalis homocysteine desulphurase catalysed both the hydrolysis of homocysteine to hydrogen sulphide, ammonia and α-ketoacid and an exchange reaction between homocysteine and 2-mercaptoethanol. Homocysteine desulphurase was detected as a single enzyme band on isoelectric focusing, whereas several isoenzymes of serine sulphydrase were found. There were large differences in serine sulphydrase isoenzyme patterns between T. vaginalis lines and between species. Several isoenzymes were amplified in cells grown with 10^{-5}M DL-propargylglycine for 24 hr. Trichomonas vaginalis grown in the presence of DL-propargylglycine (10^{-5}M) for 24 hr had no detectable homocysteine desulphurase activity but possessed markedly elevated

(approximately 3.5-fold) serine sulphydrase activity. Trichomonas vaginalis homocysteine desulphurase and serine sulphydrase activities were inhibited by bithionol, hexachlorophene and dichlorophene. These compounds also inhibited growth in vitro of T. vaginalis at concentrations similar to those that inhibited the enzymes. γ-Cystathionase (EC 4.4.1.1) activity was also detected at relatively low levels in T. vaginalis, Tritrichomonas foetus and Trichomitus batrachorum.

Trichomonas vaginalis growing in complex medium produced volatile thiols at a rate of 0.7 nmol/min/mg protein and the parasite suspended in PBS (phosphate buffered saline) with L-methionine excreted volatile thiols, including methanethiol, and α-ketoacid. Cell-free extracts of the parasite also produced volatile thiols from L-methionine, at the rate of 5.4 nmol/min/mg protein. Thiol production was not detectable with living cells or cell-free extracts of Tritrichomonas foetus, Trichomitus batrachorum or Pentatrichomonas hominis and homogenates of a range of trypanosomatids and mouse liver also failed to produce volatile thiols from L-methionine. The L-methionine-catabolising enzyme from Trichomonas vaginalis was found to be soluble with a pH optimum of 7.0 and an apparent K_m for L-methionine of 3.8 mM. The enzyme generated α-ketoacid in equimolar concentrations to volatile thiols; the release of ammonia was not detectable. The enzyme catabolised a range of substrates and was inhibited by several compounds, including bithionol and DL-propargylglycine. Parasites grown in the presence of 10^{-5}M DL-propargylglycine had no detectable L-methionine-catabolising enzyme activity. These findings indicate that T. vaginalis is significantly different from other trichomonads, a range of trypanosomatids and mouse liver in L-methionine catabolism, and that the parasite enzyme

responsible for L-methionine hydrolysis in T. vaginalis appears to be similar in several ways to bacterial L-methionine-γ-lyase (EC 4.4.1.11) and trichomonal homocysteine desulphurase (EC 4.4.1.2).

S-Adenosylmethionine (SAM) levels in trichomonads, a range of trypanosomatids and mouse liver were measured using HPLC techniques. The concentrations were found to be similar in each with the exception of Herpetomonas muscarum ingenoplastis, which contained approximately 10-fold more. Living trichomonads were found to incorporate exogenous L-methionine into intracellular SAM and its methyl carbon was also detected in lipids and nucleic acids, presumably through its involvement in transmethylation reactions. Norleucine and cycloleucine inhibited L-methionine uptake and incorporation into living T. vaginalis. Differences were observed between T. vaginalis and Tritrichomonas foetus with respect to the rates of incorporation of exogenous L-methionine into intracellular SAM and its involvement in transmethylation reactions. The results suggest that Trichomonas vaginalis and other trichomonads contain enzymes equivalent to SAM synthetase (EC 2.5.1.6) and SAM-dependent methyltransferases (EC 2.1.1).

A range of inhibitors of enzymes catalysing the metabolism of sulphur-containing amino acids were tested for efficacy against T. vaginalis in vitro. Sinefungin, tubercidin, ara A, bithionol, hexachlorophene, dichlorophene and 5-azacytidine were found to be effective antitrichomonal agents. Combinations of any two of these inhibitors were, in most cases, no more effective than one inhibitor used alone, but marked synergy was apparent with monothioglycerol and methionine. None of the inhibitors investigated was as potent as metronidazole, the drug of choice for the treatment of trichomoniasis.

The activities of pyruvate: methyl viologen oxidoreductase (EC

1.2.7.1), hydrogenase (EC 1.18.99.1), NADH: methyl viologen oxidoreductase (EC 1.6.99.3), NADPH: methyl viologen oxidoreductase (EC 1.6.99.1), NADH oxidase (EC 1.6.99.3) and NADPH oxidase (EC 1.6.99.1) were determined for T. vaginalis, Tritrichomonas foetus and Trichomitus batrachorum. The three trichomonad species were found to differ significantly, especially with respect to NADH oxidase and NADH: methyl viologen oxidoreductase activities. The species differences in ferredoxin-linked and oxygen-metabolising enzymes may be related to the ways in which the trichomonads are adapted for growth in their respective hosts.

1.0. INTRODUCTION

1.1. Classification of trichomonads

Trichomonads are classified under the phylum Protozoa, subphylum Sarcomastigophora, superclass Mastigophora, class Zoomastigophora, order Trichomonadida, family Trichomonadidae. Three genera of trichomonads are recognised in accordance to the number of anterior flagella present. They are Tritrichomonas, Trichomonas and Pentatrichomonas with three, four and five anterior flagella, respectively. The most recent detailed review of the systematics of trichomonads is that of Honigberg (1963).

1.1.1. General biology of the species

Trichomonads are aerotolerant, anaerobic, flagellate protozoa. They are actively motile and usually round, ovoid or pyriform-shaped. They reproduce by binary fission and generally do not produce cysts. Cyst (Holz 1953) and "pseudocyst" (Mattern and Wendell 1980; Fari et al. 1986) forms, however, have been reported. These forms are probably dying or degenerate trichomonads, although the latter form has been suggested to play a role in the transmission of trichomoniasis (Mattern and Wendell 1980; Fari et al. 1986).

Trichomonads are usually found in the buccal cavity, nasal passage, urogenital or digestive tract of their respective hosts which include man, cattle, birds and pigs. Most of these flagellates are thought to be harmless commensals but some are parasites of their hosts.

Trichomonad species differ from each other in morphology, physiology, biochemistry and immunologically. Figure 1 (a-d) represents the typical features of four species of trichomonads investigated in this project. Under phase contrast microscopy, the species can be easily identified as they display different characteristics (see Table 1). Trichomonads, in general, possess the

following:

(a) A prominent nucleus situated towards the anterior end of the organism.

(b) A skeletal axostyle which protrudes from the posterior end.

(c) A costa underneath the undulating membrane.

(d) Anterior flagella which originate from a large kinetosome complex. In Pentatrichomonas hominis, however, the fifth anterior flagellum originates from another kinetosome located ventrally to the base of the bundle of the typical anterior flagella (see Honigberg 1978a).

(e) A parabasal body where the Golgi apparatus is located.

(f) An endoplasmic reticulum network which is found as a corona around the nucleus.

(g) Free ribosomes which are distributed throughout the cytoplasm.

(h) Food vacuoles and some electron-dense vesicles which are situated at the caudal end of the body.

(i) Paraxostylar and paracostal granules which are membrane-bound organelles called hydrogenosomes (Lindmark and Muller 1973). The nature and biochemistry of these organelles are discussed in section 1.5.2..

The details of these trichomonal structures (a-i) can be seen in the manuscripts by Muller (1973), Honigberg (1978a, 1978b) and Benchimol and DeSouza (1983). Interestingly, trichomonads lack morphologically recognisable mitochondria and such biochemical attributes of mitochondrial metabolism as cytochromes and oxidative phosphorylation (Wellerson et al. 1959; Nielson et al. 1966; Lloyd et al. 1979; Muller 1980).

Trichomonas vaginalis (Donne 1836), Trichomonas tenax (Muller 1773), Pentatrichomonas hominis (Davaine 1860) and Trichomitus

fecalis (Cleveland 1928) are found exclusively in humans. Trichomonas vaginalis is the only species of four trichomonads known to be pathogenic to man; it parasitises the human urogenital tract. There have been reports on the pathogenicity of Pentatrichomonas hominis and Trichomonas tenax in causing dysentry or diarrhoea (Wenyon 1926) and pulmonary trichomoniasis (Hersh 1985), respectively. Nonetheless, the two trichomonads are generally considered to be harmless commensals in the intestine (colon/caecum) and buccal cavity of man, respectively. Trichomitus fecalis, as the name suggests, was found persistently in the stool of a patient (Cleveland 1928) and is not a common trichomonad of man.

Tritrichomonas foetus (Riedmuller 1928), Trichomonas gallinae (Rivotta 1878), T. gallinarum (Martin and Robertson 1911) and Tritrichomonas suis (Gruby and Delafond 1843) are four common trichomonads of veterinary importance. The latter trichomonad is probably similar, if not identical to T. foetus in morphology (Hibler et al. 1960), physiology (Doran 1957) and immunology (Stepkowski 1966; De Carli and Guerrao 1975). However, unlike T. foetus which parasitises the bovine urogenital tract, T. suis is a parasite of the swine nasal passages and digestive tract. Trichomonas gallinae and T. gallinarum, on the other hand, are parasites of the avian upper digestive tract and ceca, respectively. In chronic infections, these parasites may be found in the liver (T. gallinarum), vicera and central nervous system (T. gallinae) of their respective hosts.

According to Honigberg (1963), Trichomitus batrachorum is the most primitive member of the Trichomonadidae. Frogs and toads are hosts to this protozoan which lives essentially in the intestine.

Bovine and human urogenital trichomoniasis are sexually transmitted diseases. The diseases are world wide problems which

impart social and economic damages. In this respect, trichomonads are important in the fields of human and veterinary medicine.

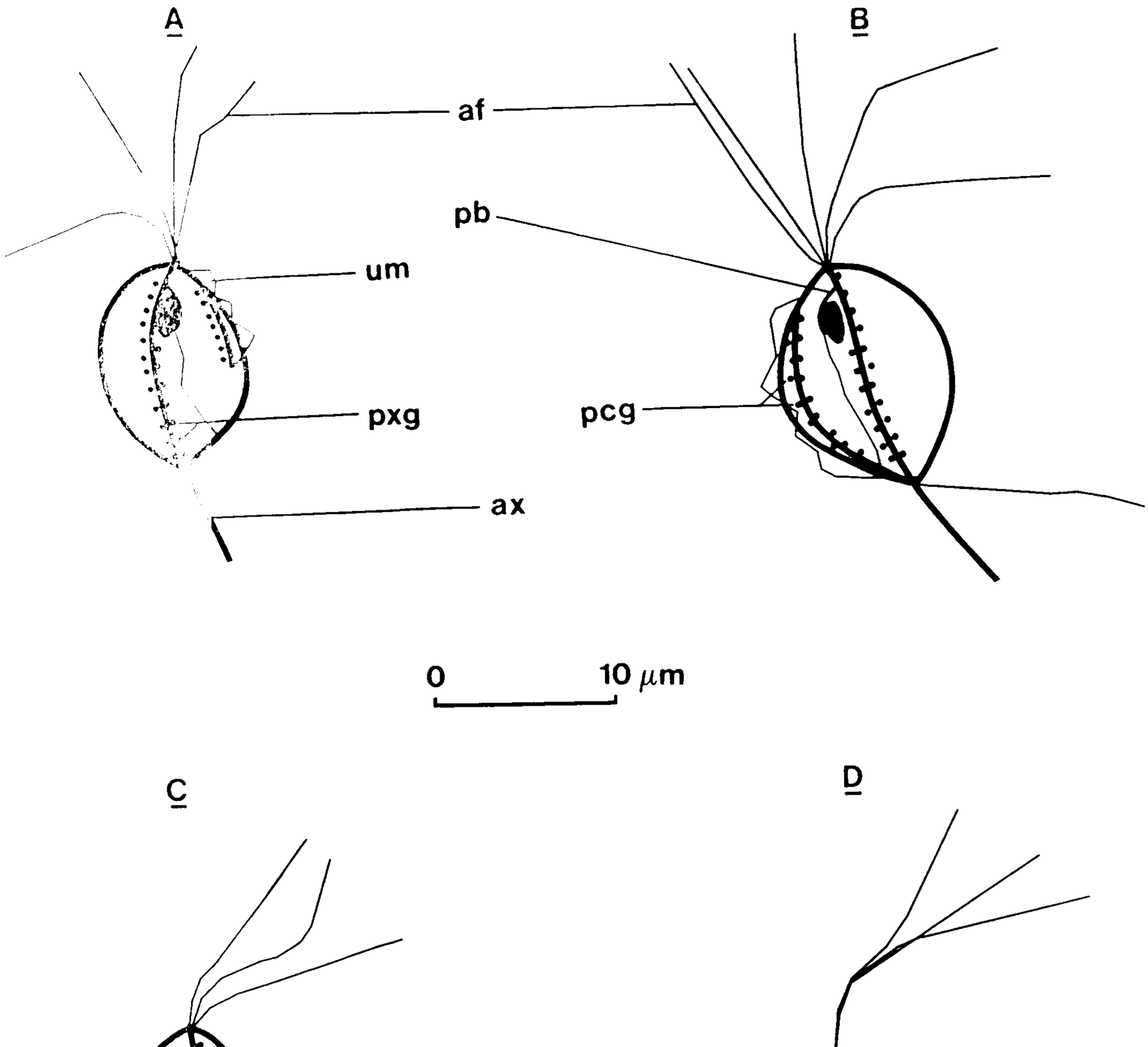


Fig. 1 Line diagrams of Trichomonas vaginalis (A), Pentatrichomonas hominis (B), Tritrichomonas foetus (C) and Trichomitus batrachorum (D) (Modified from Honigberg 1978a, 1978b; Farmer 1980). Key: af, anterior flagella; pb, parabasal body; um, undulating membrane; pxg, paraxostylar granules; pcg, paracosta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

Table 1 Some characteristics of the live trichomonads as judged by phase contrast microscopy.

Organism	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	-
Pentatrichomonas hominis	ellipsoidal or pyriform	(6-20 x 4-7)	fast jerky movements	prominent, long (about half body length)	full body length, with a free posterior flagellum	-
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 posterior flagellum	large bright vesicles/ vacuoles usually visible at the posterior end of the body
Trichomitus batrachorum	elongate or pyriform	(8-18 x 4-10)	fast jerky movements, twisting or rolling of the cell on its axis	less prominent, short	full body length, with a free posterior flagellum	form pseudocysts which occasionally display pseudopodia

[+] in all cases, whip-like actions of flagella and wave-like movements of undulating membrane and rotation of the cells on their axes can be seen.

1.2. Human trichomoniasis and treatment

Trichomonas vaginalis is the causative agent of human urogenital trichomoniasis. The parasite is transmitted during sexual intercourse, although non-venereal routes of transmission including physical contact of genitalia with infected towels or toilet seats, and infection during birth in infants are thought to occur (see Honigberg 1978b). In contrast, avian trichomonads (T. gallinae and T. gallinarum) are passed from infected parent to offspring through feeding, and by contamination of drinking water. Trichomonas tenax and Pentatrichomonas hominis, similarly, may be transmitted through direct oral-contact, and by contamination of drinking water and food. Human urogenital trichomoniasis is never life-threatening but may cause physical and psychological disturbances to the victims, especially the female. The reported incidences of T. vaginalis infections range from one to seventy percent (see Diamond 1983). This large range is probably indicative of the problems of diagnosis and difficulty in obtaining information on a sexually transmitted disease. Nonetheless, females are more frequently recognised as being infected than males, who are frequently asymptomatic.

The primary site of infection in the woman is the vagina and may extend to the cervix in latent infections. In some cases, the parasite may be found in the bladder and Skene's ducts (Grys 1973), and urinary passages (Soszka et al. 1973). Infections of the genital tract above the cervix are very rare because the cervic mucus presents a barrier to the parasite (Grys 1966). Typically, human urogenital trichomoniasis does not prevent pregnancy or cause abortions (Trussell 1947). Moreover, there is no substantial evidence for associating cervical cancer with trichomonal infections although such a link has been suggested (see Koss and Wolinska 1959; John 1986; Danilos et al. 1986). The main sites of infection in the

man are the urethra, prostate and epididymis.

The symptoms of human urogenital trichomonasis vary with individuals. More men are frequently asymptomatic than women. In spite of that, the infected male may develop any of the three stages as identified by Jira (1958): (i) primary latent (encountered most frequently): usually without any symptoms although this may lead to both acute (ii) and subchronic (iii) stages; (ii) primary acute: characterised by rapid onset and accompanied by a copious urethral discharge. This may develop into a subacute stage; (iii) primary subchronic: with a slow onset and characterised by relatively slight discharge. Again, this may lead to a secondary latent stage. Moreover, any of the three stages can give rise to the chronic stage. This stage is characterised by a slight itching sensation inside the penis and/or slight moistness at its tip. In other cases, purulent discharge may be observed before passing early morning urine or else scanty secretion throughout the day. Acute trichomonad urethritis and prostatitis are usually associated with purulent urethral discharge and inflammation of the external meatus. Furthermore, external ulceration in the area of the root of the penis has been known to occur (Kashchenko 1985). In women with trichomonal vaginitis, the two stages of the disease are acute and chronic. The acute form is marked by copious, frothy, foul-smelling discharge which may be associated with considerable inflammation and even ulceration of the genitalia (Rein and Chapel 1975; Honigberg 1978a; Spence et al. 1980; Kreiger 1981). The latter two conditions may cause severe itching or irritation in the genital region for some patients. The chronic form of the disease is noted with few symptoms except for an atypical discharge.

Trichomonal vaginitis is associated with changes in the

bacterial and mycotic flora in the vagina (Robinson and Mirchandani 1965; Teras et al. 1966). One well known feature is the disappearence of vaginal Lactobacillus species. Anaerobes and facultative bacteria like Lactobacillus and Peptococcus species are normally the dominant bacteria in the healthy vagina ecosystem (Bartlett et al. 1977). The precise nature of the associated changes in the vaginal micro-flora during trichomonal infections is not fully understood. Undoubtedly, any studies leading to the understanding of such a relationship between the parasite and the normal vagina micro-flora will provide potential targets for the chemotherapeutic attack of trichomoniasis.

Little is known, however, about specific events during the early stages of infection or about the mechanisms of T. vaginalis pathogenicity (Ackers 1982). Nielsen and Nielsen (1975) suggested that the interaction between T. vaginalis and the vaginal epithelium took place primarily at a distance probably by means of substances released into the vaginal fluid and, secondly, by a direct cell-cell contact mechanism. More recent findings on the pathogenicity of T. vaginalis, similarly, indicate the importance of releasing factors (or toxins) and/or cell-cell mediated mechanisms (Honigberg 1979; Alderete and Garza 1985; Krieger et al. 1985; Pindak et al. 1986). Nonetheless, a natural body defence or resistance against these parasites has been shown to occur involving the cytotoxicity of human monocytes and polymorphonuclear neutrophils (Rein et al. 1980; Mantovani et al. 1981) and production of antibodies IgG and IgM (Ackers et al. 1975; Mason 1979; Su 1982).

The most reliable method for the diagnosis of human trichomoniasis, as well as other trichomonal infections, is the cultivation and microscopic identification of the parasites (see Diamond 1983). Diagnosis by serological identification systems using

complement (see Honigberg 1978a, 1978b) and antibodies (Ackers et al. 1975) have also been employed. These methods, however, were not completely satisfactory because the body's immune response to T. vaginalis is poor and variable. A biochemical diagnosis of vaginitis based upon the determination of diamines in the vaginal fluid has also been described (Chen et al. 1982). This method exploits the claim that trichomoniasis is the cause of the increase in polyamine levels in the vaginal fluid (Chen et al. 1982; Sanderson et al. 1983).

The drug of choice for the treatment of human urogenital trichomoniasis is the 5'-nitroimidazole, metronidazole or "Flagyl", with cure rates of 90-95%. Other derivatives of nitroimidazoles including tinidazole, nimorazole, ornidazole and econazole have been used with considerable success (see Honigberg 1978a; Bloch and Symth 1985; Zrubek and Szymanski 1986; Valent et al. 1986; Ogunbanjo et al. 1986). The suggested treatment regimes involve treating both the infected person and partner(s). The use of metronidazole apparently offers little protection against future infection (reinfection) by T. vaginalis (Andrial and Pavic 1986). Moreover, the widespread use of this drug has caused the emergence of metronidazole-resistant T. vaginalis lines (Lossick et al. 1986). The treatment of patients infected with the drug-resistant parasites, therefore, is very difficult. This, together with the reported carcinogenic (Rustica and Shubik 1972) and mutagenic (Voogd et al. 1974, 1975; Speck et al. 1976) properties of metronidazole have stimulated the search for new drugs or other means to prevent or cure the infection. Incidentally, there are at present no other effective drugs (other than the nitroimidazoles) for the treatment of trichomoniasis. The pressing need for such new developments have been discussed

(Meingassner and Heyworth 1981; North 1985).

One such development is the discovery of Solco Trichovac (Basel Solco Ltd., Switzerland). This is a vaccine produced from killed variants of Lactobac illus acidophilus isolated from the vaginal secretion of patients with trichomoniasis and has been used with considerable success in treating non-specific vaginitis and trichomoniasis (Andrial and Pavic 1986; Soszka et al 1986; Stencl et al. 1986; Stojkovic and Bossart 1986; Hatala et al. 1986). There is as yet no clear-cut explanation for the mechanism of action of the vaccine. Antigenic cross-reactivity between Lactobacillus strains and T. vaginalis has been indicated (Stojkovic and Bossart 1986; Stencl et al. 1986), however, it may simply be acting as a non-specific adjuvant. Solco Trichovac has the advantage over drug therapy in that it confers protection against reinfection by the parasite for a period of one to two years (Andrial and Pavic 1986). Moreover, its powers of restoring the vaginal micro-flora milieu to normality in the infected patient has been suggested to play an important role in the cure. Further studies are required before it will be possible to assess the true value of Solco Trichovac.

1.3. Bovine trichomoniasis and chemotherapy

Tritrichomonas foetus is the causative agent of bovine urogenital trichomoniasis. The parasite is transmitted during coitus. Tritrichomonas foetus, unlike Trichomonas vaginalis, can cause infertility and early embryonic death leading to abortions in cattle (Bartlett 1949). Bovine urogenital trichomoniasis is of world wide distribution and in the United States is ranked third (next to leptospirosis and brucellosis) as the disease most responsible for causing abortions in cattle (see Honigberg 1978a). The disease is a great threat to the economy of beef industries, sometimes causing

losses to the value of millions of pounds (Honigberg 1978a).

The main site of infection in the cow is the uterus, but sometimes the parasite remains in the vagina causing low-grade inflammation accompanied by vaginal catarrh (Morgan 1946). The invasion of the uterine cells by Tritrichomonas foetus may precipitate low-grade endometritis, uterine and cervical catarrh which subsequently prevent fertilization of the ovum. In severe endometritis, permanent sterility ensues. Abortions usually occur following conception of an infected cow. The cow may have a complete or incomplete abortion. The earlier occurs if the foetus is expelled together with the placental membranes: in such circumstances the cow recovers and is able to conceive again. On the other hand, incomplete abortion takes place when the membranes are still retained in the uterus: this condition normally leads to chronic endometritis and permanent sterility.

The main site of infection in the bull is the prepucial cavity (Hammond and Bartlett 1943). Only rarely are the parasites found in the urethra or deeper in the urogenital tract. The bull, unlike the cow, usually harbours the parasites for life. Acute inflammation, swelling of the prepuce and mucopurulent discharge are some of the early symptoms of the bull. This event usually subsides after two weeks of infection and the bull becomes asymptomatic. Some bulls, however, may develop chronic infections leading to orchiditis.

The characteristic swelling and discharges from the prepuce of the bull are often diagnosed as trichomonal infection. However, in order to confirm the diagnosis, the collection and culturing of preputial smegma for the presence of T. foetus are required (Todorvic and McHutt 1967; Clark et al. 1971). In contrast, bovine vaginitis is not diagnostic unless trichomonads are discharged.

Accounts of the pathological changes accompanying T. foetus

infection have been described in some detail (Levin 1973; Honigberg 1979). Nevertheless, the mechanism of pathogenicity of the parasite is poorly understood. The different consequences of T. foetus and Trichomonas vaginalis infections, however, suggest that the pathogenic mechanisms of the parasites are likely to differ at least in some aspects.

There is no fully effective treatment for bovine urogenital trichomoniasis as yet. The following drugs have been used to treat infected bulls: topical boroflavine-saalble (Bartlett 1948); topical acriflavine and berenil (Fitzgerald et al. 1963); oral dimetridazole (McLaughlin 1965); intravenous dimetridazole (McLaughlin 1968); intramuscular ipronidazole with pretreatment of procaine penicillin (Skirrow et al. 1985). In general, dimetridazole and ipronidazole treatments are effective but the earlier is expensive, and topical treatment is usually not effective. In veterinary medicine, more attention is given to treating infected bulls than cows because the infection in the cow is by nature self-limiting, whereas the bull harbours the parasites for life. In addition, infected bulls used for the breeding programmes may cause widespread trichomonal infections and subsequently enormous losses for cattle farmers.

1.4. Parasite cultivation

An essential prerequisite to the cultivation of a trichomonad in vitro is the isolation of the organism from its host. Clinical specimens of trichomonads may be collected using various techniques (see Diamond 1983). With T. vaginalis for example, sterile cotton swabs are used frequently to obtain material from the vagina or urethra. Sediments obtained after centrifugation of vaginal washings are usually inoculated into appropriate semi-defined media supplemented with antibiotics to axenize the cultures which are

maintained at suitable conditions for growth of the organism.

Trichomonads can be cloned using micromanipulation, serial dilution or agar plating techniques (see Diamond 1983) and their cultivation axenically *in vitro* in semi-defined media containing antibiotics and animal sera has made possible many biochemical studies for which large numbers of uncontaminated organisms are essential. The semi-defined media most used routinely for the cultivation of trichomonads are modifications of Diamond's medium (Diamond 1957), Bushby's medium (Bushby and Copp 1955) and Johnson's CPLM (Johnson 1947). These media unfortunately contain serum, peptones and protein digests of various kinds (Diamond 1983); such ill defined substances make studies to elucidate the nutrition of the parasite very difficult. The recent development of defined media (Linstead 1981) for the cultivation of trichomonads has provided some information on the nutritional requirements of these flagellates (Linstead 1981; Wang *et al*. 1984a). We are, however, still far from knowing the complete range of nutritional factors required by trichomonads. This is mainly due to the lack of studies in this area. Nevertheless, the importance of vitamin B_{12} for the growth and iron for the maintenance of maximal activities of hydrogenosomal enzymes of *T. vaginalis* have been reported recently (Gorrell 1985; Hollander and Leggett 1985).

The growth of trichomonads *in vitro* is dependent upon various physical factors, including oxygen tension and temperature. Trichomonads, being anaerobes, cannot tolerate high oxygen concentrations (see Diamond 1957). Their growth under aerobic conditions is very poor compared to that under anaerobic conditions, although the microaerophilic conditions provided by deep cultures of the appropriate medium with a limited gas phase of air are suitable for the routine cultivation of most trichomonads (Diamond 1983). The

mammalian trichomonads grow optimally at 37°C; T. vaginalis grows considerably slower at 32°C than at 37°C, a feature that can be used for culture maintenance. Prolonged cultivation of trichomonads in vitro results in a decrease in virulence (Lindgren and Ivey 1964; Dwyer and Honigberg 1970). The virulence of trichomonads, however, can be maintained over long periods by cryopreservation in liquid nitrogen (Diamond et al. 1965; Ivey 1975).

Trichomonads can be grown intravaginally (see Honigberg 1978b; Coombs et al. 1986), subcutaneously or intraperitoneally (Honigberg 1979) in animals (e.g. rats, mice, hamsters). These models have been used for the investigation of the mechanisms of pathogenicity of the parasites as well as for the screening of potential antitrichomonal compounds.

1.5. Biochemical aspects

The aspect of trichomonad biochemistry that has been most studied is carbohydrate metabolism, especially how it relates to the hydrogenosome. This area of metabolism has been described in some detail in sections 1.5.1., 1.5.2. and 1.5.3..

Other areas of the biochemistry of trichomonads that have been studied in some detail include purine and pyrimidine metabolism, proteolysis, and polyamine metabolism. There have been few reports, however, concerning lipid and amino acid metabolism.

Purine and pyrimidine metabolism of trichomonads have been studied in great detail (Miller and Linstead 1983; Wang and Aldritt 1983; Wang and Cheng 1984; Wang et al. 1983a, 1983b, 1984a, 1984b). It is now established that trichomonads, at least Tritrichomonas foetus and Trichomonas vaginalis, are similar to Giardia lamblia in that they do not have the capacity to synthesise pyrimidines and purines de novo but depend solely on salvage pathways to meet their

requirement for nucleotides (Wang 1983; Wang et al. 1983a, 1983b; Wang and Cheng 1984). Trichomonas vaginalis and Tritrichomonas foetus differ considerably in the ways in which they salvage purines (Wang 1983). The latter generates nucleotides by salvaging purine bases with phosphoribosyl transferases (PRTases), unusually a single enzyme accounts for the PRTase activity towards hypoxanthine, guanine and xanthine, whereas Trichomonas vaginalis converts adenosine and guanosine to their respective monophosphates through the action of kinases. Trichomonas vaginalis cannot interconvert the two mononucleotides, whereas nucleotide interconversions do occur in Tritrichomonas foetus. Giardia and Entamoeba salvage their purine requirements in similar ways to T. foetus and Trichomonas vaginalis, respectively.

Pyrimidines can be synthesised de novo by most parasitic protozoa, although not by trichomonads or Giardia species, which also lack thymidylate synthetase. Trichomonads obtain TMP from thymidine through the action of thymidine phosphotransferase. This is an unusual enzyme, is surface-membrane bound and clearly represents a prime target for chemotherapeutic attack as it is responsible for the supply of all the thymidine nucleotides for DNA synthesis. Trichomonas vaginalis salvages cytidine and uridine similarly to thymidine, despite its possession of very high and as yet unexplained activities of cytidine deaminase and uridine phosphorylase, whereas Tritrichomonas foetus uses these enzymes to convert both cytidine and uridine to uracil which is salvaged by uracil PRTase. The UTP finally produced can be converted to CTP to satisfy the cytidine nucleotide requirements. Tritrichomonas foetus contains ribonucleotide reductase but Trichomonas vaginalis lacks it and obtains the required deoxyribonucleotides directly from

deoxyribonucleosides through the action of deoxyribonucleoside phosphotransferases, an enzyme very similar to and possibly the same as thymidine phosphotransferase (Wang & Cheng 1984).

Interestingly, all other protozoan parasites investigated to date with the exception of Acanthamoeba (Hassan and Coombs 1986a) similarly lack the de novo purine synthetic pathway (Wang and Aldritt 1983; Hassan and Coombs 1985, 1986b, 1986c). It is suggested that this common deficiency among parasites may provide potential targets for chemotherapeutic attack (Wang 1983; Wang et al. 1984b; Hassan and Coombs 1985).

There have been relatively few publications on proteinases of trichomonads (McLaughlin and Muller 1979; Coombs and North 1983; Lockwood et al. 1984); in contrast to the abundance of information available on the proteinases of other eukaryotic microorganisms (North 1982; North et al. 1983; North and Walker 1984; Pupkis and Coombs 1984). Nevertheless, the investigations carried out so far on trichomonads have produced interesting results. Lockwood et al. (1984) investigated the proteolytic activity in three species of trichomonads, namely T. vaginalis, Tritrichomonas foetus and Trichomitus batrachorum. They found that there were multiple forms of cysteine proteinases in lysates of these parasites. Trichomonas vaginalis and Tritrichomonas foetus were more similar to each other than to Trichomitus batrachorum in the complexity of the proteinase band patterns. The physiological role of proteinases in trichomonads is not fully understood, but it has been suggested that the proteolytic system in trichomonads may be related to the pathogenicity of the parasites (Lockwood et al. 1984; Bremner et al. 1986a); a role in supplying amino acids is another possibility. In other cell types they are involved in many functions including protein translocation, sporulation, germination and pathogenesis (see

North 1982).

The metabolism of polyamines in trichomonads is discussed in section 1.5.4.. This section also incorporates the catabolism of the amino acid arginine since it is a direct precursor to the biosynthesis of polyamines. Apart from the information available on arginine catabolism (Linstead and Cranshaw 1983), very little is known about amino acid metabolism in trichomonads. The recent identification and characterisation of aspartate: 2-oxoglutarate aminotransferase from Trichomonas vaginalis (Lowe and Rowe 1985) has provided a little more information on this area of metabolism. The parasite enzyme was found to be similar to both the vertebrate cytoplasmic and mitochondrial isoenzymes in molecular and catalytic properties, however the enzyme's very high rates of activity with aromatic amino acids as donors and 2-oxoglutarate as acceptor suggests that it is very different from most eukaryotic aspartate aminotransferases (Lowe and Rowe 1985).

Another area of trichomonad biochemistry that has been studied little until recently is lipid metabolism. It is known, however, that trichomonads are unable to carry out oxygen-dependent steps of lipid biosynthesis and lipid degradation, hence they are natural fatty acid and sterol auxotrophs (Holz Jr. et al. 1986). This correlates well with the finding that lipids and fatty acids are essential for the growth of trichomonads in vitro (Shorb and Lund 1959; Linstead 1981). Analysis of intact trichomonads show that they contain 30% (of the total) neutral lipids (free cholesterol > cholesteryl esters > triacylglycerols > unesterified fatty acids > wax esters), 65% phospholipids (phosphatidylethanolamine > phosphatidylcholine > phosphatidylglycerol > unknown acid lipid > phosphatidylinositol > phosphatidylserine > sphingolipid >

lysophospholipids > polyphosphoinositides) and 5% glycolipids (Holz Jr. et al. 1986), indicating the importance of cholesterol and phospholipids in particular for cellular composition. There is no evidence to support the presence of conversion or retro-conversion reactions of fatty acids and cholesterols in trichomonads (Holz Jr. et al. 1986). Trichomonads, however, can convert saturated fatty acids to fatty alcohols, perhaps by a reductase-dehydrogenase mechanism, and that the alcohols act as precursors in the synthesis of glyceryl ether lipids (Holz Jr. et al. 1986). Moreover, the synthesis of phosphatidylcholine from phosphatidylethanolamine by methylation reactions involving the use of methionine as the methyl donor has been described (Linstead and Bradley 1985).

1.5.1. Carbohydrate and energy metabolism

Trichomonads utilise endogenous and exogenous carbohydrates for energy generation. Glycogen is the main endogenous carbohydrate store and is abundant in trichomonads (10-30% of the dry weight) (Muller 1976). A variety of exogenous carbohydrates can be used, as listed in Table 2, but in general, glucose and maltose are the two major substrates catabolised by trichomonads.

The energy metabolism of all trichomonad species studied is similar, although there are differences including the end-products. For example, lactate is produced by Trichomonas vaginalis, T. gallinae and Pentatrichomonas hominis but not Tritrichomonas foetus (see Table 2). On the other hand, T. foetus produces succinate which none of the other three can generate. It has been discovered recently that glycerol is a metabolic end-product of glucose catabolism in Trichomonas vaginalis and Tritrichomonas foetus (Chapman et al. 1985a; Steinbuckel and Muller 1986a). The amounts of glycerol produced compensated almost exactly for the deficits in

fermentation products recognised earlier (Mack and Muller 1980). Other end-products of carbohydrate metabolism detected in trichomonads include traces of butyrate, propionate and isovalerate (Saeki et al. 1984). Interestingly, it has been shown that Trichomonas vaginalis growing in complex medium (CPLM) produced methane, ethane, propane, ethylene, carbon monoxide, carbon dioxide and other unidentified gases (Ishiguro 1985). It was suggested that these gases were produced from the catabolism of amino acids (Ishiguro 1985).

Typically, trichomonads possess a glycolytic pathway similar to most prokaryotes and eukaryotes. Phosphoenolpyruvate (PEP) produced from glycolysis is partly carboxylated and reduced to succinate; part of it is also decarboxylated and oxidised to acetate. Substrate level phosphorylation occurs when PEP is converted to oxaloacetate (GDP → GTP) or pyruvate (ADP → ATP). The conversion of pyruvate to acetate leads to further substrate level phosphorylation.

Carbohydrate catabolism and energy generation mostly occurs in two cytoplasmic compartments, the cytosol and the hydrogenosome. The subcellular organisation of the main reactions occurring has been elucidated for Tritrichomonas foetus (Fig. 2) and Trichomonas vaginalis (Fig. 3) (see Lindmark 1976; Muller 1976; Steinbuchel and Muller 1986a, 1986b). The metabolism with the hydrogenosome is discussed in more detail in section 1.5.2..

Recently some attention has been focused on the study of carbohydrate metabolism in Pentatrichomonas hominis. Although our knowledge is still far from complete, the proposed metabolic map of the metabolism in this protozoan is illustrated in Fig. 4.

Overall, therefore, trichomonads possess pathways of energy generation and carbohydrate catabolism which are characteristic of organisms adapted to an anaerobic environment. Trichomonads cannot

oxidise carbohydrates to carbon dioxide and water under aerobic or anaerobic conditions which indicates that their metabolism is basically fermentative (Muller 1976). The presence of oxygen, however, affects the energy metabolism of trichomonads with respect to end-product formation and energy production (see Table 3). In Tritrichomonas foetus, oxygen decreases the production of succinate but stimulates the generation of acetate with concomitant cessation of hydrogen production (Muller 1976). The decrease in the former end-product was accounted for by the diversion of the reducing equivalents to oxygen (the electron acceptor for NADH oxidase) which prevented the reduction of oxaloacetate to succinate (see Fig. 2). The increase in acetate production and hence substrate level phosphorylation would appear to be an advantage to this parasite; trichomonads growth in vitro, however, is adversely affected by high oxygen concentrations (see Diamond 1957) which suggest that other factors than the apparent higher energy yield are involved. The inhibition of hydrogen production is probably due to oxygen scavenging all the electrons from the reduced ferredoxin (Muller 1976).

A significant feature of trichomonads in the presence of oxygen is the intense respiration (oxygen consumption) that occurs (Muller 1976; Mack and Muller 1980). The trichomonal enzymes mainly responsible for metabolising or scavenging oxygen and its metabolites are NADH oxidase, NADPH oxidase, catalase and superoxide dismutase (see Honigberg 1978a, 1978b). These enzymes are thought to play a vital role in maintaining a low redox potential, an essence of anaerobic living. Trichomonad hydrogenosomes require anaerobic conditions to function effectively (Muller 1976). The presence of oxygen affects energy metabolism (as noted above) as well as the

reductive activation of 5'-nitroimidazoles in the hydrogensome of trichomonads. The importance of the latter interference is discussed in section 1.5.2..

Table 2 Trichomonads: utilisation of exogenous carbohydrates and end-products formed.

Species	Carbohydrate substrates	End-products	Reference
Trichomonas vaginalis	glucose, maltose, glycogen, galactose, dextrin, trehalose and pyruvate	lactate, acetate, glycerol, malate, pyruvate, ethanol, glycerol-1-phosphate, alanine, hydrogen, carbon dioxide and other unidentified organic acids	Ninomiya and Suzuoki (1952); Read (1957); Muller (1976); see Honigberg (1978b); Mack and Muller (1980); Chapman *et al.* (1985a); Steinbuchel and Muller (1986a, 1986b)
Tritrichomonas foetus	glucose, maltose, glycogen, galactose, mannose, fructose, sucrose, trehalose, lactose, mannitol and raffinose	succinate, acetate, glycerol, ethanol, malate, hydrogen, carbon dioxide and other unidentified organic acids	Doran (1959); Shorb (1964); Muller (1976); see Honigberg (1978a); Saeki *et al.* (1984); Steinbuchel and Muller (1986a, 1986b)
Pentatrichomonas hominis	glucose, maltose, amylopectin, galactose, glycogen, ribose, fructose, sucrose, saccharose and lactose	acetate, lactate, hydrogen, carbon dioxide and other unidentified organic acids	Solomon (1957); Saeki *et al.* (1984); Lo *et al.* (1984)
Trichomonas gallinae	maltose, glucose, fructose, mannose, ribose, galactose and arabinose	acetate, lactate, hydrogen, carbon dioxide and other unidentified organic acids	Saeki *et al.* (1984)

Table 3 The effects of anaerobic and aerobic incubations on end-products formation by *Trichomonas vaginalis* and *Tritrichomonas foetus*.

Organism	Anaerobic	Aerobic
Tritrichomonas foetus	H_2, CO_2, glycerol and equimolar concentrations of acetate and succinate were produced. 5ATP/mol glucose formed.	O_2 consumed and no H_2 or glycerol production. More acetate and little succinate were produced. 7ATP/mol glucose formed.
	(References: Muller 1976; Honigberg 1978b; Steinbuchel and Muller 1986a, 1986b)	
Trichomonas vaginalis	H_2, CO_2, glycerol and equimolar concentrations of lactate and acetate were produced.	O_2 consumed and no H_2 production. Lactate, acetate and glycerol were produced.
	(References: Honigberg 1978a; Mack and Muller 1980; Chapman *et al*. 1985a Steinbuchel and Muller 1986a, 1986b)	

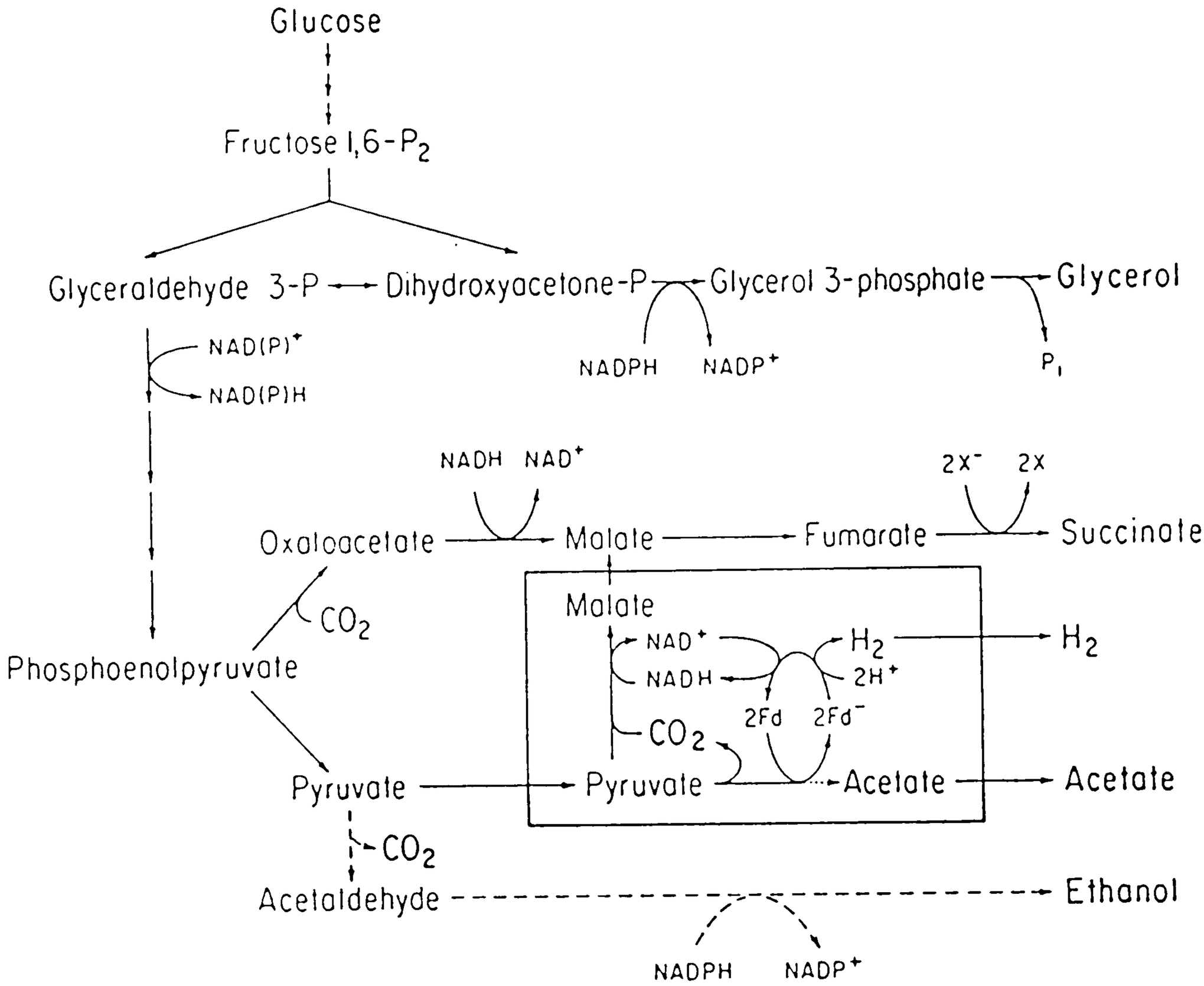


Fig. 2 Metabolic map of Tritrichomonas foetus. The formation of acetate from acetyl-CoA in the hydrogenosome (box) is simplified. For detailed information see (Muller 1980; Steinbuchel and Muller 1986b; section 1.5.2.). Pathway only active in T. foetus strain KV_1 - 1MR - 100 (---→). Arrows indicate the assumed physiological directions in vivo. The direction of flow is not indicated where uncertain (Diagram obtained from Steinbuchel and Muller 1986a).

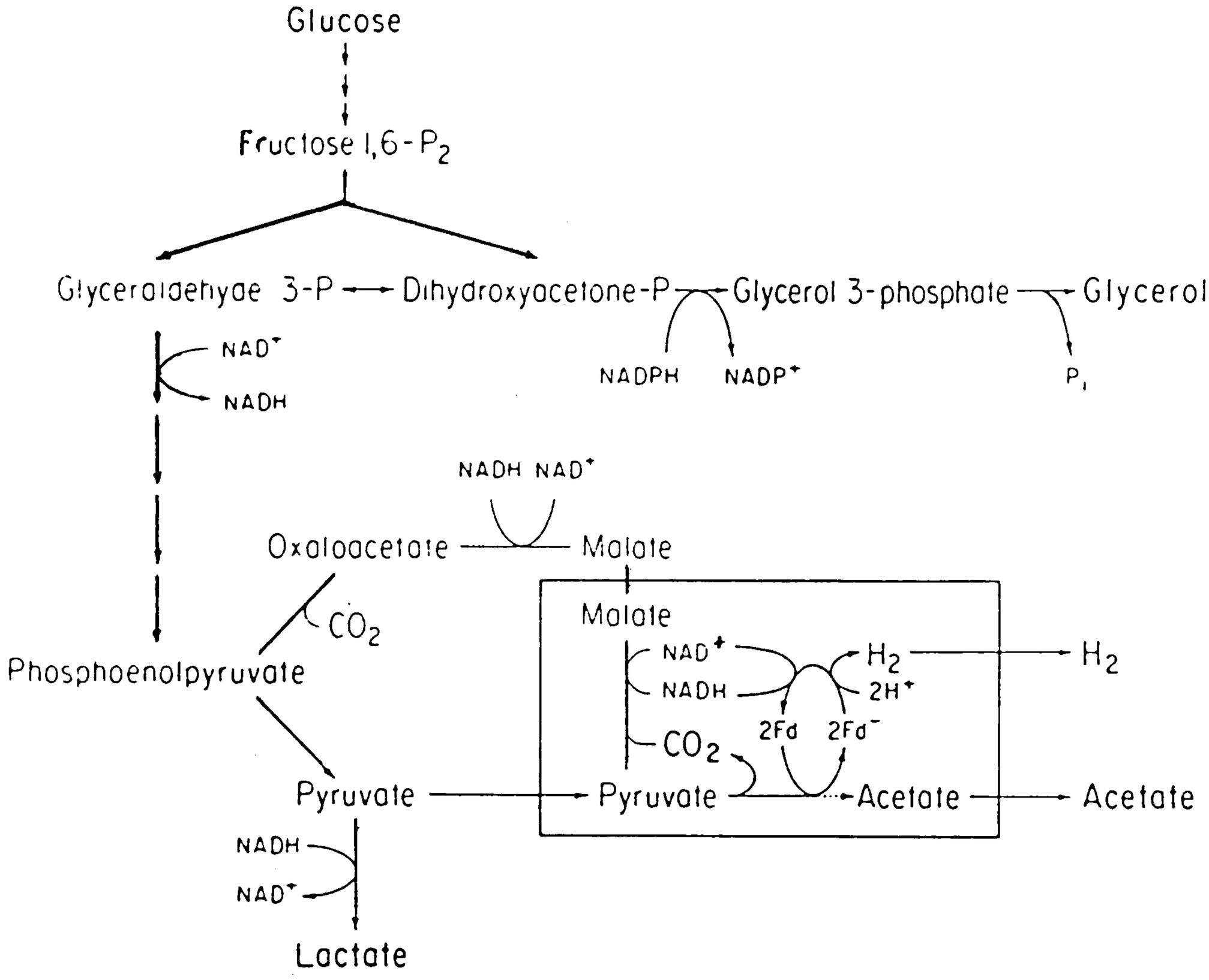


Fig. 3 Metabolic map of Trichomonas vaginalis. The formation of acetate from acetyl-CoA in the hydrogenosome (box) is simplified. For detailed information see (Muller 1980; Steinbuchel and Muller 1986b; section 1.5.2.). Arrows indicate the assumed physiological directions in vivo. The direction of flow is not indicated where uncertain (Diagram obtained from Steinbuchel and Muller 1986a).

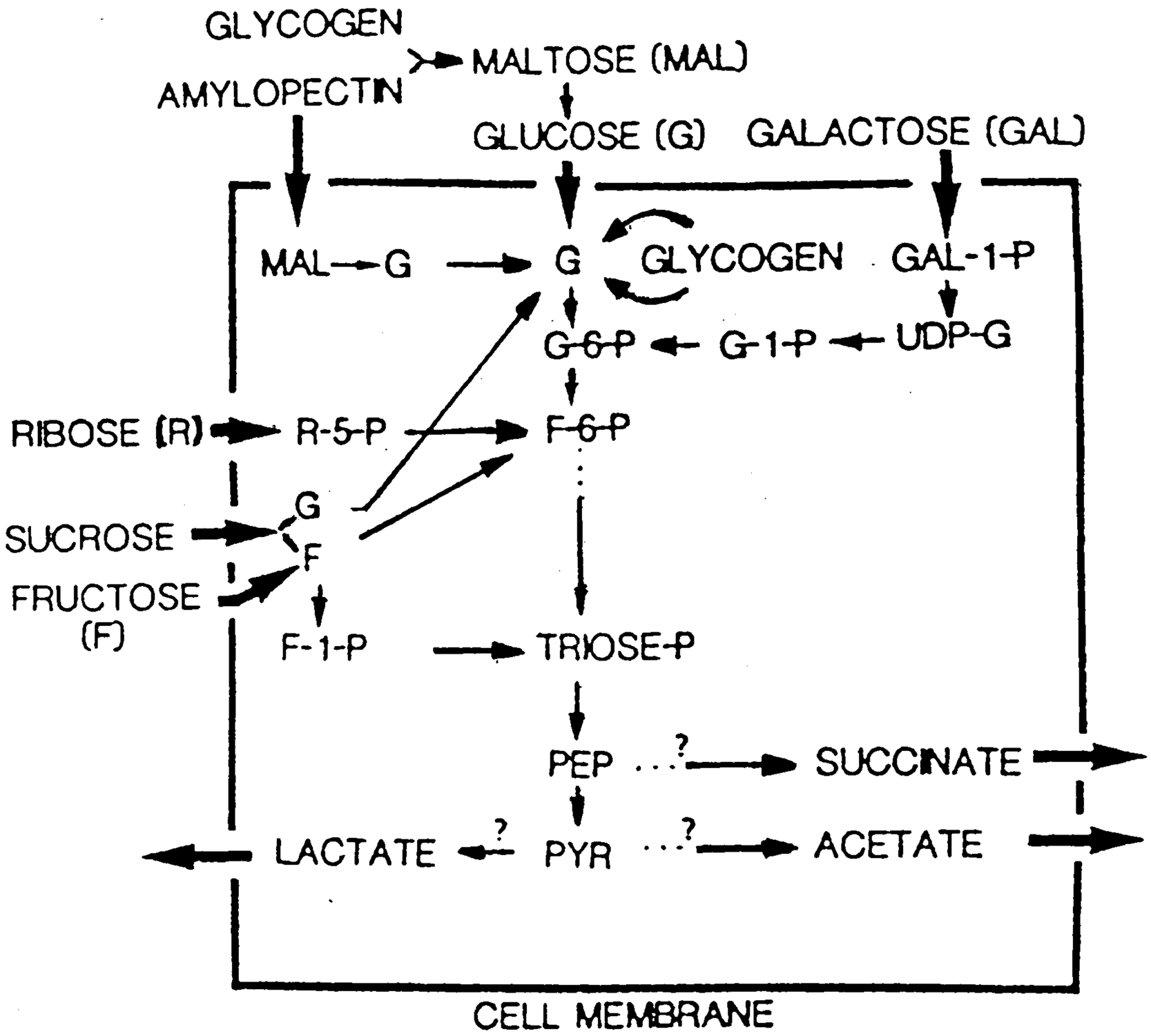


Fig. 4 Postulated pathways of carbohydrate metabolism in Pentatrichomonas hominis. Arrows indicate the assumed physiological directions in vivo. The box represents the whole cell. Abbreviations used are: UDP-G, uridine diphosphate-glucose; G-1-P, glucose 1-phosphate; G-6-P, glucose 6-phosphate; F-6-P, fructose 6-phosphate; F-1-P, fructose 1-phosphate; Triose-P, triose phosphate; PEP, phosphoenolpyruvate; PYR, pyruvate; R-5-P, ribose 5-phosphate (Diagram obtained from Lo et al. 1984).

1.5.2. Hydrogenosomal metabolism

Trichomonads possess characteristic membrane-bound organelles called hydrogenosomes which resembles neither mitochondria or peroxisomes (Muller 1973; Lindmark and Muller 1973). Hydrogenosomes are round bodies about 0.5 μm in diameter with a granular matrix and surrounded by two closely apposed 6 nm thick membranes (Benchimol and DeSouza 1983). The equilibrium density of the organelle in sucrose gradients is about 1.24 g/ml (Muller 1973). Similar organelles are also present in the rumen protozoan Dasytricha ruminantium (Yarlett et al. 1981) and other ciliates living in anaerobic marine and fresh water sediments (see Muller 1986). Entamoeba histolytica and Giardia lamblia are also aerotolerant, anaerobic protozoa which lack mitochondria and possess some of the typical hydrogenosomal enzymes (see Fig. 5). With these two parasites, however, the enzymes are located freely in the cytoplasm; hydrogenosomes have not been detected (Reeves et al. 1977; Lindmark 1980). At present, the evolutionary origin of the organelles is a mystery, as is their cellular origin. It was suggested previously that hydrogenosomes contain DNA (see Cerkasov et al. 1978), however, it is now confirmed that DNA is not present in the organelle (Muller 1985).

Over the past few years, a lot of information has been published on the biochemistry of the hydrogenosome, especially with respect to its involvement in the sensitivity of trichomonads to 5'-nitroimidazoles (Muller and Gorrell 1983; Cerkasovova et al. 1984; Kulda et al. 1984; Chapman et al. 1985b; Lloyd and Pedersen 1985; Lloyd and Kristensen 1985; Yarlett et al. 1985; 1986). The discovery of ferredoxin, an iron-sulphur protein, in the hydrogenosomes of trichomonads has shed some light into the nature and physiological significance of the organelles (Marczak et al. 1983; Gorrell and Muller 1986; Weiss and Muller 1986).

The metabolic reactions known to occur in the hydrogenosomes of trichomonads are illustrated in Fig. 5. Ferredoxin is thought to be a physiological electron carrier in hydrogenosomal pyruvate oxidation (Marczak et al. 1983). The protein containing a [2Fe-2S] cluster and of low molecular weight (about 12,000 daltons) has been isolated from the hydrogenosome of Tritrichomonas foetus (Marczak et al. 1983) and Trichomonas vaginalis (Gorrell and Muller 1986). The amino acid sequence of the two trichomonad ferredoxins were found to be similar but they differ from mitochondrial and chloroplast ferredoxins (Gorrell and Muller 1986). Using electron paramagnetic resonance spectroscopy techniques, Chapman et al. (1986), however, was able to demonstrate that the pattern of iron-sulphur clusters in T. vaginalis appeared to be more complex than in Tritrichomonas foetus. The synthesis of the two trichomonad ferredoxins in vitro on free polysomes has been studied using a combination of antibody, SDS-PAGE and fluorography procedures (Weiss and Muller 1986). The results indicated that both ferredoxins were made as a larger precursor.

One of the main functions of the hydrogenosome is the oxidative decarboxylation of pyruvate with acetate as the major final end-product. An intricate substrate level phosphorylation, involving acetate thiokinase (ADP, GDP) (EC 6.2.1), acetate: succinate CoA transferase (EC 2.3.1) and succinate thiokinase (ADP, GDP) (EC 6.2.1.4), and which does not occur in mammals, has been detected in trichomonads (see Fig. 5, Lindmark 1976). The presence of hydrogenosomes, therefore, increases the energy yield of glucose catabolism.

The reducing equivalents generated in the hydrogenosome are removed under anaerobic conditions as hydrogen. On the other hand, under aerobic conditions, oxygen is reduced via a non-haeme terminal

oxidase of high oxygen affinity, with the concomitant cessation of hydrogen production. It is thought, however, that the organelle requires anaerobic conditions to function effectively (Muller 1976). Recently, Steinbuchel and Muller (1986b) described in some detail the NAD^+: ferredoxin oxidoreductase activity detected in T. foetus and Trichomonas vaginalis. Both trichomonads were found to contain similar activity, although the precise role of the enzyme is not known. The enzyme could be used to recycle NADH for malate dehydrogenase (decarboxylating) and/or to reoxidise ferredoxin (see Fig. 5).

Other enzymes present in the trichomonal hydrogenosomes but which are not shown in Fig. 5, include NADH oxidase, NADPH oxidase, adenylate kinase and superoxide dismutase; whereas acetyl phosphate transferase, acetate kinase, catalase, Krebs cycle and glyoxalate cycle enzymes are absent (see Muller 1973; Cerkasov et al. 1978; Takeuchi and Kobayashi 1982). Thus, although there are some similarities, hydrogenosomes clearly differ from other microbodies such as mammalian peroxisomes, mitochondria and glycosomes.

Hydrogenosomes have been implicated with the reductive activation of 5'-nitroimidazoles, the drugs of choice for treating trichomoniasis (Chapman et al.1985b; Yarlett et al. 1985). The mechanisms of drug activation and resistance of trichomonads to the 5'-nitroimidazole, metronidazole are described in section 1.5.3..

It has been reported that both T. vaginalis and Tritrichomonas foetus can grow in vitro in the absence of hydrogenosomes and their enzymes (Cerkasovova 1986a). In this study, the cells were exposed to increasing sublethal doses of metronidazole (up to 100 µg/ml) which resulted in acquired resistance to the drug and lack of all hydrogenosomal functions. The findings, therefore, indicate that the organelles are not essential for the survival of trichomonads. It is

to be presumed from their existence (i.e. hydrogenosomes), however, that they do give some benefits to the parasite.

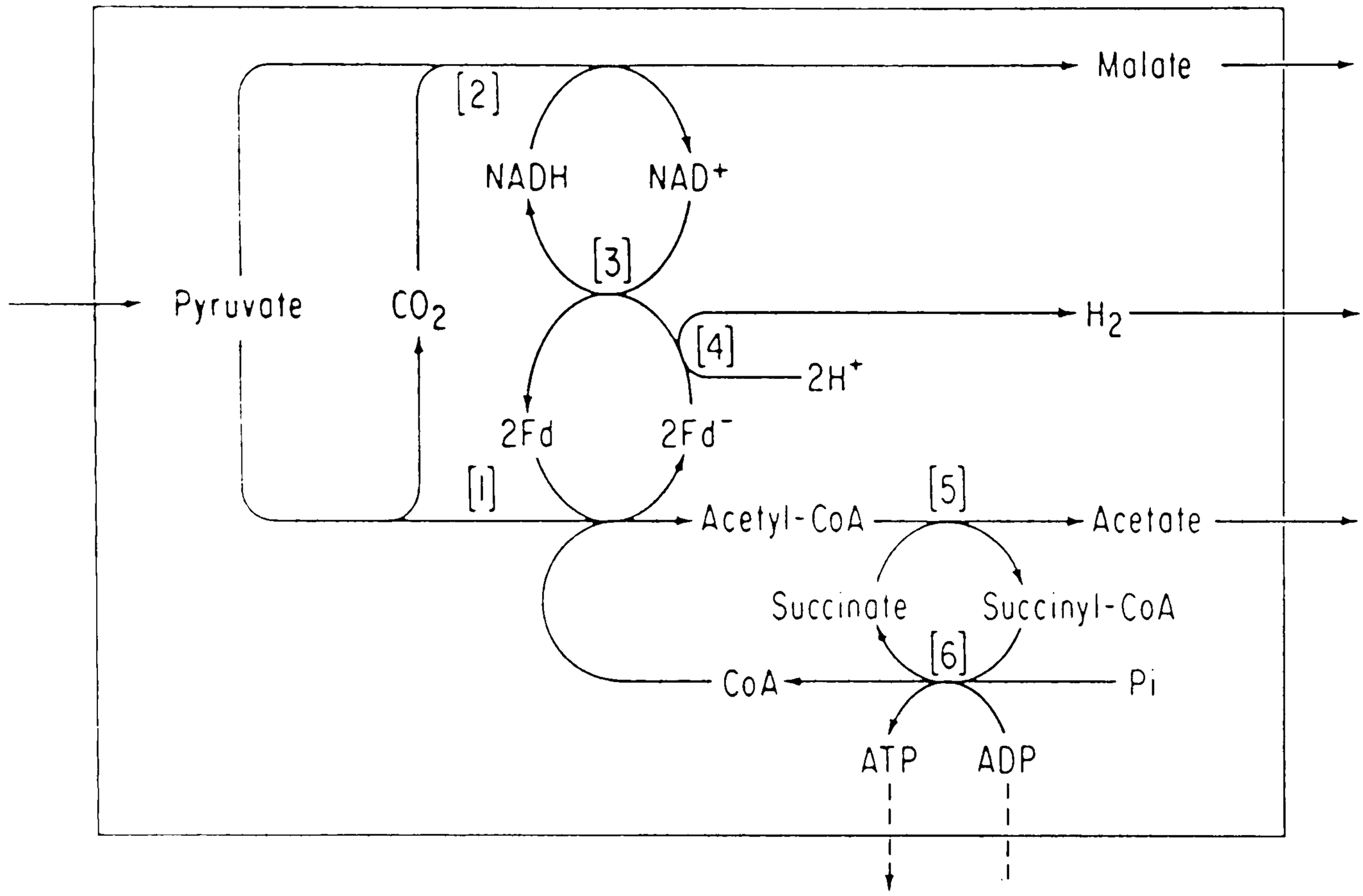


Fig. 5 Map of hydrogenosomal metabolism of Tritrichomonas foetus and Trichomonas vaginalis. Key to enzymes: [1], pyruvate: ferredoxin oxidoreductase; [2], malate dehydrogenase (decarboxylating) (NAD); [3], NAD^+: ferredoxin oxidoreductase; [4], hydrogenase; [5], acetate:succinate CoA transferase; [6],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

1.5.3. Reductive activation of 5'-nitroimidazoles and the mechanism of resistance of trichomonads to these drugs

It is now clear that the reductive activation of the antitrichomonal 5'-nitroimidazoles such as metronidazole occurs in the hydrogenosome (Chapman et al. 1985b; Lloyd and Pedersen 1985; Yarlett et al. 1985). This process and others that possibly modulate it are illustrated in Fig. 6. Pyruvate:ferredoxin oxidoreductase (reaction 5) is the major source of reduced ferredoxin which is the electron donor for the reduction of metronidazole (reaction 2) and other metabolites (reactions 1, 3 and 4). Using living Trichomonas vaginalis, Lloyd and Kristensen (1985) were able to demonstrate that hydrogen production (i.e. reaction 3) is strongly inhibited by oxygen, presumably due to the latter compound scavenging all the electrons. Under normal conditions, however, reaction (3) is the major route for reoxidising ferredoxin (Muller 1976). Lloyd and Kristensen (1985) showed that the presence of oxygen also stimulated the production of carbon dioxide by T. vaginalis which they accounted for by the enhanced rate of decarboxylation of pyruvate (reaction 5). Most interestingly, however, metronidazole was found to inhibit the production of hydrogen and carbon dioxide by T. vaginalis both under aerobic and anaerobic conditions (Lloyd and Kristensen 1985). This suggests that the drug not only successfully competes with protons for the available electrons but that the drug itself or a product of reduction actively inhibits some hydrogenosomal enzyme or electron carrier involved in hydrogen and carbon dioxide production (Lloyd and Kristensen 1985).

The reduction of the nitro group of metronidazole under anaerobic conditions produces cytotoxic intermediates which are generated according to the following pathway, as described by Muller

(1983): $(NO_2) \xrightarrow{e^-} (NO_2^-) \xrightarrow[2H^+]{e^-} (NO) \xrightarrow{e^-} (NO^-) \xrightarrow[2H^+]{e^-} NHOH.$ The mechanism of cytotoxicity is not well understood, but hypothetical schemes involving binding of these radicals to macromolecules including causing DNA lesions have been proposed (Edwards 1979, 1986). The one-electron nitro radical (NO_2^-), the first product in the metronidazole-reduction step, can be reoxidised by oxygen (reaction 6, Fig. 6) to yield the parent drug and superoxide (Muller 1983). This is thought to account, in part, for the lack of activity of 5'-nitroimidazoles against aerobes. The process of metronidazole reduction decreases the cellular level of the drug thereby increasing the gradient of diffusion of the drug into the cell; metronidazole uptake occurs by passive diffusion.

Lines of clinically isolated T. vaginalis resistant to 5'-nitroimidazoles have been reported from several countries (Meingassner et al. 1978; Thurner and Meingassner 1978; Muller et al. 1980; Lossick et al. 1986). All of these that have been studied manifest resistance in vitro only when examined in aerobic assays. Under anaerobic conditions, the lines are as sensitive to the most used 5'-nitroimidazole, metronidazole, as non-resistant lines. These findings demonstrate that resistance is related to the effect of aerobiosis. Laboratory induced anaerobic resistance to metronidazole in vitro of Tritrichomonas foetus (Cerkasovova et al. 1984; Kulda et al. 1984; Cerkasovova 1986a; Kabickova et al. 1986) and Trichomonas vaginalis (Cerkasovova 1985b; Demes et al. 1986; Kulda et al. 1986) has been reported recently. Anaerobic resistance is characterised by marked alterations of carbohydrate metabolism including the elimination of hydrogenosomal functions (Cerkasovova et al. 1984) whereas the mechanism of aerobic resistance has not yet been

clarified (Muller and Gorrell 1983). Theoretically, resistance to metronidazole could be due to modifications in any of four steps:

(a) entry of the drug into the cell.

(b) reductive metabolic activation of the drug to short-lived cytotoxic intermediates, a process strongly inhibited by aerobiosis.

(c) the oxygen-scavenging systems such as NADH oxidase thought to be the main terminal oxidase responsible for preventing permeation of oxygen into the hydrogenosome.

(d) interaction of the cytotoxic intermediates with the cellular components leading to cell death.

An investigation by Muller and Gorrell (1983) using drug-resistant and -sensitive lines of T. vaginalis failed to discover any correlation between the level of drug sensitivity and aerobic or anaerobic fermentation, respiration, anaerobic intracellular accumulation of [^{14}C] metronidazole, and activities of pyruvate: ferredoxin oxidoreductase, NADH oxidase or NADPH oxidase. Similarly, studies on the enzymology of drug activation by Cerkasovova et al. (1980) failed to uncover differences between susceptible and resistant strains. In another study, however, decreased metabolic activation and lowered NADH oxidase activity in drug-resistant lines was suggested (Clackson and Coombs 1982). More recent studies have indicated that resistant strains possess defective oxygen scavenging systems (Lloyd and Pedersen 1985; Yarlett et al. 1986) and that this deficiency is confined within the hydrogenosome (Yarlett et al. 1986). This indicates that unidentified oxygen scavengers normally present in the hydrogenosome but modified in drug-resistant lines are the key to 5'-nitroimidazole resistance in trichomonads. Thus, although the precise mechanism is yet to be discovered, the general conclusion that oxygen plays an important part in resistance of

clinical isolates of *T. vaginalis* to 5'-nitroimidazoles seems to have been confirmed.

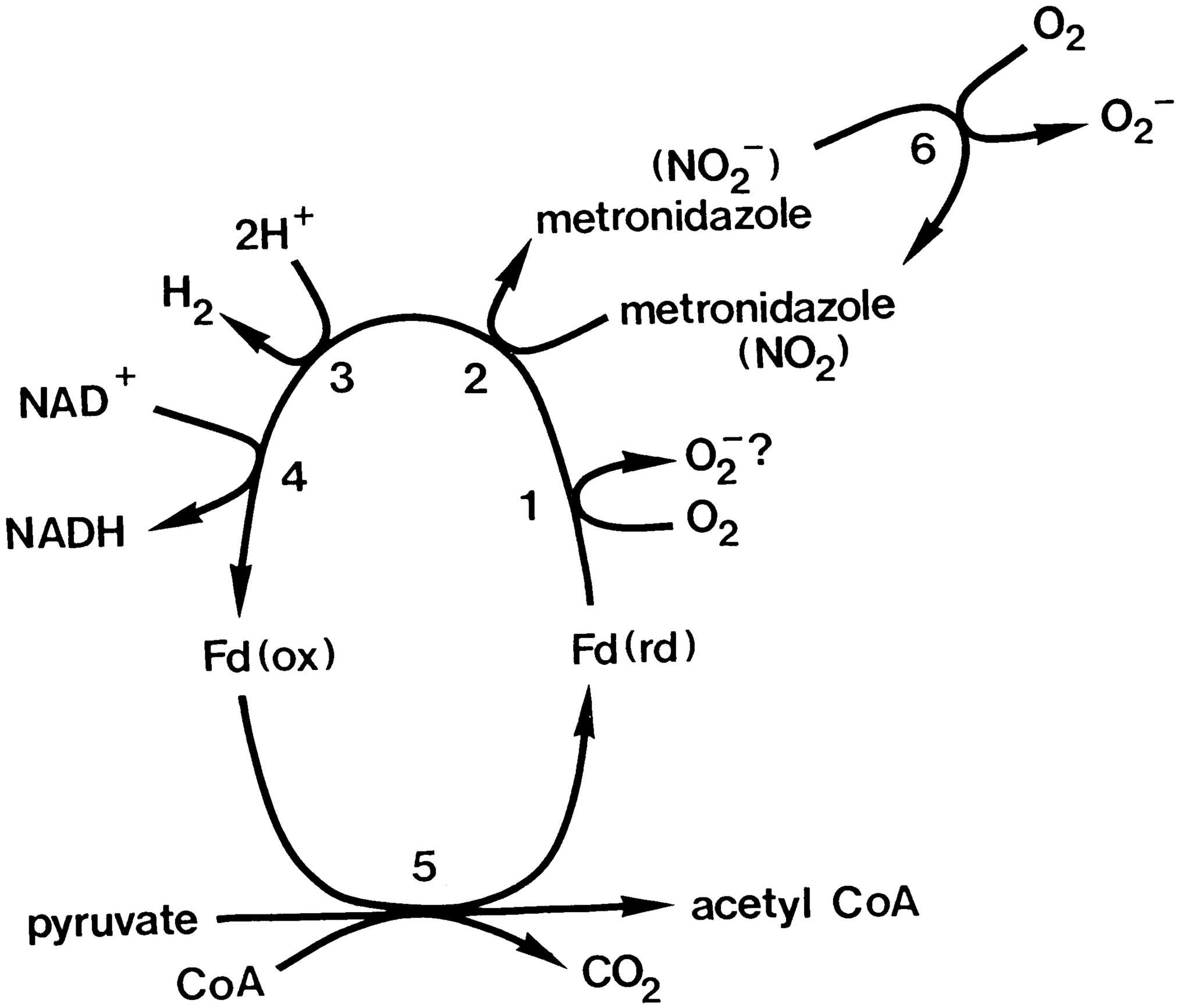


Fig. 6 Reductive activation of metronidazole and reoxidation of the nitro-free radical (NO_2^-) by oxygen (modified from Coombs 1976; Muller 1983). Key: rd, reduced; ox, oxidised; Fd, ferredoxin; 1, oxygen reduction; 2, metronidazole reduction; 3, hydrogenase; 4, NAD^+: ferredoxin oxidoreductase; 5, pyruvate: ferredoxin oxidoreductase; 6, reoxidation of the one-electron nitro-free radical (NO_2^-) of metronidazole.

1.5.4. Polyamine metabolism

Putrescine, spermidine and spermine are three naturally occurring polyamines found in many living organisms. These compounds are associated with cellular growth and differentiation processes but their exact functions are not fully understood (Raina and Janne 1975; Tabor and Tabor 1985). Nonetheless, the levels of polyamines probably vary from cell to cell. In general, eukaryotes contain both spermine and spermidine but have only trace amounts of putrescine, whereas prokaryotes possess higher concentrations of putrescine than spermidine and lack spermine (Tabor and Tabor 1976; Stevens and Winther 1979). Deviating from this generality is the phylum Protozoa. The trypanosomatids apparently have higher concentrations of spermidine than putrescine while spermine was either absent or present in only small amounts (Bacchi 1981; White et al. 1983). On the other hand, trichomonads contain high concentrations of putrescine and lower concentrations of spermidine and spermine (White et al. 1983; North et al. 1986).

Ornithine is the direct precursor to the synthesis of putrescine. The enzyme that catalyses this reaction, ornithine decarboxylase (EC 4.1.1.17), is present in Trichomonas vaginalis, Tritrichomonas foetus and Trichomitus batrachorum (North et al. 1986). The dihydrolase pathway which converts arginine in a two step reaction to ornithine has also been elucidated in Trichomonas vaginalis (Linstead and Cranshaw 1983). This, together with ornithine decarboxylase and the absence of arginine decarboxylase (EC 4.1.1.19) (North et al. 1986), would probably be the major route of polyamine biosynthesis in trichomonads. Alternatively, these flagellates may obtain putrescine and spermidine by uptake from the medium (North et al. 1986). Interestingly, trichomonads can also secrete putrescine into the culture medium (White et al. 1983).

The synthesis of spermidine from putrescine and spermine from spermidine requires decarboxylated SAM. This compound is derived from SAM by decarboxylation and the enzyme responsible is SAM decarboxylase (EC 4.1.1.50) (see Tabor and Tabor 1984). This enzyme is found in T. vaginalis and Tritrichomonas foetus but apparently absent from Trichomitus batrachorum (North et al. 1986). North et al. (1986) suggested that the human and bovine urogenital trichomonads, in general, were very much the same in polyamine metabolism, whereas the intestinal protozoan of the frog differed from these two. A schematic diagram of the metabolism of polyamines in Trichomonas vaginalis is given in Fig. 7. At present, the exact functions of polyamines in trichomonads are not known (White et al. 1983; North et al. 1986), although they probably play a part in regulating cell growth. Nonetheless, a possible link between polyamines and pathogenicity of T. vaginalis has been indicated (Bremner et al. 1986b).

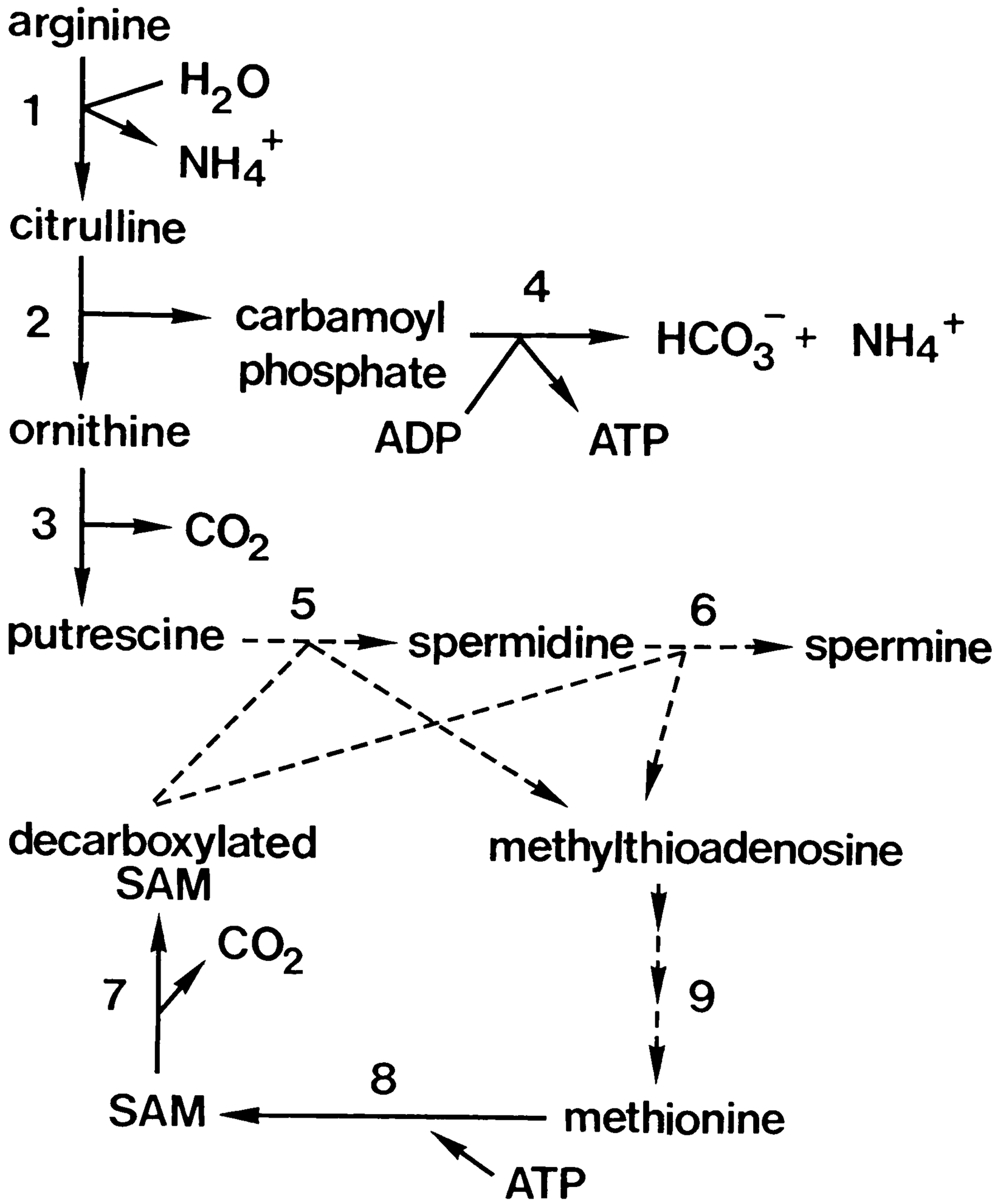


Fig. 7 Polyamine metabolism in Trichomonas vaginalis. Pathways that are present (——→) and not known (- - →). Key to enzymes: 1, arginine deiminase (EC 3.5.3.6); 2, ornithine carbamoyltransferase (EC 2.1.3.3); 3, ornithine decarboxylase (EC 4.1.1.17); 4, carbamate kinase (EC 2.7.2.2); 5, putrescine aminopropyltransferase or spermidine synthetase (EC 2.5.1.16); 6, spermidine aminopropyltransferase or spermine synthetase (EC 2.5.1.22); 7, SAM decarboxylase (EC 4.1.1.50); 8, SAM synthetase (EC 2.5.1.6); 9, conversion of methylthioadenosine to methionine.

1.5.5. Metabolism of sulphur-containing amino acids

Little has been published concerning the area of metabolism in protozoa in general, and the Trichomonadidae in particular. Nevertheless, there are indications that sulphur-containing amino acid metabolism not only occurs in many protozoa but also is of special importance to trichomonads:

(a) Methionine is an essential amino acid for Plasmodium knowlesi (Mckee et al. 1947) and Tetrahymena pyriformis (Kidder and Dewey 1945). The enzymes catalysing the transsulphuration pathway (i.e. the conversion of methionine to cysteine involving SAM synthetase, SAH hydrolase, cystathionine-β-synthetase and γ-cystathionase) have been identified in T. pyriformis (Dang and Cook 1977) and T. thermophila (Murphy and Fall 1985).

(b) Trichomonas vaginalis, Tritrichomonas foetus, Entamoeba histolytica and E. invadens lack dihydrofolate reductase (Wang and Aldritt 1983; Garrett et al. 1984) and so possibly all tetrahydrofolate-linked reactions. Consequently, all methylations that occur are likely to involve SAM as one-carbon donor. In contrast, dihydrofolate reductase activity has been detected in a range of protozoa including Plasmodium, Trypanosoma, Crithidia, Tetrahymena, Eimeria, Euglena and Leishmania (Ferone and Roland 1980; Garrett et al. 1984). In these organisms, the enzyme activity is due to a dihydrofolate reductase: thymidylate synthetase bifunctional polypeptide which is believed to be widespread among the protozoa (Garrett et al. 1984).

(c) Trichomonads are known to have an active polyamine metabolism involving ornithine decarboxylase, SAM decarboxylase and the secretion of large amounts of putrescine (see section 1.5.4.). The presence of polyamines in other protozoa including

Leishmania and Trypanosoma has been reported (Bacchi 1981; McCann et al. 1983). The importance of polyamine metabolism in regulating cell growth indicates that this area of metabolism is a potential target for chemotherapeutic attack on parasitic protozoa (McCann et al. 1983).

(d) Protein methylases I and III, two of the enzymes involved in the methylation of proteins using SAM as the methyl donor (see (viii) SAM-dependent transmethylases), have been detected in Leishmania (Paolantonacci et al. 1985).

(e) It has been suggested that Entamoeba histolytica, Giardia lamblia and Trichomonas vaginalis require thiols (particularly cysteine) to generate, maintain, and/or protect thiol groups on the parasites' external surface which are important for their survival (Gillin et al. 1984). This compound may be involved in countering the toxic effects of oxygen. Interestingly, cysteine appears to be essential for the growth of Trypanosoma brucei blood stream forms in vitro (Duszenko et al. 1985).

In contrast to the situation with respect to protozoa, the biochemistry of sulphur-containing amino acids in mammalian cells and other microorganisms is well documented (for review see Cooper 1983). The main reactions involved are shown schematically in Figs. 8 and 9, and will be considered in turn.

(i) SAH hydrolase (EC 3.3.1.1)

SAH hydrolase (also called S-adenosylhomocysteinase) is a central enzyme in this area of metabolism. The enzyme catalyses both the forward (hydrolysis) and reverse (synthesis) reactions of SAH to homocysteine and adenosine. The equilibrium of the reaction favours synthesis of SAH, although in vivo the reaction is pulled in the hydrolytic direction by the enzymatic removal of both homocysteine

and adenosine (De La Haba and Cantoni 1959; Palmer and Abeles 1979).

Based upon the investigation on a range of mammalian cells, bacteria, plants and birds, Walker and Duerre (1975) came to the conclusion that most eukaryotes possess SAH hydrolase whereas the enzyme is usually absent from prokaryotes. The detection of SAH hydrolase in Tetrahymena pyriformis and T. thermophila (Murphy and Fall 1985), however, is the only report concerning protozoa. In prokaryotes, SAH catabolism is brought about by SAH nucleosidase (SAHN, EC 3.2.2.9) and S-ribosylhomocysteine-cleavage enzyme (SRHE, EC 3.3.1.3). These reactions are illustrated below.

SAHN

(i) SAH + H_2O ⟶ adenine + S-ribosylhomocysteine.

SRHE

(ii) S-ribosylhomocysteine ⟶ homocysteine + 4,5-dihydroxy-2,3-pentendione

Both reactions are non-reversible and SAH nucleosidase was found to be present at high activities in a range of bacteria including Escherichia coli (Walker and Duerre 1975). Interestingly, SAH nucleosidase also catalyses the cleavage of 5'-methylthioadenosine to methylthioribose and adenine in bacteria (Walker and Duerre 1975; see Cooper 1983). 5'-Methylthioadenosine is a by-product of spermidine and spermine synthesis from putrescine and decarboxylated SAM, and spermidine and decarboxylated SAM, respectively (see section 1.5.4., Fig. 7).

All mammalian SAH hydrolases investigated to date have been found to be soluble (see Kajander et al. 1976; Usdin et al. 1979). The native protein of the enzyme is probably a pentamer of molecular weight ranging from 180,000-229,000 daltons (see Kajander and Raina 1981). The investigation of Palmer and Abeles (1979) on the purified

bovine liver SAH hydrolase has revealed some interesting aspects of this mammalian enzyme. It was found to contain a tightly bound NAD^+ (approximately 1 mol NAD^+ bound per subunit) and was inhibited by adenosine at concentrations greater than 0.14 mM. Addition of adenosine to the enzyme causes an increase in the absorption at 327 nm which was attributed to NADH formation. The spectrum of the enzyme shows a pronounced shoulder in the region between 300 and 400 nm. The enzyme also catalyses the rapid exchange of the adenosine 4'-proton with solvent water. They postulated, however, that the mechanism for the reversible hydrolysis of SAH involved the oxidation of position 3' of adenosine followed by α, β -elimination of homocysteine to give 3'-keto-4',5'dehydro-5'-deoxy adenosine. This compound reacts with water in a Michael-type addition to form 3'-keto adenosine which is then reduced to adenosine. In addition, the purified SAH hydrolase from rat liver on isoelectric focusing resulted in a major band of pI 5.6 (Kajander and Raina 1981).

It is thought that SAH hydrolase plays an important role in many eukaryotic cells in removing SAH, the product of transmethylation reactions involving SAM (Cantoni and Scarano 1954). SAH is an inhibitor of most SAM-dependent transmethylases (Deguchi and Barchas 1971; Chang and Coward 1975; Pugh et al. 1977; Robert-Gero et al. 1980) and it has been suggested that SAH hydrolase plays a crucial role in the regulation of biological transmethylations (Cantoni and Scarano 1954; De La Haba and Cantoni 1959; Kamantani et al. 1983). In contrast, the high activity of SAH nucleosidase and non-reversible catabolism of SAH that occurs in prokaryotes indicates that SAH probably plays no bioregulation function in these organisms.

In recent years, SAH hydrolase has emerged as a potentially useful target for the design of chemotherapeutic agents (Chiang and

Cantoni 1979; Chiang et al. 1980). Indeed numerous synthetic and natural analogues of adenosine and SAH, including ara A (Helland and Ueland 1982), adenosine dialdehyde (Patel-Thombre and Borchardt 1985), purine carbocyclic nucleosides (Houston et al. 1985a, 1985b), 3-deazaadenosine (Chiang and Cantoni 1979), 2'-deoxyadenosine (Hershfield 1979) and neplanocin A (Borchardt et al. 1984), that are good inhibitors of SAH hydrolase, have been found to possess interesting biological properties. Ara A, for example possesses oncostatic (Cass 1979), antitrichomonal (Wang et al. 1984b) and antiviral (Cass 1979) activities. 3-Deazaadenosine, 5'deoxy-5'-(isobutylthio)-3-deazaadenosine (SIBA) and sinefungin were found to be potent inhibitors of the growth of Plasmodium falciparum in vitro (Trager et al. 1980). Sinefungin, a weak inhibitor of SAH hydrolase, has good antileishmanial (Paolantonacci et al. 1985), antitrypanosomal (Dube et al. 1983), antifungal (Gordee and Butler 1973), antiviral (Robert-Gero et al. 1980) and antiamoebal (Ferrante et al. 1984) activities although these may be related to its inhibition of transmethylations (see (viii) SAM-dependent transmethylases).

(ii) Homocysteine desulphurase (EC 4.4.1.2)

Homocysteine desulphurase catabolises homocysteine to α-ketobutyrate, ammonia and hydrogen sulphide. The enzyme activity was first observed in cell-free extracts of Proteus morganii (Kallio 1951) and the only other report of the enzyme is of its detection in rat liver (Rosin and Chatagner 1969). In both cases the enzyme was found to be soluble and required pyridoxal phosphate as the cofactor.

The purified rat liver enzyme has a molecular weight of about 161,000 daltons and a K_m for L-homocysteine of 20 mM. The absorption spectrum of the enzyme revealed a minimum and maximum wavelength

at 255 and 280 nm, respectively. Based upon these and other properties of the rat liver homocysteine desulphurase, Rosin and Chatagner (1969) postulated that this enzyme is probably identical to mammalian γ-cyst athionase (EC 4.4.1.1). The catabolism of homocysteine to α-ketobutyrate, ammonia and hydrogen is thought to be of little significance in mammals (Cooper 1983).

The homocysteine desulphurase of Proteus morganii, on the other hand, has not been characterised to any great extent. It is known, however that this bacterial enzyme is very specific for pyridoxal phosphate for activity; none of pyridoxal, pyridoxamine or pyridoxine, alone or in combination with ATP was effective. Interestingly, cell-free extracts of P. morganii also catabolised the hydrolysis of cysteine to pyruvate, ammonia and hydrogen sulphide. The enzyme responsible for this reaction has been called, appropriately, cysteine desulphurase (EC 4.4.1.1). Kallio (1951) provided some evidence that the two desulphurases may be different enzymes in P. morganii. Furthermore, it was suggested that in P. morganii the sulphur-carbon cleavage in the desulphuration of homocysteine might occur first to yield α-aminobutyrate which by a subsequent step was deaminated to α-ketobutyrate and ammonia (Kallio 1951).

(iii) γ-Cystathionase (EC 4.4.1.1)

Mammalian cystathionase (also called homoserine hydrolase-deaminating enzyme) requires pyridoxal phosphate as a cofactor to catalyse the deamination of homoserine (α,β-elimination), the degradation of cystathionine (γ-elimination) and the desulphuration of cysteine (β-elimination) (Matsuo and Greenberg 1958a, 1958b; Brown et al. 1969). The catabolism of homoserine results in the production of α-ketobutyrate and ammonia. The degradation of

cystathionine generates α-ketobutyrate, ammonia and cysteine. The products of cysteine desulphuration are pyruvate, ammonia and hydrogen sulphide. These findings indicate the substrate diversity of the mammalian enzyme.

Apart from mammalian cells, γ-cystathionase has been detected in Aspergillus nidulans (Paszewski and Prazmo 1977), Tetrahymena pyriformis (Dang and Cook 1977) and T. thermophila (Murphy and Fall 1985).

The rat liver γ-cystathionase was found to have an absorption maximum at 420 nm (Brown et al. 1969). When the enzyme was combined with α-aminobutyrolactone, homocysteine thiolactone, cycloserine or α-aminobutyro-γ-selenolactone, the absorption maxima appeared in the range 495 to 530 nm. It is thought that these compounds are competitive inhibitors of the enzyme by forming complexes with the cofactor pyridoxal phosphate.

In mammals, γ-cystathionase together with cystathionine-β-synthetase (see Fig. 8, reaction (5)) are involved in the control of cellular methionine concentrations (see Cooper 1983). Excess methionine, which is toxic to most mammalian cells, is removed rapidly via homocysteine by these enzymes. However, when there is a need to conserve methionine the activities of the two enzymes are depressed to allow the methylation of homocysteine to methionine to occur.

DL-Propargylglycine, an irreversible inhibitor of pyridoxal phosphate-dependent enzymes, inhibits mammalian γ-cystathionase both in vivo and in vitro (Abeles and Walsh 1973; Kodama et al. 1982). Other inhibitors of γ-cystathionase include DL-penicillamine and hydroxylamine (Braunstein et al. 1971).

(iv) Serine sulphydrase (EC 4.2.1.22)

Serine sulphydrase (also called serine sulphydrylase, cysteine synthetase or serine hydrolase-adding hydrogen sulphide) catalyses the reversible interconversion of serine and cysteine. The enzyme has been detected in bacteria (Bruggemann et al. 1962), fungi (Bruggemann et al. 1962; Pieniazek et al. 1973), chicken liver (Braunstein et al. 1969, 1971), plants (Bruggemann et al. 1962), Tetrahymena thermophila (Murphy and Fall 1985) and mammals (Bruggermann et al. 1962).

The most detailed account on the characteristics of serine sulphydrase was given by Braunstein et al. (1969, 1971). They purified the enzyme from chicken liver and found that it possessed the following properties. It is a pyridoxal phosphate-dependent enzyme, soluble with a pH optimum of 8.4, and has a molecular weight of approximately 125,000 daltons (as compared to 175,000 daltons for Aspergillus nidulans serine sulphydrase, Pieniazek et al. 1973). The chicken liver serine sulphydrase has a pI of 6.0 as demonstrated by isoelectric focusing. The enzyme-catalysed production of hydrogen sulphide from cysteine was shown to increase greatly with the addition of 2-metcaptoethanol or other thiols (e.g. cysteamine and homocysteine) to the reaction mixture. This is indicative of a β-exchange or replacement reaction occurring between the substrate and added thiol to yield the corresponding L-cysteine thioether. The enzyme also catalyses the synthesis of cystathionine from serine and homocysteine but at a lower rate than its catabolism of cysteine to hydrogen sulphide. The enzyme, however, does not catalyse α,β-elimination reactions (e.g. γ-cystathionase catalysed reactions). The chicken liver enzyme was strongly inhibited by hydroxylamine and aminooxyacetate (both at 10^{-3}M gave 100% inhibition), a carbonyl reagent and a substrate analogue, respectively. Interestingly, it

was practically insensitive to DL-cycloserine and D- or DL-penicillamine (both up to 10^{-2}M). These two compounds are potent inhibitors of aminotransferases and α,β-eliminating enzymes (e.g. γ-cystathionase). The finding indicates that the purified chicken liver serine sulphydrase catalytic mechanisms probably does not involve ketimine intermediates. It is thought that the enzyme is probably identical to mammalian cystathionine-β-synthetase (see reaction (5), Fig. 8), one of the enzymes involved in the conversion of homocysteine to cysteine. Similarly, Asperigillus nidulans serine sulphydrase is apparently identical to the organism's cystathionine-β-synthetase (Pieniazek et al. 1973). Moreover, the serine sulphydrase activity (ranging from 2.6 to 54 nmol/min/mg protein) detected in Tetrahymena thermophila has been suggested to be an alternative activity of the protozoan's cystathionine-β-synthetase (Murphy and Fall 1985). It is probable, therefore, that the observed serine sulphydrase activity in mammals reported by Bruggemann et al. (1962) was a different expression of its cystathionine-β-synthetase. Nevertheless, although its presumed physiological function in some cells is to synthesise cystathionine (Braunstein et al. 1971) the exact role of serine sulphydrase in mammals, and other organisms is at present not known.

(v) Cystathionine-β-synthetase (EC 4.2.1.22)

Cystathionine-β-synthetase catalyses the synthesis of cystathionine from serine and homocysteine. The enzyme has been detected in mammalian cells (Braunstein et al. 1971; Cooper 1983), Aspergillus nidulans (Pieniazek et al. 1973; Paszewski and Prazmo 1977), Tetrahymena pyriformis (Dang and Cook 1977) and T. thermophila (Murphy and Fall 1985).

The purified enzyme from rat liver was found to require

pyridoxal phosphate as a cofactor and contained a major band at pI 5.5 on isoelectric focusing (Braunstein et al. 1971). The K_m values for L-serine and DL-homocysteine were 0.8 mM and 18 mM, respectively. In addition, the enzyme was found to be highly sensitive to hydroxylamine and aminooxyacetic acid (both at 10^{-3}M produced 100% inhibition) but practically insensitive to DL-cycloserine and DL-penicillamine (both up to 10^{-2}M). The rat liver cystathionine-β-synthetase, unlike γ-cystathionase, does not catalyse α, β-elimination reactions, and it is similar in several ways to chicken liver serine sulphydrase (Braunstein et al. 1971; see (iv) above).

In mammals, cystathionine-β-synthetase plays a key role in the control of plasma levels of homocysteine (Mudd et al. 1965). It was shown that cystathionine-β-synthetase deficiency is an inborn error of metabolism in some mentally retarded patients (see Mudd et al. 1965). These patients excrete homocysteine in their urine and have abnormally high concentrations of both homocysteine and methionine in the plasma. The increased cellular methionine concentrations presumably occurred as a result of the unchecked methylation of homocysteine to methionine by methionine synthetase (see (vi) below). This indicates the importance of cystathionine-β-synthetase in controlling cellular methionine as well as homocysteine.

(vi) Methyltetrahydrofolate (mTHF): homocysteine methyltransferase (EC 2.1.1.13) and betaine: homocysteine methyltransferase (EC 2.1.1.5)

Methionine is an essential amino acid in mammals and the recycling of the methionine pool can be achieved by methylation of homocysteine using either mTHF or betaine as the one-carbon donor (Billings et al. 1981; Skiba et al. 1982; Tautt et al. 1982). Due to the activity of mTHF: homocysteine methyltransferase (also called

methionine synthetase), methionine requirements of whole animals and cells cultured *in vitro* can be met by nutrients containing homocysteine, folic acid and vitamin B_{12} without added methionine. This enzyme, which contains a cobalamin (vitamin B_{12}) prosthetic group tightly bound to the apoprotein, is found in almost all types of mammalian cells, bacteria, plants and fungi (see Taylor and Weissbach 1973). There are two isoenzymes of mTHF: homocysteine methyltransferase in *Escherichia coli*. The activity of one of the isoenzymes is greatly stimulated by inorganic phosphate and Mg^{2+}, whereas the other isoenzyme depends on SAM and a reducing system (created *in vitro* by the use of reduced FMN and 1,4-dithiothrietol or 2-mercaptoethanol alone; however the use of the former system was found to be consistently better than the latter) for activity (Taylor and Weissbach 1973). SAM was used in the assay conditions to prevent propylation of the prosthetic group which is essential for catalytic activity. It has been demonstrated that methionine, through its conversion to SAM by SAM synthetase, regulates folate-dependent reactions in isolated rat hepatocytes by increasing the activity of mTHF: homocysteine methyltransferase which subsequently leads to an increased concentration of tetrahydrofolate (Billings *et al*. 1981).

Betaine: homocysteine methyltransferase is found primarily in mammalian liver cells (Skiba *et al*. 1982; Awad Jr. *et al* 1983), although its presence in fungi and bacteria is known (Balinska and Paszewski 1979). The purified human betaine: homocysteine methyltransferase was found to have an apparent molecular weight of 270,000 daltons, no requirements for cofactors and, in particular, neither folate, cobalamin nor pyridoxine was associated with the enzyme (Skiba *et al*. 1982). These findings suggest that this enzyme is different from mTHF: homocysteine methyltransferase. Studies on

the fungus Aspergillus nidulans, however, have revealed a common enzyme protein for the mTHF: homocysteine methyltransferase and betaine: homocysteine methyltransferase activities (Balinska and Paszewski 1979).

Isovalerate and 3,3-dimethylbutyrate are good inhibitors of the human betaine: homocysteine methyltransferase (Awad Jr. et al. 1983), whereas mTHF: homocysteine methyltransferase is inhibited by propyl iodide (Taylor and Weissbach 1973).

(vii) SAM synthetase (EC 2.5.1.6)

SAM is probably present in all cell types and in most cases is synthesised from L-methionine and ATP by SAM synthetase (also called methionine adenosyltransferase). SAM synthetase has been found in a variety of organisms including mammals, bacteria, plants, fungi and the protozoan Tetrahymena (Dang and Cook 1977; Tabor and Tabor 1984; Shiozaki et al. 1984; Giovanelli et al. 1985; Murphy and Fall 1985). Interestingly, certain mammalian cells and yeasts can actively take up exogenous SAM (Zappia et al. 1978).

SAM synthetase has fascinated a number of investigators over the years. The reaction is most unusual in that there is a complete dephosphorylation of the ATP, and the energy of ATP is used to form the energy-rich sulphonium compound (SAM). Moreover, the ATP is split in a non random fashion: that is, pyrophosphate originates from the α-and β-phosphates of ATP, and orthophosphate from the γ-phosphate. Neither free tripolyphosphate nor ADP is an intermediate in this reaction (Cantoni and Durell 1957). The mechanism of this reaction became even more interesting when evidence was presented for an enzyme-bound tripolyphosphate intermediate and is now widely recognised that the mechanism of action of SAM synthetase is as shown below.

$$\text{Enzyme} + \text{Methionine} + \text{Adenosine} - \underset{\alpha\beta\gamma}{\text{PPP}} \longleftrightarrow \underset{\alpha\beta\gamma}{\text{Enzyme} - \text{PPP}} + \text{SAM}$$

$$\underset{\alpha\beta\gamma}{\text{Enzyme} - \text{PPP}} \longrightarrow \underset{\alpha\beta}{\text{Enzyme} + \text{PPi}} + \underset{\gamma}{\text{Pi}}$$

SAM synthetase in mammals have been shown to exist in three different forms. These isoenzymes have been referred to as α, β, and γ (see Tabor and Tabor 1984). Immunological studies of the three forms indicated that α-and β-forms are immunologically very similar, whereas the γ-form differed from the two. It is, however, not certain at present whether the α-and β-forms represent completely different proteins, or if one form is derived from the other. Nonetheless, these forms show distinctness in location and biochemistry (see Tabor and Tabor 1984).

The α-form is found in liver, but represents only about 5% of the total SAM synthetase activity of the liver, and has a molecular weight of 210,000 daltons. This enzyme requires dithiothreitol (DTT) and is completely inhibited by p-chloromercuribenzoate. In contrast to the β-form, it is only slightly activated by either dimethylsulphoxide or glycerol, and is inhibited by tripolyphosphate and cycloleucine.

The β-form is the predominant form in liver but is not found in non-hepatic tissues. It has a molecular weight of 100,000 daltons and also requires DTT and is inhibited by p-chloromercuribenzoate. The enzyme, however, is strongly activated (about 17-fold) by dimethylsulphoxide or glycerol.

The γ-form is the only one found in non-hepatic tissues (e.g. kidney, heart, brain and lung). The amount of γ-form activity is highest in kidney and pancreas, and is comparable to the α-form

levels in liver. It has a molecular weight of about 190,000 daltons; does not require DTT and is only slightly inhibited by p-chloromercuribenzoate. In contrast to the stimulation observed with the α- and β-forms, the γ-form is slightly inhibited by dimethylsulphoxide and glycerol. Tripolyphosphate and cycloleucine also inhibit the enzyme activity.

SAM synthetase activity in Escherichia coli is due to a single protein whereas two isoenzymes of SAM synthetase have been detected in yeasts (see Tabor and Tabor 1984). The significance of the two forms in yeasts is at present not known and awaits results of further investigations before a full assessment can be made. Hence, mammalian, bacteria and yeasts SAM synthetases are apparently quite different from each other.

In regard to substrate specificity, liver SAM synthetase is relatively specific for L-methionine. Ethionine is a weak substrate, whereas homocysteine is not utilised at all. Selenomethionine, apparently, is a very good substrate.

It has been demonstrated that SAM synthetase levels increase if cell cultures are grown in methionine-deficient media, and decrease upon addition of methionine to the culture media (see Tabor and Tabor 1984).

The importance of SAM synthetase in generating SAM, the biologically important methyl donor and precursor of polyamine synthesis, is well documented (Usdin et al. 1979; Cooper 1983; Tabor and Tabor 1984). Studies on the fate of SAM in rat liver extracts have revealed that more than 99% of SAM was demethylated and exclusively catabolised further by SAH hydrolase (i.e. involved in transmethylation and transsulphuration reactions), whereas less than 1% was decarboxylated and immediately utilised totally for polyamine biosynthesis (Eloranta and Kajander 1984). Moreover, it has been

suggested that hypermethioninemia in infants may be related to deficiency of SAM synthetase activity in the liver (see Tabor and Tabor 1984).

Cycloleucine, L-2-amino-4-hexynoic acid and L-2-amino-4-methoxy-but-3-enoic acid are among the numerous analogues of L-methionine developed to exploit the importance of SAM synthetase in biological methylations. These compounds were found to be effective inhibitors of the enzyme (Coulter et al. 1974a, 1974b, 1974c). Interestingly, selenite toxicity in mice has been suggested to involve inactivation of SAM synthetase and subsequent depletion of cellular SAM (Hoffman 1977).

(viii) SAM-dependent transmethylases (EC 2.1.1.)

SAM-dependent transmethylases (or methyltransferases) are a group of enzymes that catalyse the transfer of the methyl moiety of SAM to small molecules (such as dopamine, noradrenaline, histamine, melatonin, serotonin, tryptamine) and macromolecules (such as proteins, nucleic acids, lipids) thereby altering the activities of these molecules (see Usdin et al. 1979; Paik and Kim 1980). SAM-dependent transmethylation is also involved in xenobiotic metabolism (Loo and Smith 1985).

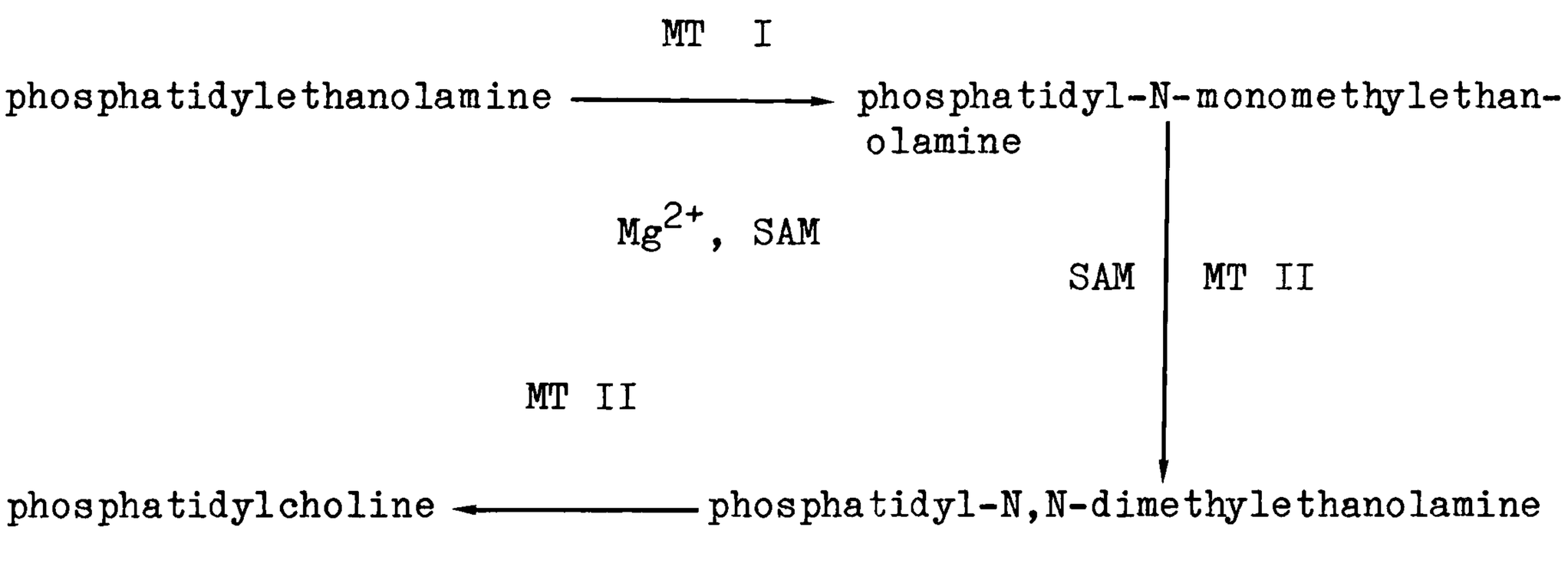
In mammals, methylations of low molecular weight amines or catecholamines usually occur at the N- or O- position of the substrate. The enzyme catechol O-methyltransferase (COMT, EC 2.1.1.6) catalyses the transfer of the methyl group from SAM to one of the phenolic hydroxyl moieties of catechol or substituted catechols. This reaction plays an important role as one of the primary pathways for the enzymatic inactivation of catecholamines, steroidal catechols and xenobiotic catechols (see Creveling et al. 1979). The enzyme phenylethanolamine N-methyltransferase (PNMT, EC

2.1.1.28) catalyses the N-methylation of noradrenaline to produce adrenaline, the physiologically important hormone in mammals (Pendleton 1979). Histamine, which plays an important part in allergy, is metabolised primarily by methylation of the imidazole ring in the central nervous system of mammals. This reaction is catalysed by the enzyme histamine N-methyltransferase (HMT, EC 2.1.1.8) (Duch et al. 1979).

There are three protein-specific methyltransferases whose properties have been well characterised: protein methylase I (SAM: protein-arginine N-methyltransferase, EC 2.1.1.23) methylates the guanido groups of arginine residues; protein methylase II (SAM: protein-carboxyl O-methyltransferase, EC 2.1.1.24) methyl esterifies the carboxyl groups of glutamyl and aspartyl residues; protein methylase III (SAM: protein-lysine N-methyltransferase, EC 2.1.1.43) methylates the ϵ-NH_2 groups of lysine residues (Paik and Kim 1980). These enzymes from mammalian sources are cytosolic and they display substrate specificity: protein methylase I is very specific for histone; protein methylase II has a broad substrate specificity with gelatin and oxidised pancreatic ribonuclease as the two best substrates found. The enzyme also methylates γ-globulin and histone at relatively high rates; protein methylase III is specific for histone including arginine-rich histone (Paik and Kim 1980). Similar enzymes have also been detected in fungi (Paik and Kim 1980) and protein methylases I and III were detected in the flagellate protozoan Leishmania (Paolantonacci et al. 1985). In general, protein methylation is intimately related to many cellular functions including exocytotic secretion, sperm motility, bacterial chemotaxis, the assembly of flagellin in bacterial flagella, the visual function of rhodopsin in the retina and carnitine biosynthesis (Bouchard et

al. 1980; Paik and Kim 1980).

The methylation of lipids, particularly membrane phospholipids has been studied in some detail (Hirata et al. 1979; Garcia-Gil et al. 1982; Hashizume et al. 1983a, 1983b). It is now widely recognised that two enzymes, namely methyltransferase I (MT I, EC 2.1.1.17) and methyltransferase II (MT II, EC 2.1.1.17), are involved in the synthesis of phosphatidylcholine from phosphatidylethanolamine. The reactions that are involved, based upon work with mammalian erythrocytes (Hirata et al. 1979) are illustrated below:



The two enzymes are located in the particulate fractions of mammalian cells (Hirata et al. 1979; Hashizume et al. 1983a, 1983b). Methyltransferase II has also been detected in Dirofilaria immitis and in this case it was recovered mainly in the microsomal fraction (Srivastava and Jaffe 1985). The two enzymes were found to be asymmetrically distributed in the mammalian red blood cell membrane, with MT I localised on the cytoplasmic side of the membrane while MT II is on the outer surface (Hirata et al. 1979). This indicated that the product of MT I was embedded in the membrane and that there was a transmembrane movement of phospholipids from the cytoplasmic to the external membrane surface as they are successively methylated. Studies with rat liver plasma membrane methyltransferases

demonstrated that MT I is inhibited by GTP whereas the activity of MT II was stimulated by this compound (Hashizume et al. 1983a), suggesting a regulatory role of GTP in phospholipid methylation. Phospholipid methylation is important in the maintenance of membrane fluidity, lipid translocation, coupling of the β-adrenergic receptor to adenylate cyclase and phagocytosis (Hirata et al. 1979; Garcia-Gil et al. 1982; Hashizume et al. 1983a). Interestingly, the principal fungal and plant sterols differ from the major animal sterol, cholesterol, in two important ways. They are unsaturated in the side chain between carbons -22 and -23, and they are methylated at carbon -24. The latter reaction is catalysed by Δ^{24}-sterol methyltransferase (SMT, EC 2.1.1.41) which plays a vital role in cell growth (Parks et al. 1979). This enzyme is apparently absent in mammals. The enzymatic methylation of N-oleoylmercaptoethylamide, a non-polar lipid, using SAM as the methyl donor has also been reported (Zats et al. 1982).

The enzymes that catalyse nucleic acid methylation reactions are divided into two major groups: (i) RNA methyltransferases of which there are three types i.e. mRNA-, rRNA- and tRNA- methyltransferases; (ii) DNA methyltransferases. An in-depth account of these enzymes has been described (see Usdin et al 1979; Hoffman 1985). In brief, nucleic acids are normally methylated at specific base or nucleotides residues. Methylation at the following positions are known to occur: C-3 methylcytidine; C-5 methylcytidine; C-5 methylcytosine; N-6 methyladenine; C-1 methyladenine; C-1 methyladenosine; C-2 methyladenosine; N-6 methyladenosine; C-1 methylguanosine; N-2, N-2 dimethylguanosine; C-7 methylguanosine (Usdin et al. 1979; Hoffman 1985). The enzymes concerned have been detected in a variety of mammalian cells, bacteria and viruses (see Usdin et al. 1979).

Methylation at specific base sequences of nucleic acids can be important in protecting these sites from cleavage by restriction endonucleases. Alternatively, methylation can identify sites of targets for an endonuclease to mediate excision of certain sequences which are not required in replication (Christman 1979; Grantt and Schreider 1979). Methylation of nucleic acids plays a part in regulating gene expression during differentiation and cell growth.

A common feature of most methyltransferases is the inhibition produced by the demethylated product of transmethylation, SAH. This compound has been shown to inhibit bacterial DNA cytosine-5-methyltransferase (Crooks et al. 1984), yeast Δ^{24}-sterol methyltransferase (Parks et al. 1979), sulphydryl xenobiotic transmethylases in rat liver (Loo and Smith 1985), protein methylase III of Neurospora crassa (Paik and Kim 1980) and Leishmania protein methylases I and III (Paolantonacci et al. 1985), to name but a few. The activities of methyltransferases in vivo are dependent on the intracellular level of SAH and therefore the activity of the enzyme SAH hydrolase. This is not the only means of regulation of transmethylation reactions. A natural peptide inhibitor (called methinin) of transmethylations in the rabbit has been discovered recently (Lyon et al. 1982). Similarly, there is evidence for the existence of two natural protein inhibitors in the rat liver cytosol that regulate methylation of phospholipids (Hazhizume et al. 1983b). The importance of the regulatory system for SAM-dependent methyltransferases has attracted considerable attention as a target for drug design. This is particularly so in cancer chemotherapy, as the altered methionine metabolism and transmethylation reactions in cancerous cells are well understood (Hoffman 1985). Two general approaches have been used: (a) the synthesis of analogues of SAH that

function directly on a particular methyltransferase and (b) the synthesis of inhibitors of SAH hydrolase which cause an elevation of cellular levels of SAH and subsequently produce inhibition of methyltransferases. The design of synthetic analogues of the natural peptide inhibitors is another possibility; there are, however, no reports of this approach as yet.

The chemotherapeutic properties of sinefungin, a natural analogue of SAH and a potent inhibitor of transmethylation, was described earlier (see (i) SAH hydrolase). Other known inhibitors of SAM-dependent methyltransferases include 5-azacytidine, an analogue of cytosine (Groudine et al. 1981), S-N-6-methyladenosylhomocysteine, an analogue of SAH (Hoffman 1978) and, a series of base- and amino acid- modified analogues of S-aristeromycinyl-L-homocysteine, a carbocyclic nucleoside (Houston et al. 1985c).

(ix) L-Methionine-γ-lyase (EC 4.4.1.11) and transamination of methionine

Cellular methionine can be metabolised in several ways. One route involves the initial synthesis of SAM and subsequent transmethylation and transsulphuration of the compound, as described above. There are also two ways in which methionine can be catabolised.

(a) The simultaneous deamination-demethiolation of L-methionine by L-methionine-γ- lyase to produce, in a single step, α-ketobutyrate, ammonia and methanethiol. The enzyme is pyridoxal phosphate-dependent and has been detected in bacteria (Tanaka et al. 1977), rumen ciliate protozoa (Merricks and Salsbury 1976; Onodera and Migita 1985) and fungi (Pezet and Pont 1980). As far as it is known the enzyme is not present in mammals. Bacterial L-methionine-γ-lyase has been studied in some detail

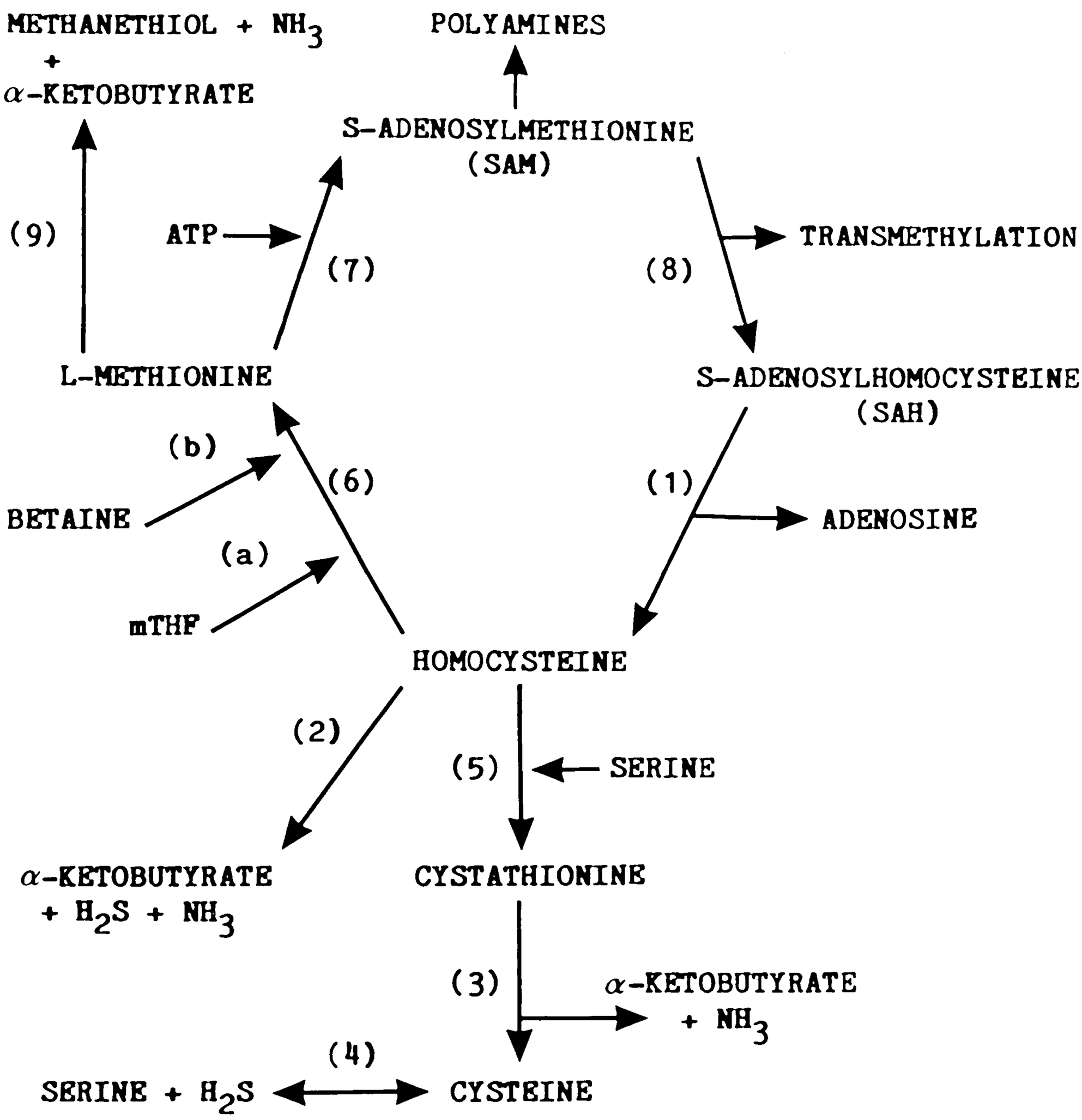
(Laakso and Nurmikko 1976; Tanaka et al. 1977; Ferchichi et al. 1985). The enzyme from Pseudomonas ovalis was found to be soluble with molecular weight of about 173,000 daltons and consisted of non-identical subunits (molecular weights 40,000 and 48,000 daltons) (Tanaka et al. 1977). The enzyme was found to have absorption maxima at 278 and 420 nm, and a shoulder around 330 nm, which were independent of the pH (from 6.0 to 10.0), and contain 4 mol of pyridoxal-5'-phosphate per mol of the enzyme. Typically, L-methionine-γ-lyase from bacteria catalyses α,γ-elimination (with substrates such as methionine, ethionine, homocysteine) and α,β-elimination (with substrates such as cysteine and S-methylcysteine) as well as γ-exchange (of the thiomethyl group of methionine with various alkanethiols or arylthioalcohols or derivatives of ethanethiol e.g. cysteamine and 2-mercaptoethanol, to yield the corresponding S-substituted homocysteine) and β-exchange (of the thiol group of cysteine with various alkanethiols to yield S-alkylcysteines) reactions (Ito et al. 1975; Tanaka et al 1977). α-Keto-γ-methiolbutyrate, an important intermediate in mammalian transamination of methionine (see below), however, is not a substrate for L-methionine-γ-lyase (Tanaka et al. 1977). Interestingly, the absorption spectrum of Pseudomonas L-methionine-γ-lyase (λmax 420 nm with a shoulder at about 330 nm) closely resembles those of some other pyridoxal phosphate-dependent enzymes such as L-leucine aminotransferase (λmax 326 and 414 nm), L-ornithine aminotransferase (λmax 330 and 420 nm), tyrosine phenol-lyase (λmax 340 and 430 nm) and kynureninase (λmax 337 and 430 nm) (see Tanaka et al. 1977), which may reflect their common requirement for pyridoxal phosphate. L-Methionine-γ-lyase is highly sensitive to thiol

reagents like N-ethylmaleimide, iodoacetate, p-chloromercuribenzoate and mercury chloride (concentrations from 0.1 - 1.0 mM produced 80-100% inhibition of activity). The enzyme is also inhibited 70-100% after a 10 min preincubation, at concentrations of 0.1 - 1.0 mM, with hydroxylamine, L-penicillamine or L-cycloserine, which are typical inhibitors of pyridoxal phosphate-dependent enzymes.

(b) The transaminative pathway, which occurs primarily in mammalian cells (see Fig. 9, Case and Benevenga 1976; Steele and Benevenga 1978). Transamination of methionine to its keto analogue, α-keto-γ-methiolbutyrate, and further oxidation to carbon dioxide has been shown to account for a substantial amount of methionine methyl carbon oxidation in rat liver homogenate (see Steele and Benevenga 1978). The transaminase(s) responsible for the formation of α-keto-γ-methiolbutyrate *in vivo* remain unknown; glutamine transaminases are probably not responsible even though methionine has been demonstrated to be a substrate of purified preparations of rat liver and kidney glutamine transaminases (see Cooper 1983). It is known, however, that α-keto-γ-methiolbutyrate is a better percursor for methanethiol production than is methionine (see Cooper 1983), in contrast to bacterial L-methionine-γ-lyase (see above). It seems that the intermediate(s) generated by the transaminative pathway may be responsible for the observed growth and tissue defects of animals fed with high levels of methionine (Steele and Benevenga 1978; see Finkelstein and Benevenga 1986).

Methanethiol is a product of both pathways (a and b above) and it is a pungent, volatile gas that has been found to be

toxic to mammals (Shults et al. 1970; Zieve et al. 1974) and pathogenic fungi (Walker et al. 1937) and to inhibit mammalian mitochondrial respiration (Waller 1977) and catalase activity (Finkelstein and Benevenga 1986); nonetheless, it is reported to be essential for cell division in certain cell lines (Toohey 1977). It is known that higher plants produce volatile thiols to repel potential predators and parasites (see Kadota and Ishida 1972). Thus, there is evidence that in some organisms at least, methionine catabolism may play an important role in the ecology of the organism. The relative importance of the transaminative pathway versus the transulphuration pathway (i.e. the use of methionine to produce SAM which is subsequently metabolised as illustrated in Fig. 8) of L-methionine breakdown awaits results of further study, although there are indications that the latter route greatly predominates.



ENZYMES: (1) SAH HYDROLASE
(2) HOMOCYSTEINE DESULPHURASE
(3) γ-CYSTATHIONASE
(4) L-SERINE SULPHYDRASE (or L-CYSTEINE SYNTHETASE)
(5) CYSTATHIONINE-β-SYNTHETASE
(6) (a) mTHF: HOMOCYSTEINE METHYLTRANSFERASE
(6) (b) BETAINE: HOMOCYSTEINE METHYLTRANSFERASE
(7) SAM SYNTHETASE
(8) TRANSMETHYLASES
(9) L-METHIONINE-γ-LYASE

Fig. 8 Metabolism of sulphur-containing amino acids.

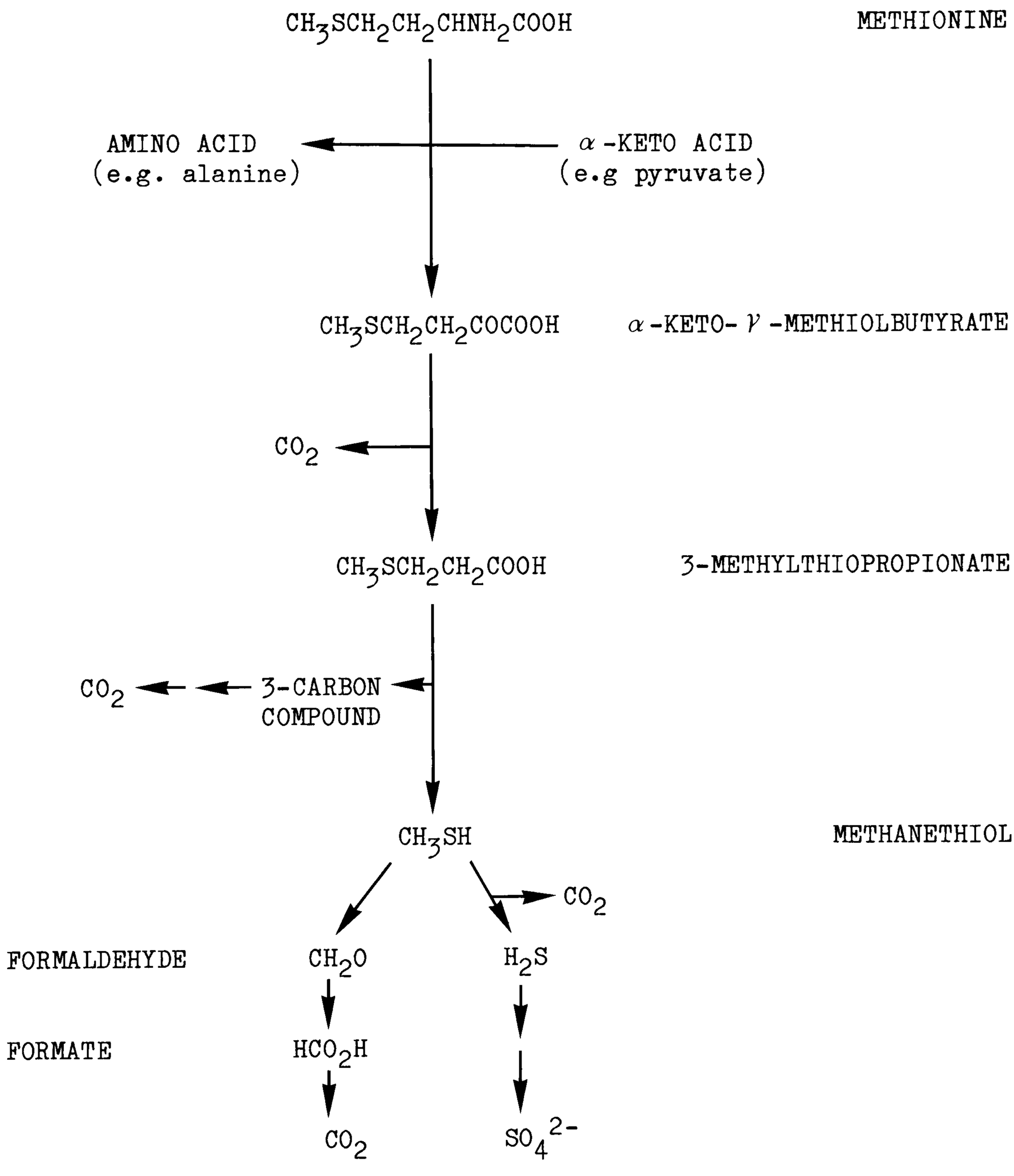


Fig. 9 Transaminative pathway of methionine catabolism (modified from Steele and Benevenga 1978; Cooper 1983; Finkelstein and Benevenga 1986).

1.6. Aims of the project

The primary aim of the project was to investigate the enzymes and pathways of sulphur-containing amino acid metabolism in trichomonads and to elucidate the parts they play in the growth of the parasites and the host-parasite interaction. There were several reasons for studying this area of metabolism in trichomonads, as detailed in 1.5.5.. The information available at the time indicated that this is an area of metabolism of special importance to trichomonads. Four species of trichomonads have been used: Trichomonas vaginalis, Pentatrichomonas hominis, Tritrichomonas foetus and Trichomitus batrachorum. These differ from each other in many features including pathogenicity and locations in their respective hosts, making them ideal for comparative biochemical studies which should help pin-point the functional significance of sulphur-containing amino acid metabolism as well as providing an insight into the special adaptations of each organism. In addition, it was hoped that the study would provide information on potential sites for chemotherapeutic attack on human and bovine trichomoniasis.

The project was approached in two ways: (i) biochemical studies which involved detection and characterisation of the pertinent enzymes; (ii) chemotherapeutic studies which involved screening inhibitors of mammalian sulphur-containing amino acid metabolising enzymes for antitrichomonal activity using the human parasite, Trichomonas vaginalis, as a model. It was anticipated that the results obtained from the two approaches would complement each other.

A second aim of this project was to investigate and compare the ferredoxin-linked and oxygen-metabolising enzymes in T. vaginalis, Trichomitus batrachorum and Tritrichomonas foetus. These enzymes are central to the functioning of the hydrogenosome, an organelle of special interest. A lot has been published concerning these enzymes

in T. foetus and Trichomonas vaginalis, but very little is known in Trichomitus batrachorum. It was anticipated that this study would produce information about these enzymes in the latter organism and so provide further insight into the roles they play in the adaptation of trichomonads to their respective hosts.

2.0. MATERIALS AND METHODS

2.1. Parasites, cultivation, harvesting and fractionation

2.1.1. Parasite lines

Eleven lines of Trichomonas vaginalis were used. Clone G3 has been cultivated in vitro for several years in our laboratory (Coombs 1976); this metronidazole-sensitive line was used for all experiments except where indicated otherwise. Lines IR78 and Fall River are metronidazole-resistant (Thurner and Meingassner 1978; Muller et al. 1980) and were originally obtained from Dr J.G. Meingassner (Sandoz Forschungsinstitut, Vienna, Austria). Line Boston, although reported to be metronidazole-resistant when isolated (Muller et al. 1980) was found to be sensitive to the drug when tested in our laboratory, was also obtained from Dr J.G. Meingassner. Lines 45, 55, 61 and 64 are recent metronidazole-sensitive isolates that have been maintained as stabilates since isolation from patients attending the Genito-Urinary Clinic, Royal Infirmary, Glasgow and were obtained from Dr I.B. Tait. Line 69506, a metronidazole-sensitive line, had been maintained intravaginally in mice, being passaged approximately every 2 weeks over a 12 month period (Bremner et al. 1986b) before use in this study, was originally obtained from Dr V.C. Latter (Wellcome Research Laboratories, Beckenham, Kent, England). Lines 2755 and 45733 are recent metronidazole-resistant isolates that had been maintained as stabilates since isolation and were obtained from Dr S. Al-Egaily (Huddersfield Royal Infirmary, Huddersfield, England) and Dr B.L. Radford (Public Health Laboratory, Exeter, England), respectively.

Trichomitus batrachorum (clone B2) was derived from an isolate obtained from the intestine of the leopard frog Rana pipiens (Coombs 1976). Tritrichomonas foetus (clone F2) was cloned in our laboratory from the Pfizer strain originally obtained from Dr D.J. Linstead

(Wellcome Research Laboratories, Beckenham, Kent, England). Pentatrichomonas hominis (ATCC 30098) was obtained from Dr A. Yule of the London School of Hygiene and Tropical Medicine (University of London, London, England). Entamoeba histolytica (strain 200:NIH) was obtained from Mr W.D. Strachan (Department of Zoology, University of Glasgow, Glasgow, Scotland). Leishmania mexicana mexicana (M 379), L. tarentolae (LV 414), Crithidia fasciculata (ATCC 11745), Herpetomonas muscarum muscarum (ATCC 30260), H. m. ingenoplastis (ATCC 30269) and Tetrahymena pyriformis (CCAP 1630/1f) were obtained from Dr G.H. Coombs (see Coombs 1982; North et al. 1983; Mallinson and Coombs 1986). Escherichia coli (ATCC 15224/ML 308) was obtained from Dr I.D. Hamilton (Department of Biochemistry, University of Glasgow, Glasgow, Scotland).

2.1.2. Cryopreservation of trichomonads

Trichomonads were cryopreserved using a procedure which involved adding sterile dimethylsulphoxide to 5% (v/v) to late-log phase cultures of trichomonads growing in MDM. Samples were transferred to plastic tubes (1 ml) and sealed with screw tops. These were wrapped in cotton wool, put into a card box and exposed to liquid nitrogen vapour in a sealed container over a period of 24 hr, and stored subsequently in liquid nitrogen. Trichomonads were removed from cryopreservation by thawing the samples in a 37°C water bath, and the samples (1 ml) were inoculated into fresh MDM (25 ml).

2.1.3. Cultivation of trichomonads in vitro

All trichomonads were routinely cultured axenically in modified Diamond's medium (MDM) [Table 4] supplemented with 10% (v/v) heat-inactivated horse serum, 1,000 units/ml benzylpenicillin and 1 mg/ml streptomycin sulphate. These cultures were checked routinely for contamination by microscopic observation and plating out on nutrient

agar. Contaminated cultures were discarded. Trichomitus batrachorum was grown at 25°C and the other trichomonads at 37°C. Cultures of T. batrachorum, Trichomonas vaginalis and Tritrichomonas foetus were initiated at a starting density of about 10^4 organisms/ml and were subpassaged every 4, 2 and 3 days, respectively. Cultures of Pentatrichomonas hominis (with starting density of about 10^5 organisms/ml) were subpassaged every 3 days. It was observed that the latter trichomonad grew better with foetal calf serum (instead of horse serum) and a supplement of galactose (10 mg/ml) in MDM.

Table 4 MDM (Diamond 1957)

Ingredients	g
Trypticase	40
Yeast extract	20
Maltose	10
Ascorbic acid	2
KCl	2
$KHCO_3$	2
KH_2PO_4	2
K_2HPO_4	1
Ferrous sulphate	0.2

The ingredients were dissolved in 1.8 l distilled deionised water, pH adjusted to 6.35 and dispensed into glass bottles. These bottles were sterilised for 15 min at 15 lb/in^2 in an autoclave. The sterilised medium without any furher addition is stable at room temperature for several months. Sterile heat-inactivated horse serum (10%, v/v), benzylpenicillin (1,000 units/ml) and streptomycin sulphate (1 mg/ml) were added to the sterile medium under aseptic conditions prior to use.

2.1.4. Cultivation of other protozoa and Escherichia coli in vitro

Tetrahymena pyriformis was grown in NPLD (2% (w/v) neopeptone, 0.1% (w/v) neutralised liver digest) at 25°C. Leishmania mexicana mexicana, L. tarentolae, Crithidia fasciculata, Entamoeba histolytica, Herpetomonas muscarum muscarum and H. m. ingenoplastis were cultivated using published methods and media (Diamond et al. 1978; Coombs 1982; North et al. 1983). Escherichia coli was grown in minimum medium (Hamilton and Holms 1970) at 37°C. Amastigotes of L. m. mexicana were obtained as described by Mottram and Coombs (1985).

2.1.5. Harvesting protozoa and other cells

Cells at late-log phase of growth were harvested by centrifugation at 2,300 x g for 10 min at 4°C, washed twice with 0.25 M sucrose and stored as pellets at -70°C until required or used immediately.

2.1.6. Production of cell-free homogenates, soluble and particulate fractions

Cell-free homogenates of L. m. mexicana, L. tarentolae, C. fasciculata, H. m. muscarum, H. m. ingenoplastis, T. pyriformis and Entamoeba histolytica in 0.25 M sucrose were produced using 0.1% (v/v) Triton X-100. Escherichia coli lysates in 0.25 M sucrose were produced by six cycles of freezing in liquid nitrogen and thawing at 37°C. Trichomonad lysates in 0.25 M sucrose were usually produced by 2 cycles of freezing to -196°C and thawing in a water bath at 37°C. Livers of CBA mice (Department of Zoology, University of Glasgow) were excised, washed and resuspended in ice cold 0.25 M sucrose (1g wet weight liver/5 ml solution) and homogenised well using a Potter homogeniser. Homogenates were used in all experiments

unless otherwise indicated.

To investigate the subcellular location of enzymes in trichomonads, freshly harvested parasites in 0.25 M sucrose were disrupted by 30-40 strokes with a Potter tissue homogeniser fitted with a serrated Teflon plunger type A (A.H. Thomas Co., Philadelphia, U.S.A.), operating at 2,500 rpm. This caused lysis of more than 98% of the parasites as judged by microscopic observation. The parasite lysate was centrifuged at 100,000 x g for 1 hr at 4°C in order to obtain soluble and particulate fractions, the latter being resuspended in 0.25 M sucrose to the same volume as the supernatant.

2.1.7. Materials

Yeast extracts and neopeptone were obtained from Difco Laboratories, Surrey, England. Neutralised liver digest and nutrient agar were purchased from Oxoid Ltd., Basingstoke, Hampshire, England. Trypticase was obtained from BBL, Wembley, Middlesex, England. All other chemicals were of analar grade and obtained from either Sigma Chemical Co. Ltd., Poole, Dorset, England or BDH, Glasgow, Scotland.

2.2. Methods of assessing activities of inhibitors

2.2.1. Determination of antitrichomonal activity: LD_{50}; MLC

The effects of inhibitors upon the growth axenically *in vitro* of trichomonads were assessed using tripling (or otherwise as indicated) dilutions of the compounds. Cultures (5 ml) were initiated with 10^5 organisms/ml, incubation was for 24 hr at 37°C for *Tritrichomonas foetus* and *Trichomonas vaginalis* or 25°C for *Trichomitus batrachorum*, with air as the gas phase, and the number of parasites present after 24 hr was determined using an improved Neubauer haemocytometer. All inhibitors were tested in this way unless otherwise indicated. Experiments to assess the effect of oxygen upon a drug's activity

were carried out using microtitre plates with cultures of 200 µl and incubations under aerobic (air) and anaerobic ($N_2/CO_2/H_2$, 80/10/10) conditions. Controls to monitor for the effects of solvents were used in all cases. The activities of the inhibitors are presented as the LD_{50}, the concentration that reduced the number of parasites by 50%, and the MLC, the minimum concentration tested that killed all parasites by 24 hr.

2.2.2. Determination of enzyme inhibition: I_{50}

The I_{50}, the concentration that reduced the enzyme rate by 50% was determined by graphical extrapolation. Inhibitors were preincubated with enzyme or added just before the start of the enzyme assays as indicated for the individual cases. Controls containing the appropriate solvents were used in all cases.

2.2.3. Preparation of inhibitors

The following compounds were dissolved in distilled deionised water or buffer: M & B 13671 (DL-methionine sulphoxide); M & B 22487 (L-2-amino-4(2'-nitrobenzamido)-butyric acid); M & B 23837 (2-amino-4-bromobutyric acid hydrobromide); M & B 23885 (DL-ethionine); M & B 25016 (2-amino-4-butoxybutyric acid); L-cycloleucine; seleno-L-methionine; sinefungin; methylglyoxal bis(guanylhydrazone); 5-azacytidine; S-adenosyl-L-homocysteine (SAH); adenine-9-β-D-arabinofuranoside (ara A); tubercidin; 2'-deoxyadenosine; DL-propargylglycine; L-cycloserine; hydroxylamine; DL-penicillamine; isovaleric acid; 3,3-dimethylbutyric acid; N,N-dimethylglycine; monothioglycerol; S-methylthioglycerol; norleucine; methionine sulphoximine; betaine; L-cysteine; S-methyl-L-cysteine; DL-homocysteine; L-cysteine sulphinic acid; L-methionine; metronidazole; choline chloride; 2,2'-dithiodiethanol. 4'-

Deoxypyridoxine, M & B 8416 (glutamic acid), M & B 13928 (DL-α-amino-γ-sulphamoylbutyric acid), M & B 23435 (L-2-amino-4-(2'-nitrobenzenesulphonamido) butyric acid), M & B X24038 (DL-homocystine), M & B 25049 (DL-2-amino-4-(4-chlorophenylmethane-sulphonyl) butyric acid), bithionol, dichlorophene, hexachlorophene and 2,4-dichlorophenol were dissolved in 0.1M NaOH. M & B 25062 (DL-2-amino-4-(4'-chlorophenylmethane sulphinyl)-butyric acid) was dissolved in 0.2 M NaOH. 3-Deazauracil was made in 0.03 M NaOH and chloramphenicol in 50% methanol. The solutions of inhibitors when necessary were sterilised by filtration using millipore filters (pore size 0.22 μm).

2.2.4. Materials

The M & B compounds were gifts from May & Baker Ltd., Dagenham, Essex, England. All other inhibitors were bought from Sigma Chemical Co., Ltd., Poole, Dorset, England, Aldrich Chemical Co., Ltd., Gillingham, Dorset, England or BDH, Glasgow, Scotland.

2.3. Studies on the consumption and incorporation of exogenous L-methionine by living trichomonads

Freshly harvested and washed cells of Trichomonas vaginalis Trichomitus batrachorum or Tritrichomonas foetus were resuspended in 0.25 M sucrose and used in the experiments. The incubation mixture in a final volume of 2.4 ml contained sodium phosphate buffer, pH 7.0 (100 mM), maltose (17 mM), KCl (13 mM), ascorbic acid (6 mM), trichomonads (2×10^7/ml) and one of the following radiolabelled compounds (with final concentration and specific activity used in the assays given in parentheses): [^{35}S]-L-methionine (424 μM, 9.3 μCi/μmole); [^{14}C-methyl-]-L-methionine (421 μM, 0.5 μCi/μmole); [^{14}C-methyl-]-L-methionine (4 μM, 55 μCi/μmole); [U-^{14}C]-L-leucine

(0.6 μM, 330 μCi/μmole); [^{3}H-methyl-]-thymidine (0.8 μM, 5 mCi/μmole). Incubation was at 37°C for 0-40 min. Inhibitors (cycloleucine or norleucine, 15 mM) were added just before the start of the reaction as appropriate. At the times indicated, the incubation mixture was centrifuged (2,300 x g for 10 min at 4°C) to separate the cells and spent medium. The cells were resuspended in 0.25 M sucrose, the suspension divided into two and centrifuged at 2,300 x g for 10 min at 4°C. The supernatants were discarded and the two pellets (A and B) were extracted using the following adaptations of the procedure of Gutteridge and Gaborak (1979) to produce lipid, nucleic acid and protein fractions. Pellet A was extracted three times with 0.1 ml 5% (w/v) trichloracetic acid (TCA) for 30 min at 0°C. The precipitates were harvested by centrifugation at 10,000 x g for 2 min at 18°C and washed thoroughly with ice-cold 5% (w/v) TCA. The pellet remaining was designated as the cold-TCA precipitate. Pellet B was also extracted three times with 0.1 ml 5% (w/v) TCA, and aliquots (10 μl) of the supernatant of the first TCA-extraction were used to assess for radioactivity incorporated into SAM and methionine using TLC techniques (see section 2.5.). The resultant pellet collected by centrifugation (10,000 x g for 2 min at 18°C) after three TCA-extractions was subsequently extracted two times with 0.2 ml chloroform at 37°C for 15 min. The chloroform-suspension was centrifuged at 10,000 x g for 2 min at 18°C. The supernatants were pooled to give the lipid fraction which was air-dried on filter paper, whereas the pellet was further extracted two times with 0.2 ml 5% (w/v) TCA at 90°C for 30 min. The supernatants were pooled to give the nucleic acid fraction and the resultant pellet (the protein fraction) was washed thoroughly with ice-cold 5% (w/v) TCA. Samples of the spent medium, cold TCA-precipitate, lipid, nucleic acid and protein fractions were dispensed into 5 ml of scintillant (Hydroluma,

obtained from May & Baker Ltd., Dagenham, Essex, England) and assessed for radioactivity using a liquid scintillation spectrometer (Isocap 300, Searle, England). The complete incubation mixture at zero time was used as the control in all cases. Counting efficiencies were determined using the external standard channels ratio method and usually were approximately 30% (for ^{3}H-compounds) and 70% (for ^{14}C-or ^{35}S-compounds).

2.3.1. Studies on the SAM concentrations of Trichomonas vaginalis resuspended in maltose phosphate-buffered saline solution (MPBSS) with and without exogenous L-methionine

Freshly harvested and washed cells of T. vaginalis were resuspended in 0.25 M sucrose and used in the experiments. The incubation mixture in a final volume of 1.5 ml contained sodium phosphate buffer, pH 7.0 (100 mM), maltose (17 mM), KCl (13 mM), ascorbic acid (6 mM), trichomonads (2 x 10^{7}/ml) and L-methionine (10 mM) as appropriate. Incubation was at 37°C for 0-45 min. At the times indicated, the cells were collected by centrifugation (2,300 x g for 10 min at 4°C) and analysed for SAM using HPLC techniques (see section 2.6.).

2.3.2. Materials

[^{14}C-methyl-]-L-methionine, [^{35}S]-L-methionine, [U-^{14}C]-L-leucine and [^{3}H-methyl-]-thymidine were obtained from Amersham International PLC, Buckinghamshire, England.

2.4. Determination of metabolites and protein

2.4.1. Volatile thiol

Thiol(SH)-containing compounds react readily with 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) to form aryl mercaptans which absorb

maximally at 412 nm. In the assays for volatile thiol production, DTNB was used as the trapping agent either separate from or in the incubation mixture as appropriate (see section 2.7.10.). The amount of volatile thiol produced was estimated from the standard curve constructed for L-cysteine. Gas samples collected from live trichomonads incubated in phosphate buffered saline (PBS) with or without L-methionine were also analysed qualitatively by mass spectrometry.

2.4.2. α-Ketoacid

α-Ketoacids undergo a chemical reaction with 3-methyl-2-benzothiozolone hydrazone hydrochloride (MBTH) to form azines which absorb maximally at about 324 nm (Soda 1968). Aliquots of the protein-free reaction mixtures were analysed for α-ketoacid using this method. The amount of α-ketoacid produced was determined from the standard curve constructed for α-ketobutyrate.

2.4.3. Ammonia

Ammonia reacts with alkaline hypochlorite and phenol in the presence of a catalyst sodium nitroprusside to form indophenol. The concentration of ammonia is directly proportional to the abosrbance of indophenol, which is measured spectrophotometrically at 570 nm (Horn and Squire 1967). Aliquots of the protein-free incubation mixtures were determined for ammonia by this method, using ammonium chloride as standard.

2.4.4. Protein

Protein was measured by the method of Lowry *et al.* (1951), using bovine serum albumin as standard.

2.4.5. Materials

The following were purchased from Sigma Chemical Co., Ltd., Poole, Dorset, England: α -ketobutyrate; 3-methyl-2-benzothiozolone hydrazone hydrochloride; L-cysteine hydrochloride; 5,5'-dithiobis(2-nitrobenzoic acid); phenol-nitroprusside solution; alkaline hypochlorite solution; ammonium chloride; folin and Ciocalteu's phenol reagent; bovine serum albumin (Fraction V). Cupric sulphate, sodium carbonate and potassium sodium (+)- tartrate were obtained from BDH, Glasgow, Scotland.

2.5. Identification and determination of SAM and L-methionine by thin layer chromatography (TLC)

Aliquots (10 μl) of the supernatant of the first TCA-extraction of pellet B (see section 2.3.) were applied to a TLC silica gel plastic sheet to which non-radioactive L-methionine (50 nmole) and SAM (10 nmole) had been added as carriers. The reaction components were separated by ascending chromatography in a mixture of butan-1-ol, methanol, distilled water and liquid ammonia 0.88 S.G. (60/20/20/1, v/v). Spots corresponding to L-methionine (Rf, 0.49) and SAM (Rf, 0.04) were identified using ninhydrin (0.5% (w/v) solution), cut out, dispensed into 5 ml scintillant (8.75 g PPO/125 mg POPOP/2.5 l toluene/75 ml Triton X-100) and assessed for radioactivity.

2.5.1. Materials

TLC silica gel plastic sheets (20 x 20 cm, layer thickness 0.25 mm) were obtained from Camlab, Cambridge, England. Ninhydrin was bought from BDH, Glasgow, Scotland. All other reagents were of analar grade and obtained from either Sigma Chemical Co., Ltd., Poole, Dorset, England or BDH, Glasgow, Scotland.

2.6. Identification and determination of SAM by high-performance liquid chromatography (HPLC)

Pellets of trichomonads and other protozoa, and mouse liver homogenates were extracted with 0.1 M perchloric acid (PCA) for 30 min at 0°C. These samples were centrifuged (10,000 x g for 2 min at 18°C) and the resultant supernatants were analysed for SAM using a modification of the procedure developed by Zappia et al. (1980). A Gilson pump (model 302)/manometric module (model 802) was used to generate a flow rate of 2.0 ml/min which produced a pressure drop of about 1,000 psi. A SGE microsyringe (0.25 µl) was employed to inject the sample into the Rheodyne (model 7125) sample injector valve. A Partisil SCX column (10 µm), 25 cm x 4.6 mm I.D. (obtained from HPLC Technology, Cheshire, England) was used at 18°C with filtered ammonium formate buffer, pH 4.0 (0.5 M) as the eluent. The identity of SAM was established by the elution time and, in the case of trichomonads, absorbance ratio (280 nm/254 nm) which differed from other adenosyl-compounds (see Fig. 10 and Table 5). An extraction efficiency of about 96% was obtained from the use of SAM as an internal standard. SAM concentrations were determined from the standard curve constructed using 0-3 nmole SAM.

2.6.1. Materials

Ammonium formate was bought from Aldrich Chemical Co. Ltd., Gillingham, Dorset, England. The following compounds were purchased from Sigma Chemical Co., Ltd., Poole, Dorset, England: S-adenosyl-L-homocysteine (SAH); S-adenosyl-L-methionine iodide (SAM); adenosine; adenine; methylthioadenosine (MTA); adenosine-5'-triphosphate disodium salt (ATP).

Table 5 HPLC separation of adenine and adenosyl-sulphur compounds[a].

Compound	Peak number in Fig. 10	Elution time (min)	Absorbance ratio (280nm/254nm)
ATP	1	1.6	0.30
SAH	2	2.5	0.24
ADENOSINE	2	2.5	0.23
ADENINE	3	3.6	0.38
SAM	4	5.0	0.24
MTA	5	6.1	0.25

a, operating conditions as described in section 2.6..

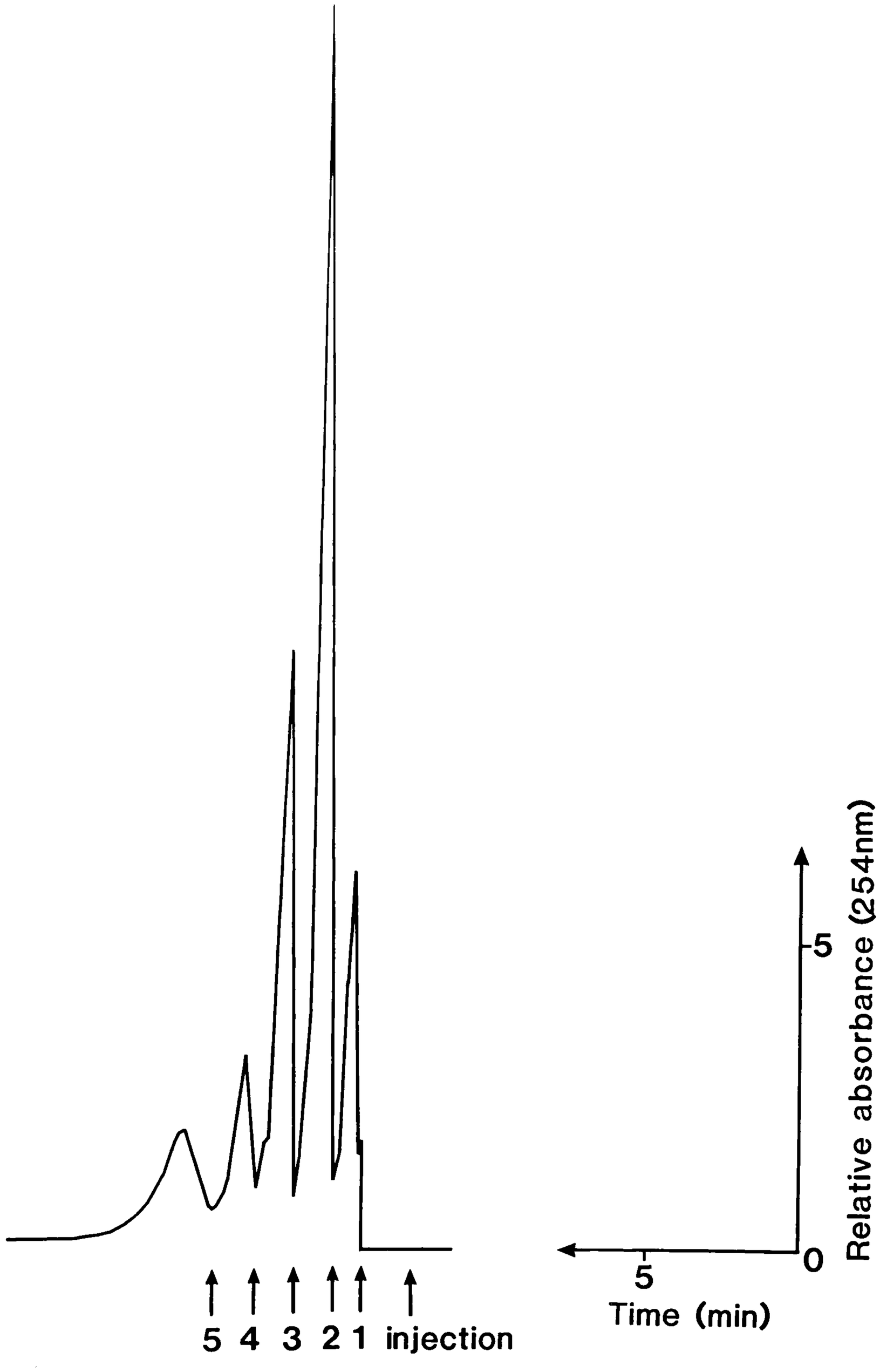


Fig. 10 HPLC separation of adenine and adenosyl-sulphur compounds. A typical trace of the compounds (all 5 nmole). Operating conditions as described in section 2.6.. Under these conditions SAH and adenosine coeluted (peak 2).

2.7. Enzyme assays

Unless otherwise indicated, all spectrophotometric enzyme assays were conducted under aerobic conditions at 37°C using either a Pye Unicam SP8000 or a Perkin-Elmer lambda 5 uv/vis spectrophotometer to monitor the changes in absorbance. For the anaerobic experiments, homogenates in sealed tubes were gently gassed with argon for 2-3 min to remove the oxygen present, and were subsequently used in the enzyme assays. Radiochemical assays were carried out as indicated in the individual cases.

2.7.1. NADH oxidase (EC 1.6.99.3) and NADPH oxidase (EC 1.6.99.1)

The oxidation of NADH or NADPH was followed by monitoring the decrease in absorbance at 340 nm. The standard reaction mixture consisted of sodium phosphate buffer, pH 6.55 (67 mM), NADH (0.1 mM) or NADPH (0.1 mM) and cell-free homogenate in a final volume of 1.5 ml. The reactions were started by addition of cell-free homogenate. The enzyme activities were calculated using the molar extinction coefficient of 6200 M^{-1} cm^{-1} for NADH and NADPH.

2.7.2. NADH: methyl viologen oxidoreductase (EC 1.6.99.3) and NADPH: methyl viologen oxidoreductase (EC 1.6.99.1)

The reduction of methyl viologen was followed by monitoring the increase in absorbance at 600 nm under anaerobic conditions. These were achieved by gassing the reaction mixture, in a 2 mm pathlength cell sealed with suba-seal, with argon for 2-3 min. Cell-free homogenate was injected into the sealed cell to start the reaction. The standard reaction mixture in a final volume of 0.53 ml contained sodium phosphate buffer, pH 6.55 (67 mM), NADH (9.4 mM) or NADPH (9.4 mM), 2-mercaptoethanol (250 mM), methyl viologen (20 mM) and cell-free homogenate. The molar extinction coefficient of methyl viologen was taken as 6300 M^{-1} cm^{-1}.

2.7.3. Pyruvate: methyl viologen oxidoreductase (EC 1.2.7.1)

The enzyme was assayed anaerobically using an assay mixture of final volume 0.53 ml containing: sodium phosphate buffer, pH 6.55 (67 mM); pyruvate (2.5 mM); coenzyme A (0.25 mM); methyl viologen (20 mM); 2-mercaptoethanol (250 mM) and cell-free homogenate. The enzyme activity was determined by taking the molar extinction coefficient of methyl viologen to be 6300 M^{-1} cm^{-1}. To study the influence of NAD^+, NADH, $NADP^+$, NADPH, oxidised glutathione (GSSG) and reduced glutathione (GSH) on the enzyme activity, anaerobic solutions of these compounds were added either approximately 1-1.5 min after or just before initiation of the reaction.

2.7.4. Hydrogenase (EC 1.18.99.1)

The incubation mixture consisted of sodium phosphate buffer, pH 6.55 (67 mM), methyl viologen (20 mM), cell-free homogenate and hydrogen (H_2) in a final volume of 0.53 ml. The H_2 was added by bubbling it through the anaerobic incubation mixture in a sealed cell for 2-3 min. The reaction was started by addition of cell-free homogenate. The increase in absorbance at 600 nm was monitored and the enzyme activity was calculated using the molar extinction coefficient of 6300 M^{-1} cm^{-1} for methyl viologen.

2.7.5. SAH hydrolase (EC 3.3.1.1)

SAH hydrolase activity was measured in the direction of SAH synthesis using a modification of the method of Kamantani et al. (1983). The standard reaction mixture consisted of sodium phosphate buffer (pH 8.0, 25 mM), DL-homocysteine (3 mM), DTT (2 mM), EDTA-Na_4 (1 mM), [2-^{3}H] adenosine (255 µM, specific activity 11.1 µCi/µmole) and enzyme (0.1-0.2 mg of extract protein) in a final volume of 200 µl. Deoxycoformycin was omitted from the assay mixture because this

compound has been found to be an inhibitor of mammalian SAH hydrolase (Cantoni et al. 1979; Hallam et al. 1984). The reaction was started by the addition of enzyme. Incubation was at 37°C, with air as the gas phase, for 1-25 (usually 5) min, whereupon 20 µl 8 M formic acid was added to stop the reaction. A 10 µl aliquot of the resultant mixture was then applied to a TLC PEI cellulose F plastic sheet to which 0.02 µmoles of non-radioactive adenosine and SAH had been added as carriers. The reaction components were separated by ascending chromatography in a mixture of butan-1-ol, methanol, distilled deionised water and liquid ammonia 0.88 S.G. (60/20/20/1, v/v). Spots corresponding to adenosine (Rf, 0.48) and SAH (Rf, 0.14) were identified under ultraviolet light, cut out, dispensed into 5 ml scintillant (PPO/POPOP/toluene/Triton X-100, 8.75 g/125 mg/2.5 l/75 ml) and counted using a liquid scintillation spectrometer. Counting efficiencies were determined using the external standard channels ratio method and usually were approximately 30%. Each experiment was carried out in duplicate and the enzyme activity is expressed as nmole SAH formed per min per mg protein.

The activity of T. vaginalis SAH hydrolase at a range of pH was determined using the following buffers: citric acid/sodium phosphate, 25 mM (pH 4.0-6.5); sodium phosphate, 25 mM (pH 6.0-8.0); Tris/HCl, 25 mM (pH 7.5-8.9).

The effects of divalent cations on the enzyme activity were studied using $CaSO_4$, $CoCl_2$, $MgCl_2$, $CuSO_4$ and $MnSO_4$. All were dissolved in distilled, deionised water and added to the assay mixture just before the reaction was initiated. The reaction was stopped after 5 min of incubation at 37°C. To study the effects of other potential inhibitors, homogenates of T. vaginalis and mouse liver were preincubated for 30 mins at 18°C with different concentrations of ara A, 2'-deoxyadenosine, tubercidin and sinefungin

or with buffer for the controls. After preincubation, the reaction was started by addition of both substrates and terminated after 5 min incubation at 37°C. All the inhibitors were dissolved in sodium phosphate buffer, 25 mM (pH 8.0).

2.7.6. SAM synthetase (EC 2.5.1.6)

SAM synthetase activity was measured by following the synthesis of radiolabelled SAM from [^{3}H-methyl-]-L-methionine and ATP. The assay system consisted of sodium phosphate buffer (pH 7.5, 100 mM), DTT (2 mM), SAM (1 mM), $MgCl_2$ (25 mM), KCl (25 mM), ATP (10 mM), [^{3}H-methyl-]-L-methionine (225 μM, specific activity 10 μCi/μmole) and extracts of trichomonads or mouse liver in a final volume of 200 μl. Incubation was at 37°C for 30 min. Controls without ATP or extract were used. The reaction was terminated by application of 20 μl of 1.1 M PCA and the protein precipitate was centrifuged at 10,000 x g for 2 min at 18°C. An aliquot (10 μl) of the supernatant remaining was then applied to a TLC silica gel plastic sheet to which non-radioactive L-methionine (50 nmole) and SAM (10 nmole) had been added as carriers. The reaction components were separated by ascending chromatography in a mixture of butan-1-ol, methanol, distilled water and liquid ammonia 0.88 S.G. (60/20/20/1, v/v). Spots corresponding to L-methionine (Rf, 0.49) and SAM (Rf, 0.04) were identified using ninhydrin (0.5% (w/v) solution), cut out, dispensed into 5 ml scintillant (8.75 g PPO/125 mg POPOP/2.5 l toluene/75 ml Triton X-100) and assessed for radioactivity. Counting efficiencies were determined using the external standard channels ratio method and usually were approximately 30%. Each experiment was carried out in duplicate.

2.7.7. Homocysteine desulphurase (EC 4.4.1.2)

Homocysteine desulphurase was assayed spectrophotometrically at 37°C using a modification of the method of Braunstein et al. (1971). The standard reaction mixture consisted of sodium phosphate buffer (pH 7.5, 100 mM), lead acetate (0.33 mM), DL-homocysteine (3.3 mM) and enzyme sample in a final volume of 1.5 ml. The production of lead sulphide was followed at 360 nm. The extinction coefficient of lead sulphide was estimated by the use of sodium sulphide to be 5205 M^{-1} cm^{-1}. The activity of T. vaginalis homocysteine desulphurase at a range of pH was determined using the following buffers (100 mM): citric acid/sodium phosphate (pH 4.0-6.5); sodium phosphate (pH 6.0-8.0); Tris/HCl (pH 7.5-8.9). The production of ammonia and α-ketoacid by trichomonad extracts were assayed as follows. The reaction mixture in a final volume of 1.5 ml contained DL-homocysteine (3.3 mM), cell-free extract and sodium phosphate buffer, pH 7.5 (100 mM). Controls without homocysteine or cell-free extract were used. Incubation was at 37°C for 15 min and the reaction was terminated by addition of 100 µl 10 M formic acid. The acid precipitate was removed by centrifugation and aliquots of the supernatant were used for the determination of ammonia and α-ketoacid, as described in sections 2.4.2. and 2.4.3.. To overcome solubility problems, Tris/HCl buffer, pH 7.5 (100 mM), rather than the standard sodium phosphate buffer was used to study the effects of bithionol, hexachlorophene, dichlorophene and 2-4-dichlorophenol on the homocysteine desulphurase activity. All inhibitors were added just before the start of the enzyme assays.

2.7.8. Serine sulphydrase (EC 4.2.1.22)

Serine sulphydrase was assayed spectrophotometrically at 37°C using a modification of the method of Braunstein et al. (1971). The

standard reaction mixture consisted of Tris/HCl buffer (pH 8.5, 100 mM), lead acetate (0.33 mM), 2-mercaptoethanol (28.4 mM), L-cysteine (3.3 mM) and enzyme sample in a final volume of 1.5 ml. The production of lead sulphide was followed at 360 nm. The activity of T. vaginalis serine sulphydrase at a range of pH was determined using the following buffers (100 mM): citric acid/sodium phosphate (pH 4.0-6.5); sodium phosphate (pH 6.0-8.0); Tris/HCl (pH 7.5-8.9); glycine/sodium hydroxide (pH 9.0-9.5); sodium carbonate/sodium hydrogen carbonate (pH 9.5-10.5). To assay for the production of ammonia and α-ketoacid by trichomonad extracts, the reaction mixture in a final volume of 1.5 ml containing L-cysteine (3.3 mM), 2-mercaptoethanol (28.4 mM), cell-free extract and Tris/HCl buffer, pH 7.5 (100 mM) was used. Controls without cysteine or cell-free extract were employed. Incubation was at 37°C for 15 min and the reaction was terminated by addition of 100 µl 10 M formic acid. The acid precipitate was removed by centrifugation and aliquots of the supernatant were determined for ammonia and α-ketoacid as described in sections 2.4.2. and 2.4.3.. All inhibitors were added just before the start of the enzyme assays.

2.7.9. γ- Cystathionase (EC 4.4.1.1)

γ-Cystathionase was estimated at 37°C by following the release of ammonia (and also α-ketoacid for T. vaginalis homogenate only) from cystathionine (3.0 mM) at pH 7.5 (sodium phosphate buffer, 100 mM). The reaction was initiated by addition of homogenate, incubated for 30 min and stopped with 100 µl 10 M formic acid. Aliquots of the protein-free supernatant were assessed for ammonia and α-ketoacid where appropriate, as described in sections 2.4.2. and 2.4.3..

2.7.10. L-Methionine-catabolizing enzyme

Estimation of thiol production

(a) Trichomonads in phosphate buffered saline, PBS. The production of volatile thiols from L-methionine by living trichomonads was measured using a modification of the method of Laakso and Nurmikko (1976) by trapping the thiols produced with DTNB and monitoring the change in absorbance at 412 nm. The reaction mixture consisted of L-methionine (15 mM), KCl (13 mM), DTNB (0.2 mM), trichomonads (approximately 1.5 x 10^7 cells) and sodium phosphate buffer, pH 7.5 (100 mM) in a final volume of 1.5 ml and was maintained at 37°C. The reaction was initiated by addition of trichomonads and controls lacking L-methionine were used. The absorbance at 412 nm was monitored continuously for 20 min. The amount of thiols produced was estimated from the standard curve constructed for L-cysteine. The cells were examined microscopically before and after each experiment (including experiments with inhibitors in the incubation mixture) and were found all to be motile except where indicated. A similar set of experiments was performed using sealed tubes. After 1 hr incubation at 37°C, the gas phase was extracted with a syringe and was analysed by mass spectrometry. The pH dependance of thiol production by T. vaginalis was determined using the following buffers (100 mM): sodium phosphate (pH 6.0-8.0); Tris/HCl (pH 7.5-8.5).

(b) Trichomonads in complex medium, MDM. Flasks containing a centre well in the main body and a side arm were used in these experiments in order to avoid direct contact of DTNB with the sulphydryl compounds present in MDM. DTNB solution (2 mM) was placed in both the centre well (0.1 ml) and the side arm (0.2 ml) to trap volatile thiols produced from the trichomonad cultures in the main body of the flask, which was sealed with a suba-seal. The reaction

mixture consisted of trichomonads (approximately 5 x 10^6 cells) in MDM (± L-methionine, 10 mM) in a final volume of 1.5 ml, and was incubated at 37°C for 1 hr. Thereafter, the DTNB was extracted from the chambers, diluted with distilled water and the absorbance of the resultant mixture at 412 nm was determined. Controls without trichomonads were used. Termination of the reaction using 100 µl 10 M formic acid did not affect the absorbance values obtained. For the detection of hydrogen sulphide, lead acetate solution (25 mM) was used instead of DTNB.

(c) Cell-free extracts of trichomonads and other protozoa. The assays of L-methionine-catabolising enzyme were carried out in a similar way to (b). The same flasks were used and DTNB was the trapping agent (but replaced by lead acetate for the detection of hydrogen sulphide). The reaction mixture consisted of L-methionine (10 mM), KCl (13 mM), cell-free extract and sodium phosphate buffer, pH 7.0 (100 mM) in a final volume of 1.5 ml. The reaction was started by addition of extract and incubated at 37°C for 60 min. The reaction was terminated by injection 100 µl 10 M formic acid into the incubation medium through the rubber suba-seal. Thereafter, the DTNB was removed, diluted with water and its absorbance at 412 nm measured. The use of 100 µl 2 M NaOH instead of formic acid to stop the reaction did not affect the absorbances obtained. Controls without L-methionine or extract were used. The activity of the L-methionine-catabolising enzyme from T. vaginalis at a range of pH was determined using the following buffers (100 mM): succinic acid/NaOH (pH 4.0-6.0); sodium phosphate (pH 6.0-8.0); Tris/HCl (pH 7.5-8.9); glycine/NaOH (8.9-10.5); sodium phosphate (pH 11.0-11.5).

Ammonia and α-ketoacid determinations. The production of ammonia and α-ketoacid from L-methionine by trichomonads in PBS was

determined using the same conditions as described in (a) above except for the omission of DTNB. After the incubation, the reaction mixture was centrifuged (2,300 x g for 10 min at 4°C) and the cell-free medium analysed. Ammonia and α-ketoacid were determined as described in sections 2.4.2. and 2.4.3.. Volatile thiols produced were determined as described in (a) above. Similarly, the acid-precipitated reaction mixtures from the cell-free extract experiments as described in (c) above were centrifuged and the resultant supernatants were analysed for ammonia and α-ketoacid.

Inhibitor studies. To overcome solubility problems, Tris/HCl buffer, pH 7.5 (100 mM), was used instead of the standard sodium phosphate buffer, pH 7.5 (100 mM), in the study of the effects of bithionol and hexachlorophene on the L-methionine-catabolising enzyme activities in parasite extracts. All inhibitors were added just before the start of the reaction, with the exception of experiments involving trichomonads in MDM for which 1 hr preincubations with DL-propargylglycine or metronidazole were involved.

2.7.11. Materials

The following chemicals were purchased from Sigma Chemical Co., Ltd., Dorset, England: DL-homocysteine; dithiothreitol (DTT); ethylene diaminetetraacetic acid (EDTA-Na_4); ethyleneglycol-bis-(β-amino ethyl ether)-N N N'N' tetraacetic acid (EGTA); 2'-deoxyadenosine; 7-deazaadenosine (tubercidin); sinefungin; adenosine; S-adenosylhomocysteine (SAH); and adenine-9-β-D-arabinofuranoside (Ara A). Butan-1-ol (chromatographic grade), Triton X-100 and TLC PEI celulose F plastic sheets (layer thickness 0.1 mm) were obtained from BDH, Glasgow, Scotland. [^{3}H-methyl-]-L-Methionine and [2-^{3}H] adenosine were obtained from Amersham

International PLC, Buckinghamshire, England. All other reagents were of analar grade and bought from either Sigma Chemical Co., Ltd., Poole, Dorset, England or BDH. Glasgow, Scotland.

2.8. Isoelectric focusing (IEF)

Flat bed agarose isoelectric focusing (IEF) was performed using carrier ampholytes as outlined in the Pharmacia guide to IEF. The agarose gel was cast on to a hydrophilic polyester sheet (Gelbond). The gel (115 x 185 mm) contained 0.28 g agarose IEF, 3.3 g sorbitol, 28 ml distilled deionised water, 1.3 ml Ampholine pH 4-6 and 0.6 ml Pharmalyte pH 3-10. The set gel was placed on the cooling plate of a flat bed electrophoresis apparatus (Pharmacia FBE 3000). The anode and cathode electrode strips were soaked in 0.05 M H_2SO_4 and 1 M NaOH, respectively. 20 µl of the sample homogenates (containing 0.2-0.4 mg protein for the detection of isoenzyme patterns and 0.07-0.1 mg protein for the protein profiles) were applied to strips of filter paper (applicators) which were placed at the centre of the gel approximately 1 cm apart. A constant power supply (LKB Bromma 2197) was set to deliver a maximum of 1500 V, 50 mA and 20 W. The sample applicators were removed after 30 min and the electrophoresis continued for a total of 1.75 hr by which time the human haemoglobin marker had focused. To stain for serine sulphydrase activity, the focused gel was submerged in 150 ml of reaction mixture containing L-cysteine (3.3 mM), lead acetate (0.33 mM), 2-mercaptoethanol (28.4 mM) and Tris/HCl buffer, pH 8.5 (100 mM) and incubated at 37°C for approximately 1-1.5 hr. The staining for homocysteine desulphurase was carried out similarly by submerging the focused gel in 150 ml of reaction mixture containing DL-homocysteine (3.3 mM), lead acetate (0.33 mM), 2-mercaptoethanol (28.4 mM) and Tris/HCl buffer, pH 7.5

(100 mM) and incubated at 37°C for approximately 1-1.5 hr. To reveal the protein patterns of trichomonads, the focused gel was fixed in 5% (w/v) sulphosalicylic acid / 10% (w/v) TCA, washed in destain solution (Ethanol/Acetic Acid/Water; 35/10/55, v/v), dried, stained with 0.2% (w/v) page blue 83 dye in destain solution and well washed in destain solution. The gels were then dried and photographed.

2.8.1. Materials

Page blue 83 dye and lead acetate were bought from BDH Chemicals Ltd., Glasgow, Scotland. Pharmalyte pH 3-10 and Ampholine pH 4-6 carrier ampholytes for isoelectric focusing were obtained from Pharmacia Fine Chemicals Ltd., Hounslow, Middlesex, England and LKB Ltd., Croydon, Surrey, England, respectively. Gelbond film (127 x 245 mm, 0.2 mm thick) was bought from Pharmacia Fine Chemicals Ltd., Hounslow, Middlesex, England.

2.9. Statistical analysis of data

2.9.1. Linear regression analysis

All apparent K_m values were calculated by the method of Lineweaver-Burk (1934). The data were analysed by linear regression analysis using the method of least squares.

2.9.2. Student's t-test.

The student's t-test was used to establish the significance of the data (with $P < 0.05$ being taken as significant).

2.10. Expression of enzyme activity and results

Enzyme activity is expressed as nmol or pmol substrate catabolised or product formed/min or hr/mg protein. The results are given as means ($\pm$ range or standard deviation) from the number of experiments (n) given in parentheses.

3.0. RESULTS

3.1. SAH hydrolase

Under the standard conditions of assay, the rate of production of SAH was linear for 10 min with Trichomonas vaginalis extract, 15 min with Trichomitus batrachorum and Tritrichomonas foetus extracts and 5 min with mouse liver homogenate. For these incubation periods, the rates were directly proportional to the amount of extract protein used up to twice that in the standard assay. Significant amounts of ^{3}H were detected only in the adenosine and SAH spots, and the latter was unlabelled if homocysteine was omitted from the reaction mixture. The absence of label in inosine (Rf, 0.24) and hypoxanthine (Rf, 0.37) spots suggests that adenosine deaminase was not active and probably had been inactivated by freezing. The pH optimum for the Trichomonas vaginalis enzyme was found to be 8.0 (Fig. 11). The mouse liver enzyme was almost equally active at pH 7.0, pH 7.5 and pH 8.0, and so subsequently was assayed at pH 8.0. SAH hydrolase activities in crude homogenates of T. vaginalis, Trichomitus batrachorum, Tritrichomonas foetus and mouse liver were found to be 14 ± 4 (n=33), 3.3 ± 0.9 (n=5), 1.2 ± 0.3 (n=4) and 38 ± 8 (n=8) nmol/min/mg protein, respectively. Approximately 93% of Trichomonas vaginalis SAH hydrolase activity was recovered in the soluble fraction of the parasite.

The apparent K_m (adenosine) of the T. vaginalis enzyme was found to be 100 ± 37 μM (n=4) (Fig. 12); adenosine concentrations greater than 450 μm inhibited the enzyme (data not shown). The apparent K_m (homocysteine) was calculated as 155 ± 41 μM (n=3) with no inhibition of the enzyme activity even with homocysteine at 6 mM. Divalent cations (at 1 mM) inhibited the T. vaginalis enzyme as follows: Co^{2+}, 32%, Cu^{2+}, 27%, Zn^{2+}, 21%, Mg^{2+}, 20%; Mn^{2+} and Ca^{2+} had no detectable effect. Both T. vaginalis SAH hydrolase and the enzyme from mouse liver were affected by EDTA (Fig. 13). In both cases, the

activity was optimal with 1 mM EDTA added to the reaction mixture, whereas higher EDTA concentrations caused considerable inhibition. EGTA up to 0.6 mM, had no effect on the T. vaginalis activity, nor did DTT up to 20 mM.

The effect of ara A, 2'-deoxyadenosine and sinefungin upon the SAH hydrolase activities of T. vaginalis and mouse liver are shown in Figs. 14 and 15. Ara A was the most effective inhibitor, but the trichomonal enzyme was less sensitive than that from mouse liver (I_{50}s of 10^{-4} M and 3×10^{-5} M, respectively, Fig. 14). The inhibition of the T. vaginalis enzyme was found to be dependent upon the length of the preincubation of drug with enzyme, such that with ara A at 0.5 mM the inhibition increased from only 19% when there was no preincubation to 75% and 83% with 15 and 30 min preincubation, respectively. The other two compounds tested both inhibited SAH hydrolase from both T. vaginalis and mouse liver; with neither, however, was there any apparent difference between the sensitivities of the two enzymes and the maximum inhibition attained was less than 50% in each case (Fig. 15). Tubercidin even at 1 mM produced little, if any, inhibition of T. vaginalis or mouse liver enzyme.

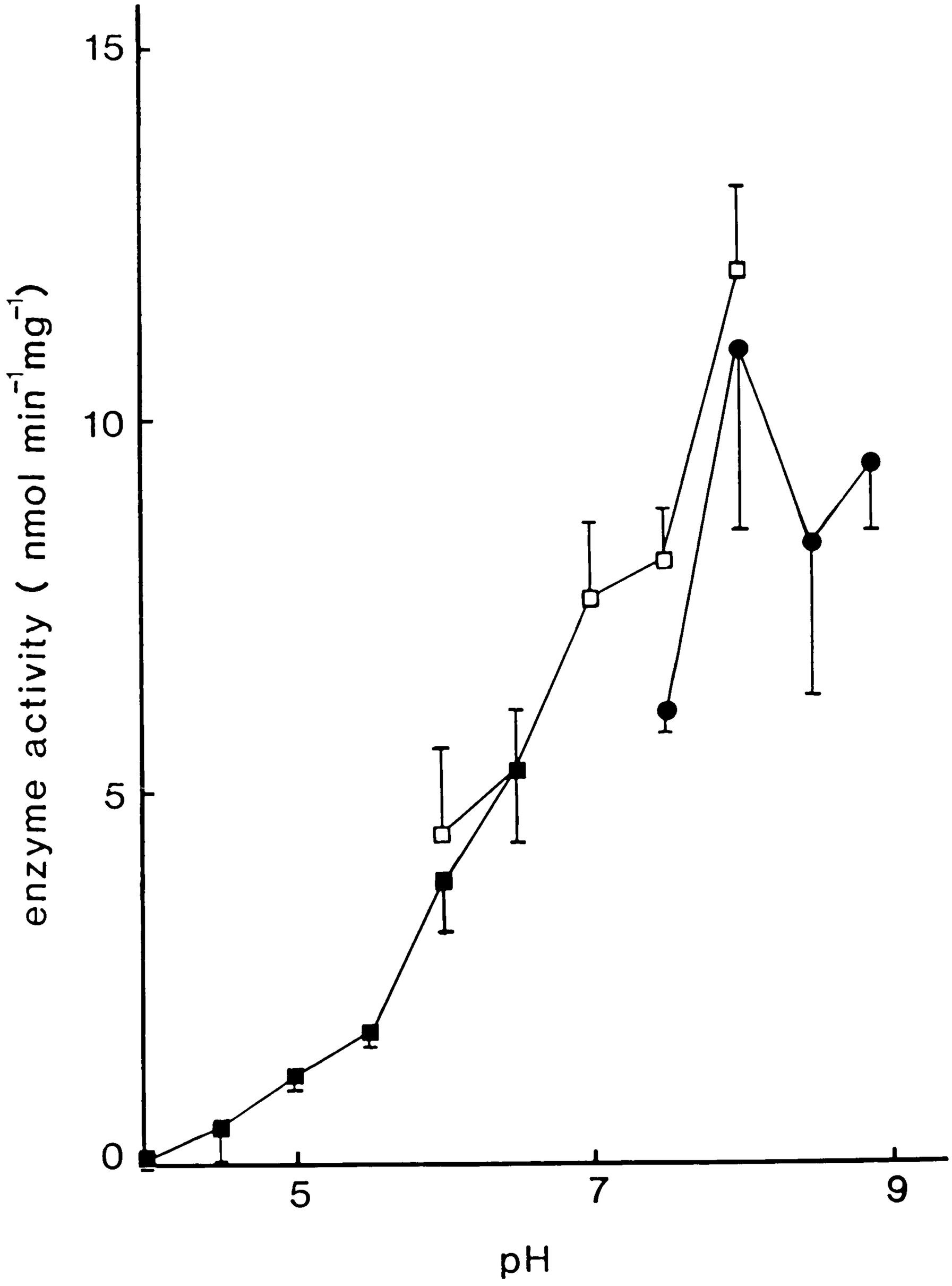


Fig. 11 The pH profile of Trichomonas vaginalis SAH hydrolase. The enzyme was assayed under standard conditions as indicated in section 2.7.5.. The buffers used were: citric acid/sodium phosphate, 25 mM (■); sodium phosphate, 25 mM, (□); Tris/HC1, 25 mM, (●). The data presented are the means (± standard deviation) from three experiments.

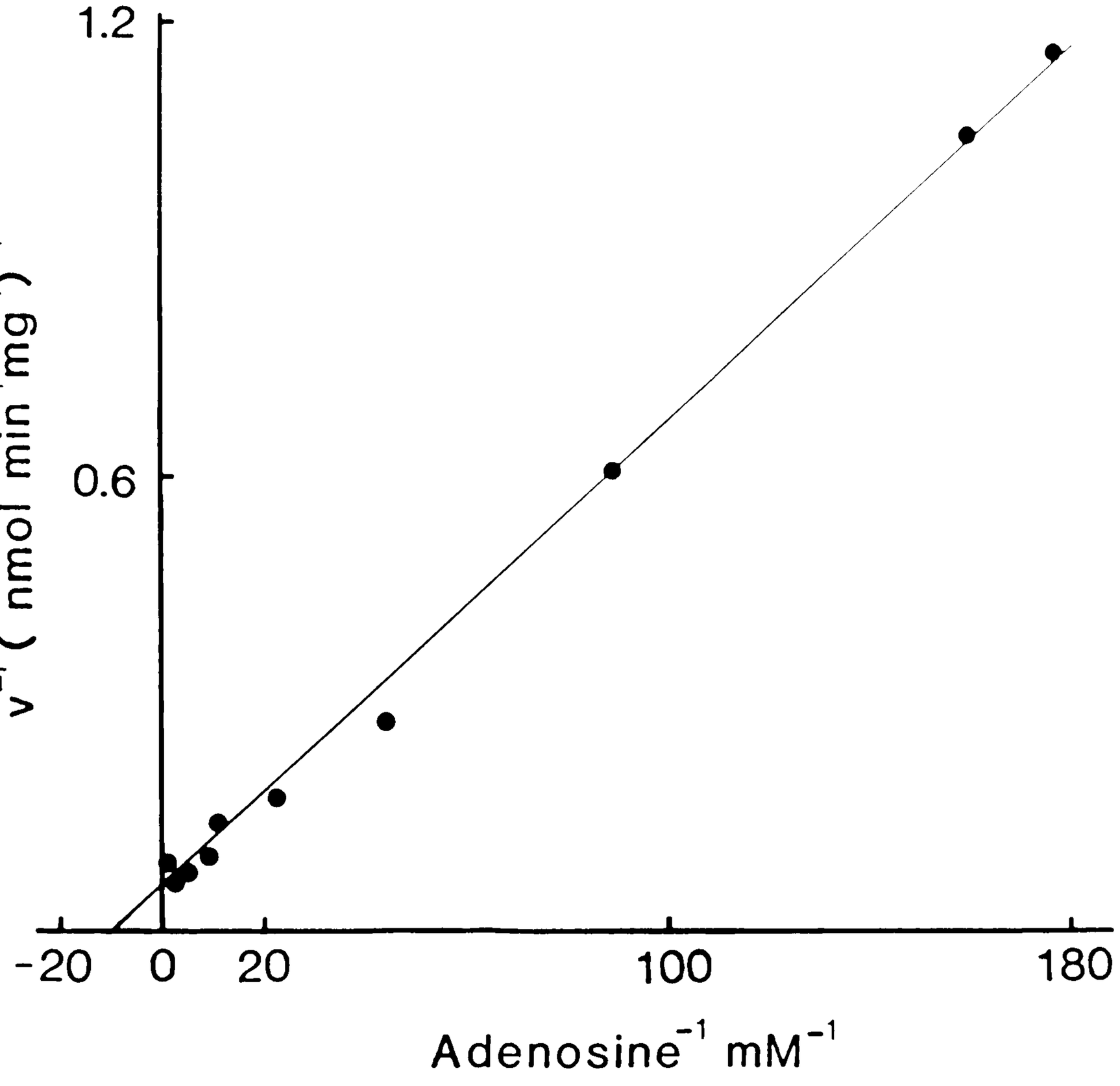


Fig. 12 A Lineweaver-Burk plot of the activity of *Trichomonas vaginalis* SAH hydrolase at a range of adenosine concentrations. The data are from one experiment which is representative of the four experiments carried out. The enzyme was assayed in the presence of 1 mM EDTA, 2 mM DTT, 3 mM homocysteine, 25 mM sodium phosphate buffer, pH 8.0, and adenosine as indicated.

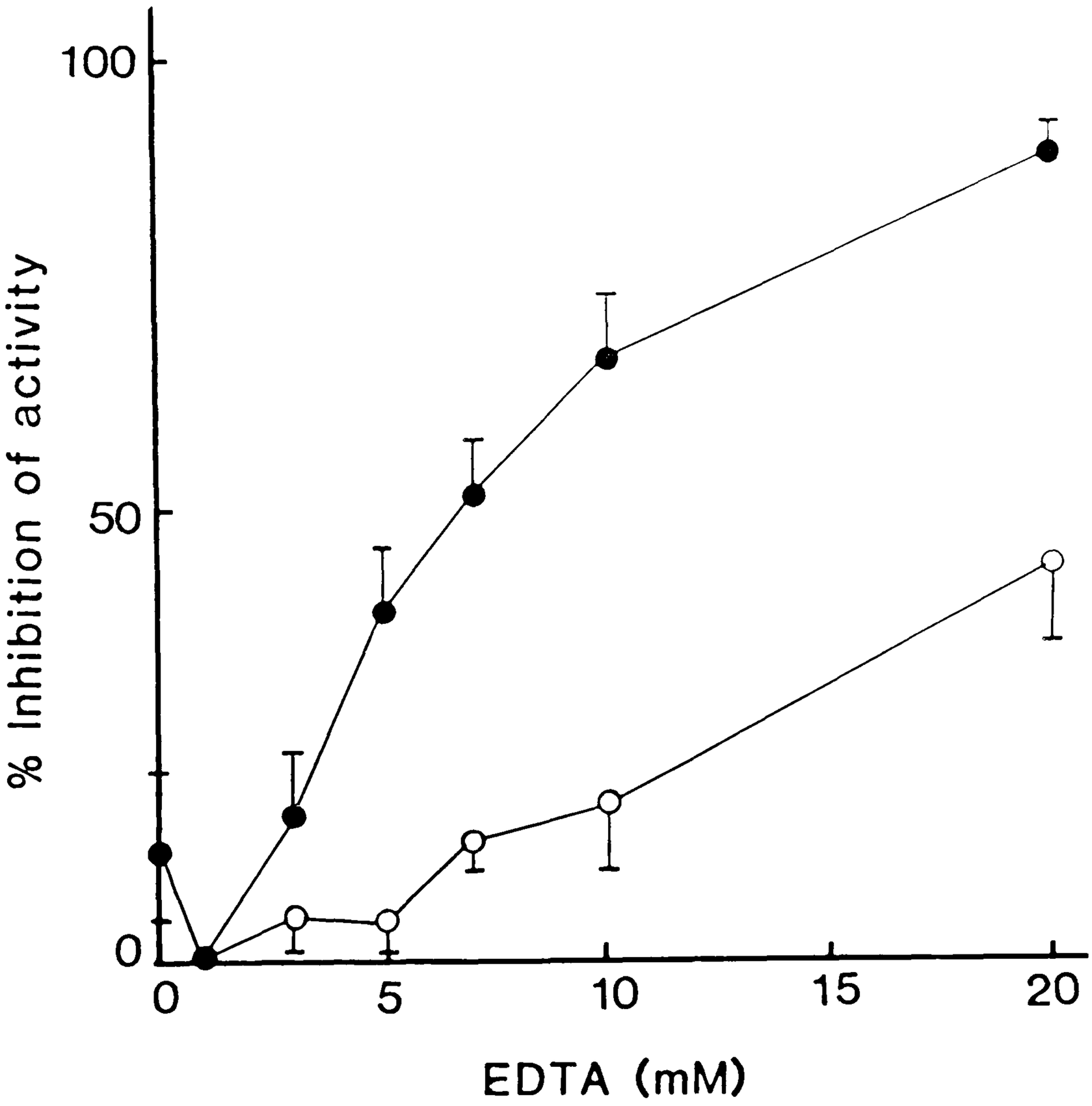


Fig. 13 The effect of EDTA on SAH hydrolase from mouse liver (○) and Trichomonas vaginalis (●). The enzymes were assayed in the presence of 2 mM DTT, 255 μM adenosine, 3 mM homocysteine, 25 mM sodium phosphate buffer, pH 8.0, and the indicated concentrations of EDTA. The data are presented as the % inhibition of the activity with 1 mM EDTA and are the means (± standard deviation) from at least three experiments.

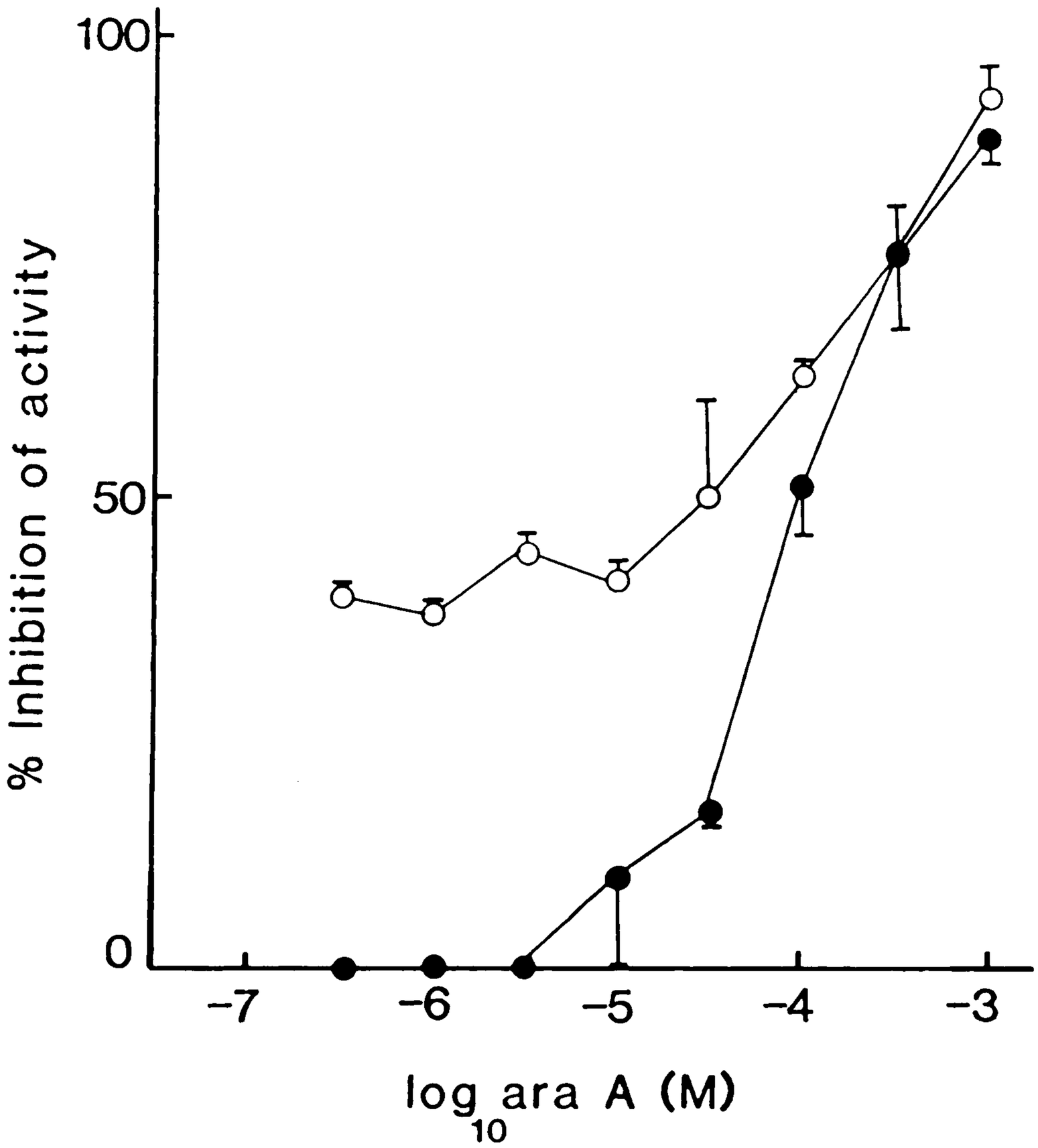


Fig. 14 The effect of ara A on mouse liver (○) and Trichomonas vaginalis (●) SAH hydrolases. Experiments were performed as indicated in section 2.7.5.. The results given are the means (± standard deviation) from three experiments.

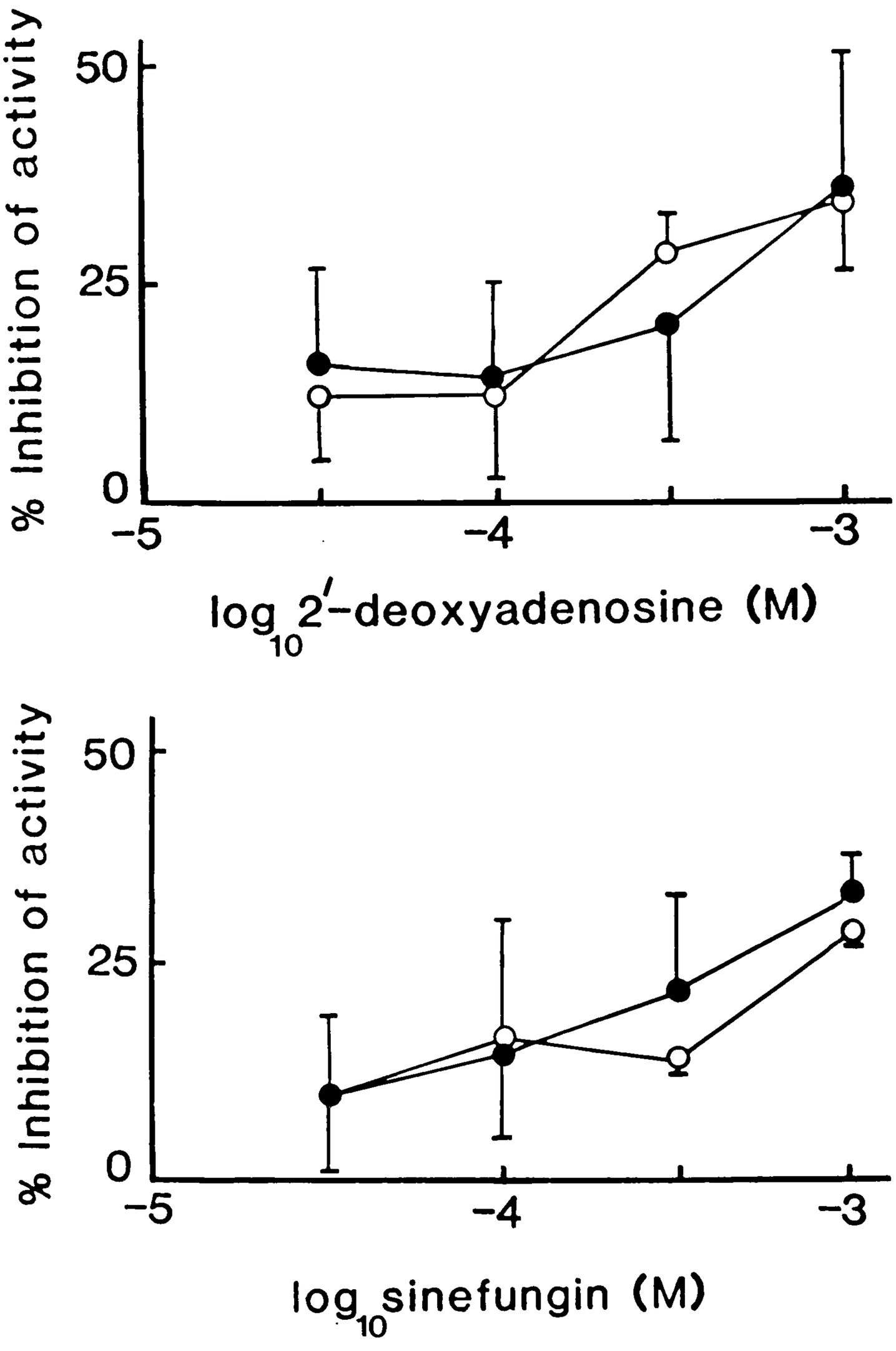


Fig. 15 The effects of 2'-deoxyadenosine and sinefungin on mouse liver (○) and Trichomonas vaginalis (●) SAH hydrolases. Experiments were conducted as indicated in section 2.7.5. The results given are the means (± range) of two experiments.

3.2. Homocysteine desulphurase and serine sulphydrase

The activities of homocysteine desulphurase and serine sulphydrase detected in homogenates and soluble fractions of trichomonads, and lysates of mouse liver, Escherichia coli and other protozoa are given in Tables 6 and 7. The activities in Trichomonas vaginalis were much higher than those in homogenates of Pentatrichomonas hominis, Trichomitus batrachorum and Tritrichomonas foetus; homocysteine desulphurase being undetectable with homogenates and soluble fractions of T. foetus and homogenates of Trichomitus batrachorum. Serine sulphydrase is apparently absent from Tetrahymena pyriformis and mouse liver. Using the standard assay conditions for Trichomonas vaginalis, homocysteine desulphurase activity was undetectable in mouse liver homogenates and lysates of Leishmania mexicana mexicana (amastigotes and promastigotes), L. tarentolae, Crithidia fasciculata, Entamoeba histolytica, Tetrahymena pyriformis, Herpetomonas muscarum muscarum and Escherichia coli (limit of detection approximately 15 nmol/min/mg protein). In contrast, the hydrolysis of cystathionine to ammonia (by γ-cystathionase) was catalysed at a greater rate by mouse liver soluble fraction (12 nmol/min/mg protein) than by homogenates Trichomonas vaginalis (7 nmol/min/mg protein), Tritrichomonas foetus (2 nmol/min/mg protein) or Trichomitus batrachorum (3 nmol/min/mg protein). Trichomonas vaginalis homogenates also catalysed the hydrolysis of cystathionine to α-ketoacid at a rate of 5 nmol/min/mg protein.

The T. vaginalis homocysteine desulphurase was found to be soluble (approximately 97% of the total activity being recovered in the soluble fraction), optimally active at pH 7.5 (Fig. 16) with an apparent K_m for homocysteine of 549 $\pm$ 23 (n=2) μM (Fig. 17). The enzyme was specific for homocysteine; none of cysteamine, 2-

mercaptoethanol, cystathionine, SAH, L-methionine, D-cysteine, monothioglycerol, S-methylthioglycerol, glutathione (both oxidised and reduced forms) and cysteine sulphinate were hydrolysed to hydrogen sulphide. The following compounds (with concentrations tested up to those indicated in parentheses) had little or insignificant effect on the rate of hydrogen sulphide production from the parasite homocysteine desulphurase: D-methionine (27 mM); pyruvate (27 mM); L-serine (107 mM); GSH (13.3 mM); GSSG (13.3 mM); DL-homoserine (107 mM); sinefungin (1.1 mM); SAH (0.02 mM); NAD^+ (0.12 mM); S-methylthioglycerol (27 mM); monothioglycerol (27 mM); cycloleucine (13.3 mM); 4'-deoxypyridoxine (33.3 mM); cysteine sulphinate (13.3 mM); metronidazole (0.94 mM); α-ketobutyrate (20 mM); DTT (2 mM); NH_4Cl (27 mM); $(NH4)_2SO_4$ (27 mM); pyridoxal-5'-phosphate (0.11 mM); cystathionine (8.3 mM); coenzyme A (0.53 mM); selenomethionine (4.4 mM); SAM (3.0 mM); M & B 8416 (4.6 mM); M & B 13671 (4.1 mM); M & B 13928 (3.7 mM); M & B 22487 (1.7 mM); M & B 23435 (1.1 mM); M & B 23837 (1.3 mM); M & B 23885 (4.1 mM); M & B X24038 (2.3 mM); M & B 25016 (3.8 mM); M & B 25049 (1.2 mM); M & B 25062 (2.4 mM). Analysis of the products of the catabolism of homocysteine by T. vaginalis and P. hominis revealed that hydrogen sulphide, α-ketoacid and ammonia were all produced by T. vaginalis extracts (Table 8). The rates of production of the latter two compounds were lower, but not significantly different ($0.05 < P < 0.1$), from that of hydrogen sulphide. Ammonia production was not detectable with homogenates of P. hominis, although the limit of detection was similar to the expected level of production. The rates of production of ammonia and α-ketoacid from homocysteine by T. vaginalis extracts were reduced by 2-mercaptoethanol (Table 9), whereas hydrogen sulphide production was

slightly potentiated, but this was not significant ($p > 0.1$).

The T. vaginalis serine sulphydrase was also found to be soluble (approximately 96% of the total activity being recovered in the soluble fraction), optimally active at pH 8.5 (Fig. 18) with an apparent K_m for L-cysteine of 420 ± 110 (n=3) μM (Fig. 19). High concentrations (> 5 mM) of the substrate were inhibitory (60 mM inhibited 100%) and the enzyme was specific for L-cysteine; none of D-cysteine, L-cysteine sulphinate or cysteamine were hydrolysed to hydrogen sulphide. The parasite enzyme was highly dependent upon 2-mercaptoethanol; the activity was reduced 15-fold if this compound was omitted from the reaction mixture. In contrast, the parasite enzyme was either only slightly affected or unaffected by the following compounds (with concentrations used and % inhibition of activity): α-ketobutyrate (33 mM, 42 ± 2% (n=2)); L-serine (33 mM, 28 ± 12% (n=2)); GSH (17 mM, 39 ± 9% (n=2)); DL-cystathionine (10.4 mM, 0% (n=2)); DL-homoserine (33.3 mM, 0% (n=2)); pyridoxal-5'-phosphate (0.1 mM, 0% (n=2)). Analysis of the products of the catabolism of cysteine by T. vaginalis serine sulphydrase revealed that hydrogen sulphide was produced; ammonia and α-ketoacid were not detected.

The various lines of T. vaginalis were assayed for homocysteine desulphurase and serine sulphydrase activities (Table 10). There was no clear correlation between enzyme activities and sensitivity to metronidazole or age in culture.

The homocysteine desulphurase and serine sulphydrase activities of T. vaginalis were inhibited by various compounds (Tables 11a and 11b) and their antitrichomonal activities are given in Tables 11a and 21a. Bithionol, hexachlorophene and dichlorophene were found to be good inhibitors of both enzymes. 2,2'-Dithiodiethanol and 2,4-dichlorophenol, compounds with some structural similarity to

bithionol, had little effect on either activity. Bithionol hexachlorophene and dichlorophene inhibited both the enzyme activities and growth in vitro at similar concentrations (Table 11a). In contrast, DL-propargylglycine was more potent against homocysteine desulphurase than serine sulphydrase and had little effect on the growth in vitro (Table 11a). Similarly, L-cycloserine and DL-penicillamine were more effective against homocysteine desulphurase than serine sulphydrase (Table 11b). Combinations of DL-propargylglycine (10^{-5} M) with bithionol and hexachlorophene were tested against the growth in vitro of T. vaginalis (Table 11a). DL-Propargylglycine did not markedly potentiate either compound.

Trichomonas vaginalis grown in the presence of DL-propargylglycine (10^{-5} M) for 24 hr had no detectable homocysteine desulphurase activities but possessed markedly elevated (approximately 3.5-fold) serine sulphydrase activity (see Table 12). Activities of the two enzymes returned to the usual levels about 24 hr after the parasite was removed (washed) from the pressure of the drug (see Fig. 20). Cell lines IR78, Boston and Fall River similarly treated with DL-propargylglycine (10^{-5} M) for 24 hr also lacked detectable homocysteine desulphurase activity. These cell lines, however, possessed only slightly elevated serine sulphydrase activity. Treatment of Tritrichomonas foetus in the same way had no effect on the serine sulphydrase activity. Despite this difference, these lines of Trichomonas vaginalis were no more sensitive to DL-propargylglycine (10^{-6} M - 10^{-2} M) than clone G3, whether tested aerobically or anaerobically. Similarly, Trichomitus batrachorum and Tritrichomonas foetus were as insensitive to DL-propargylglycine (10^{-6} M - 10^{-2} M) under aerobic conditions as the T. vaginalis lines. In addition, there were no apparent differences in sensitivity to

metronidazole (under aerobic or anaerobic conditions) between cells of T. vaginalis grown in the presence or absence of 10^{-5} M DL-propargylglycine for 24 hr.

The isoenzyme patterns of serine sulphydrase produced by IEF of extracts of T. vaginalis lines are shown in Figure 21a. This revealed that several proteins are responsible for the enzyme activity. These proteins are acidic with pIs ranging from 3.5 to 6.0. No enzyme activity was present in human blood cell lysates treated in the same way. Small differences in isoenzyme patterns were observed between clone G3, Boston and Fall River, but IR78 was the most distinct. An increase in the number of isoenzymes detectable was the result of growing Boston line in the presence of 10^{-5} M DL-propargylglycine (compare tracks 3 and 7). Cells of clone G3 and IR78 similarly treated with 10^{-5} M DL-propargylglycine did not show any increase in isoenzyme numbers. With the former line, however, several of the bands appeared much earlier and stained more intensely, indicating a large increase in enzyme activity (compare tracks 2 or 6 with 9). With IR78, DL-propargylglycine treatment caused only a slight increase in stain intensity (compare tracks 4 and 8). In contrast to line IR78, the isoenzyme banding patterns for two recently isolated drug-sensitive lines, 6950d and 45, were found to be very similar to G3. The only clear difference was that line 45 possessed an additional band with a pI or 3.9, very similar to the most acidic bands found with Boston, IR78 and Fall River. There were marked species differences in the isoenzyme patterns of serine sulphydrase (Fig. 21b).

Trichomonas vaginalis homocysteine desulphurase activity was found to be due to a single protein (Fig. 22). The enzyme band, however, was not detectable with the use of sodium phosphate buffer (pH 7.5, 100 mM) rather than the Tris/HCl buffer nor if 2-

mercaptoethanol was omitted from the reaction mixture. As well as those lines included on this gel, lines IR78, 69506 and 45 also had the same single homocysteine desulphurase band. No bands were detectable with other species. In addition, T. vaginalis grown in the presence of 10^{-5} M DL-propargylglycine for 24 hr lacked a detectable homocysteine desulphurase band (data not shown).

Total protein patterns of all T. vaginalis lines were found to be similar, irrespective of whether the cells were grown in the presence or absence of 10^{-5} M DL-propargylglycine for 24 hr (clone G3 is given as an example in Fig. 23). Three major bands characteristic of T. vaginalis have been arrowed in the Figure. The protein profiles of T. vaginalis, Trichomitus batrachorum, Tritrichomonas foetus and Pentatrichomonas hominis, however, differed considerably (Fig. 23).

Table 6 Enzyme activities in homogenates (H) and soluble fractions (S) of trichomonads.

Organism	Activity (nmol H_2S/min/mg protein)[a] Homocysteine desulphurase	Serine sulphydrase
Trichomonas vaginalis		
H	131 ± 57 (19)	251 ± 114 (13)
S	484 ± 90 (93)	664 ± 162 (59)
Tritrichomonas foetus		
H	n.d. < 4 (6)	22 ± 4 (9)
S	n.d. < 2 (10)	80 ± 15 (16)
Trichomitus batrachorum		
H	n.d. < 3 (6)	3 ± 1 (5)
S	6 ± 2 (9)	7 ± 4 (13)
Pentatrichomonas hominis		
H	7 ± 4 (12)	61 ± 15 (27)

[a] results are the means (± standard deviation) from the number of experiments given in parentheses.

n.d., not detectable.

Table 7 Serine sulphydrase activity in homogenates of other protozoa, *Escherichia coli* and mouse liver.

Organism	Serine sulphydrase activity[a] (nmol H_2S/min/mg protein)	
Leishmania mexicana mexicana (promastigotes)	96 ± 26	(16)
Leishmania mexicana mexicana (amastigotes)	8 ± 8	(9)
Leishmania tarentolae	111 ± 36	(7)
Crithidia fasciculata	492 ± 125	(8)
Herpetomonas muscarum muscarum	148 ± 15	(8)
Entamoeba histolytica	61 ± 7	(6)
Tetrahymena pyriformis	n.d. < 9	(7)
Escherichia coli	130 ± 12	(9)
mouse liver	n.d. < 9	(16)

[a] results are the means (± standard deviation) from the number of experiments given in parentheses.

n.d., not detectable.

TABLE 8 Products of homocysteine catabolism by trichomonads.

Product	Rate of production (nmol/min/mg protein)[a]	
	Trichomonas vaginalis	Pentatrichomonas hominis
Hydrogen sulphide	131 ± 57 (19)	7 ± 4 (12)
Ammonia	75 ± 27 (15)	n.d. < 4 (3)
α-Ketoacid	76 ± 23 (15)	13 ± 3 (3)

[a] results are the means (± standard deviation) from the number of experiments given in parentheses.

n.d., not detectable.

Table 9 The effect of 2-mercaptoethanol on *Trichomonas vaginalis* homocysteine desulphurase activity[a].

2-Mercaptoethanol concentration (mM)	Rate of production (% of control)[b]		
	Ammonia	Hydrogen sulphide	α-Ketoacid
4.2	50 ± 6	124 ± 23	52 ± 3
8.4	26 ± 2	127 ± 25	30 ± 8

[a] 2-mercaptoethanol was included in the assay mixture at the concentrations indicated.

[b] results are the means (± standard deviation) from three experiments and expressed at the % of the production in the absence of 2-mercaptoethanol.

Table 10 Enzyme activities of Trichomonas vaginalis lines.

Cell lines	Activity (nmol H_2S/min/mg protein)[a] Homocysteine desulphurase	Serine sulphurase
metronidazole-sensitive[b]		
G3	131 ± 57 (19)	255 ± 36 (13)
45*	58 ± 52 (3)	190 ± 78 (3)
55*	127 (1)	65 (1)
61*	124 ± 19 (3)	119 ± 23 (3)
64*	126 ± 33 (3)	136 ± 35 (3)
6950δ+	71 ± 30 (2)	363 ± 170 (2)
BOSTON	98 ± 42 (9)	125 ± 64 (7)
metronidazole-resistant[c]		
45733*	28 ± 21 (3)	20 ± 28 (3)
2755*	70 ± 22 (2)	110 ± 1 (2)
FALL RIVER	75 ± 28 (8)	157 ± 64 (7)
IR78	54 ± 16 (4)	164 ± 85 (4)

[a] results are the means (± range or standard deviation) from the number of experiments given in parentheses.

[b] with MLC values (assayed under aerobic conditions) less than 5 μg/ml.

[c] with MLC values (assayed under aerobic conditions) greater than 10 μg/ml.

* fresh isolates i.e. subpassaged in vitro less than 3 times.

+ maintained by serial subpassage intravaginally in mice.

Table 11a Effect of inhibitors on the enzyme activities and growth *in vitro* of *Trichomonas vaginalis*[+].

Inhibitor	Inhibition of growth		I_{50} (M)	
	LD_{50}(M)	MLC(M)	Homocysteine desulphurase	Serine sulphydrase
DL-Propargylglycine (DLP)	a (2)	a (2)	$(1.7 \pm 0.3)\ 10^{-6}$ (3)	$(3.9 \pm 0.3)\ 10^{-3}$ (2)
Bithionol (B)	$(8.1 \pm 2.0)\ 10^{-5}$ (3)	3.10^{-4} (3)	$(1.7 \pm 0.2)\ 10^{-5}$ (3)	$(7.7 \pm 1.8)\ 10^{-5}$ (4)
Hexachlorophene (H)	$(1.9 \pm 1.2)\ 10^{-5}$ (3)	3.10^{-4} (3)	$(6.5 \pm 0.1)\ 10^{-5}$ (3)	$(1.2 \pm 0.6)\ 10^{-5}$ (4)
Dichlorophene	$(8.7 \pm 2.4)\ 10^{-5}$ (2)	3.10^{-4} (2)	$(6.4 \pm 3.6)\ 10^{-5}$ (2)	$(7.5 \pm 0.4)\ 10^{-4}$ (2)
2,2'-Thiodiethanol	-	-	b (2)	b (2)
2,4-Dichlorophenol	-	-	c (2)	d (2)
B with DLP (10^{-5}M)	$(3.0 \pm 2.4)\ 10^{-5}$ (3)	3.10^{-4} (3)	-	-
H with DLP (10^{-5}M)	$(4.4 \pm 2.6)\ 10^{-6}$ (3)	3.10^{-4} (3)	-	-

[+] results are the means (± range or standard deviation) from the number of experiments given in parentheses.

a, 23 ± 2% inhibition at 10^{-2}M.

b, no inhibition at 4 x 10^{-3}M.

c, 46% inhibition at 2 x 10^{-3}M.

d, no inhibition at 2 x 10^{-3}M.

-, not done.

Table 11b Effects of inhibitors on the enzyme activities of *Trichomonas vaginalis*[+].

Inhibitor	I_{50}(M) Homocysteine desulphurase	Serine sulphydrase
L-Cycloserine	$(3.5 \pm 0.1)\ 10^{-5}$ (3)	a (2)
Hydroxylamine	$(6.5 \pm 0.2)\ 10^{-5}$ (3)	$(1.5 \pm 0.3)\ 10^{-2}$ (2)
DL-Penicillamine	$(5.2 \pm 0.7)\ 10^{-3}$ (4)	b (2)
L-Methionine	$(9.7 \pm 1.6)\ 10^{-3}$ (6)	$(1.5 \pm 0.1)\ 10^{-2}$ (2)
L-Cysteine	$(2.9 \pm 0.1)\ 10^{-2}$ (2)	-
S-Methyl-L-cysteine	-	$(1.7 \pm 0.8)\ 10^{-3}$ (2)

[+] results are the means (± range or standard deviation) from the number of experiments given in parentheses.

a, 37 ± 5% inhibition at 3.3×10^{-2}M.

b, 45 ± 12% inhibition at 3.3×10^{-2}M.

-, not done.

Table 12 The effects of DL-propargylglycine on the growth axenically *in vitro* and enzyme activities of *Trichomonas vaginalis*[a].

Concentration (M) of DL-propargylglycine in growth medium	% Inhibition of growth *in vitro*	Relative enzyme activity[b]	
		Homocysteine desulphurase	Serine sulphydrase
0 (control)	0	1	1
10^{-7}	0 (5)	0.6 ± 0.1 (6)	1.3 ± 0.2 (5)
10^{-6}	4 ± 6 (4)	0 (5)	3.1 ± 0.8 (5)
10^{-5}	22 ± 10 (11)	0 (11)	3.5 ± 1.3 (8)
10^{-4}	13 ± 13 (4)	0 (6)	4.6 ± 0.5 (5)
10^{-3}	27 ± 17 (4)	0 (7)	5.0 ± 1.2 (5)

[a] results are the means (± standard deviation) from the number of experiments given in parentheses.

[b] figures given are the enzyme activities (from cells grown in the presence of different concentrations of DL-propargylglycine for 24 hr) relative to the enzyme activity detected in parasites grown in the absence of the drug (control).

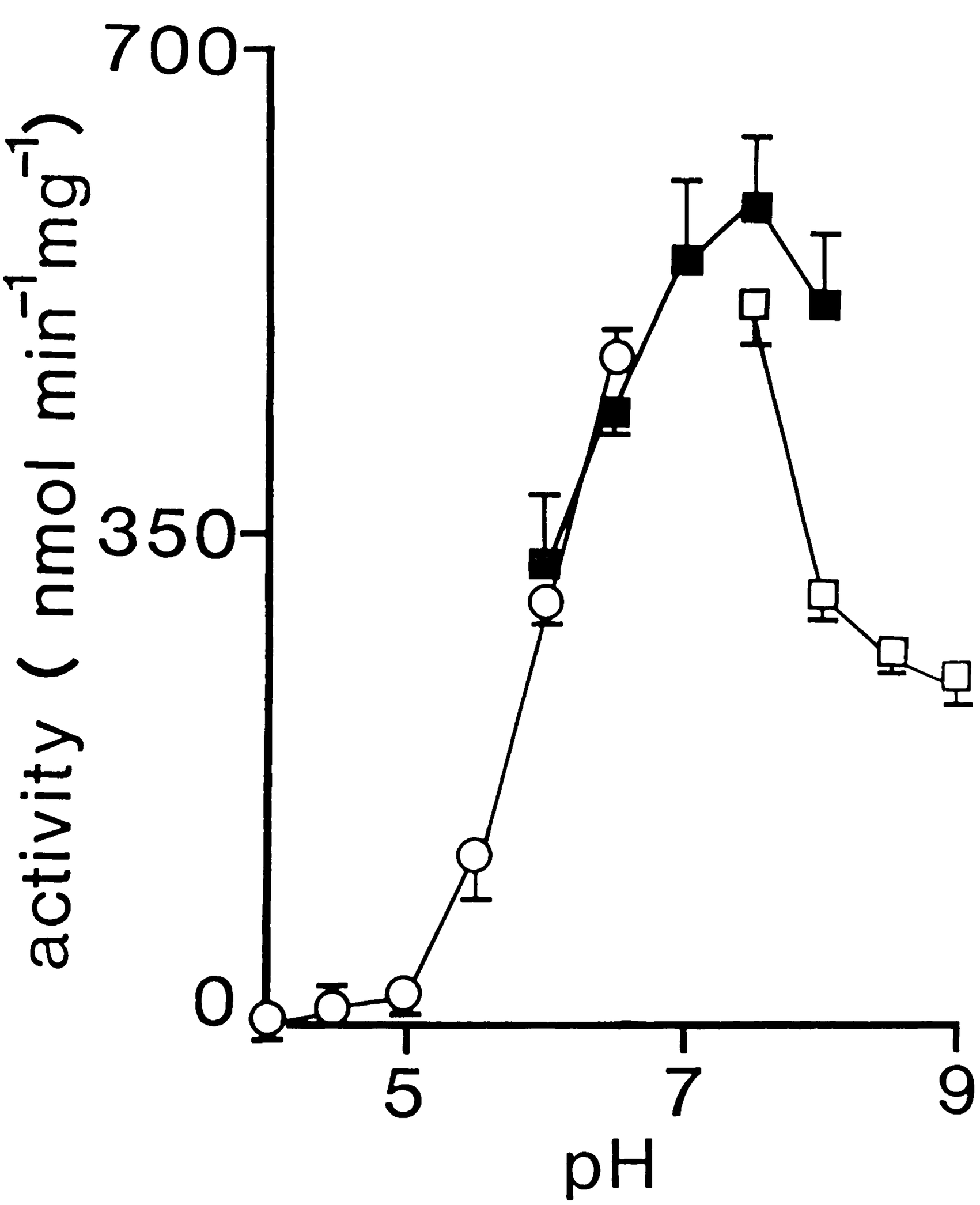


Fig. 16 The pH profile of Trichomonas vaginalis homocysteine desulphurase (means ± standard deviation from three experiments).

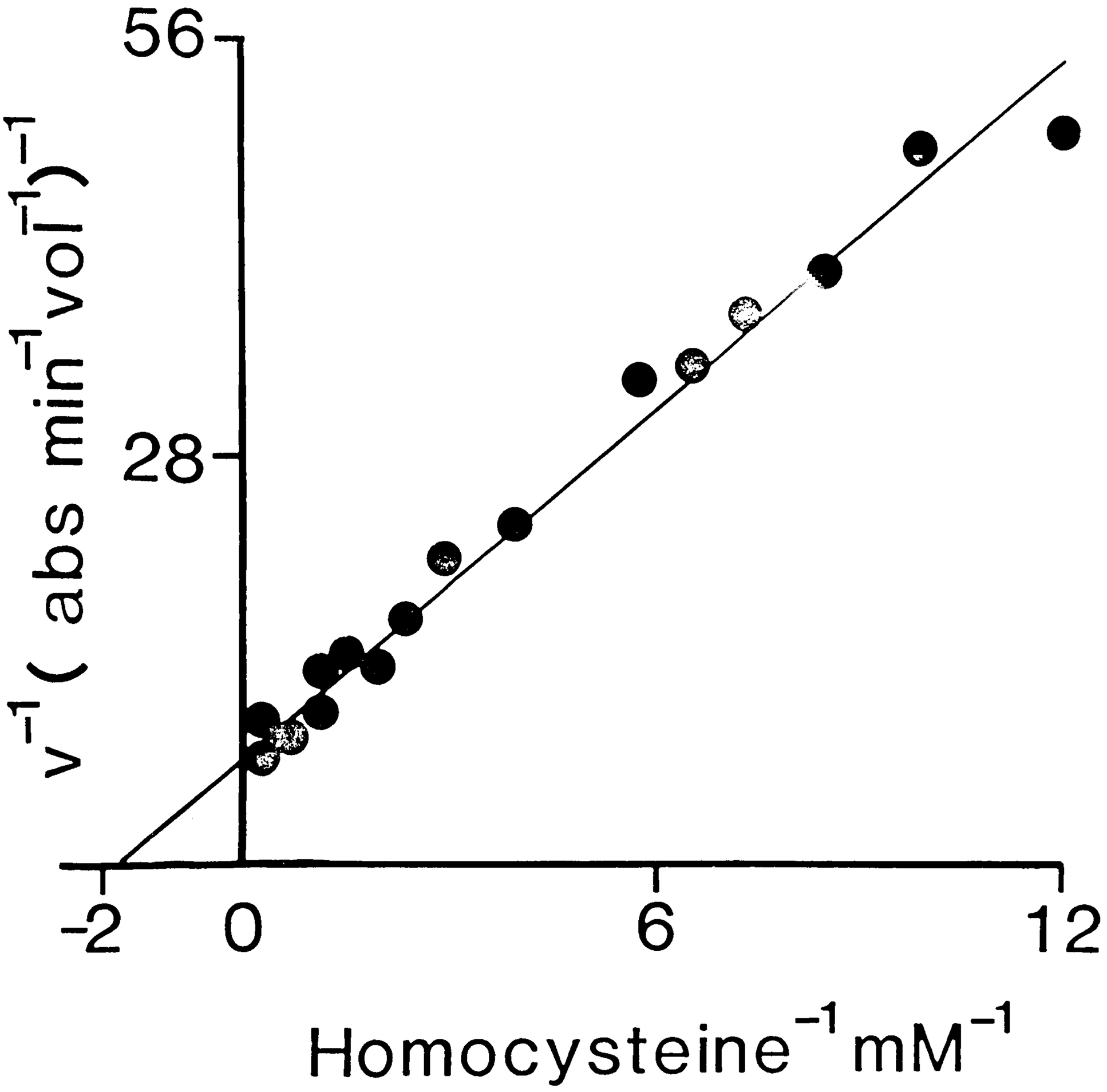


Fig. 17 A Lineweaver-Burk plot of the activity of Trichomonas vaginalis homocysteine desulphurase at a range of homocysteine concentrations. The data are from one experiment which is representative of the two experiments carried out.

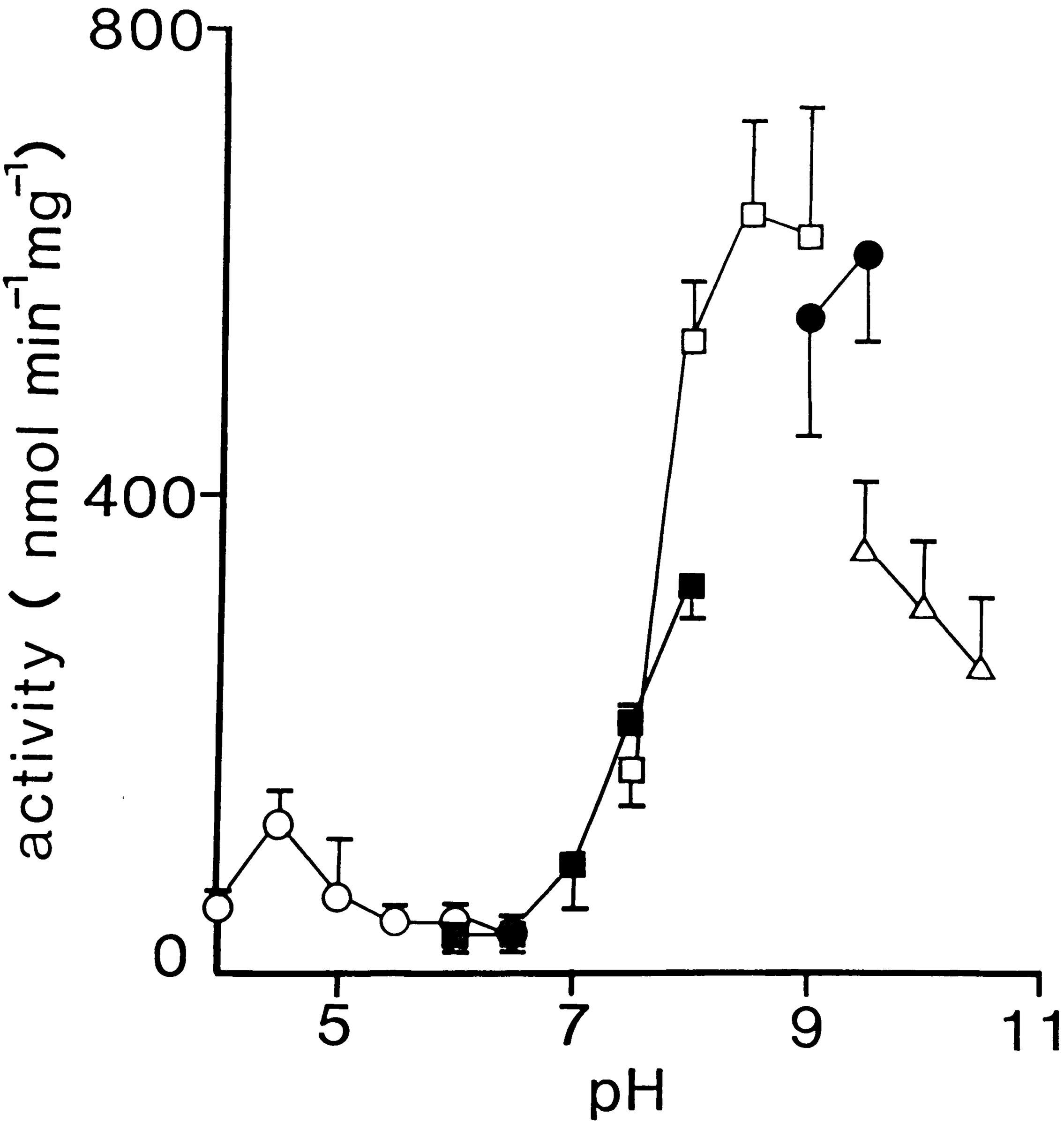


Fig. 18 The pH profile of Trichomonas vaginalis serine sulphydrase (means ± standard deviation from three experiments).

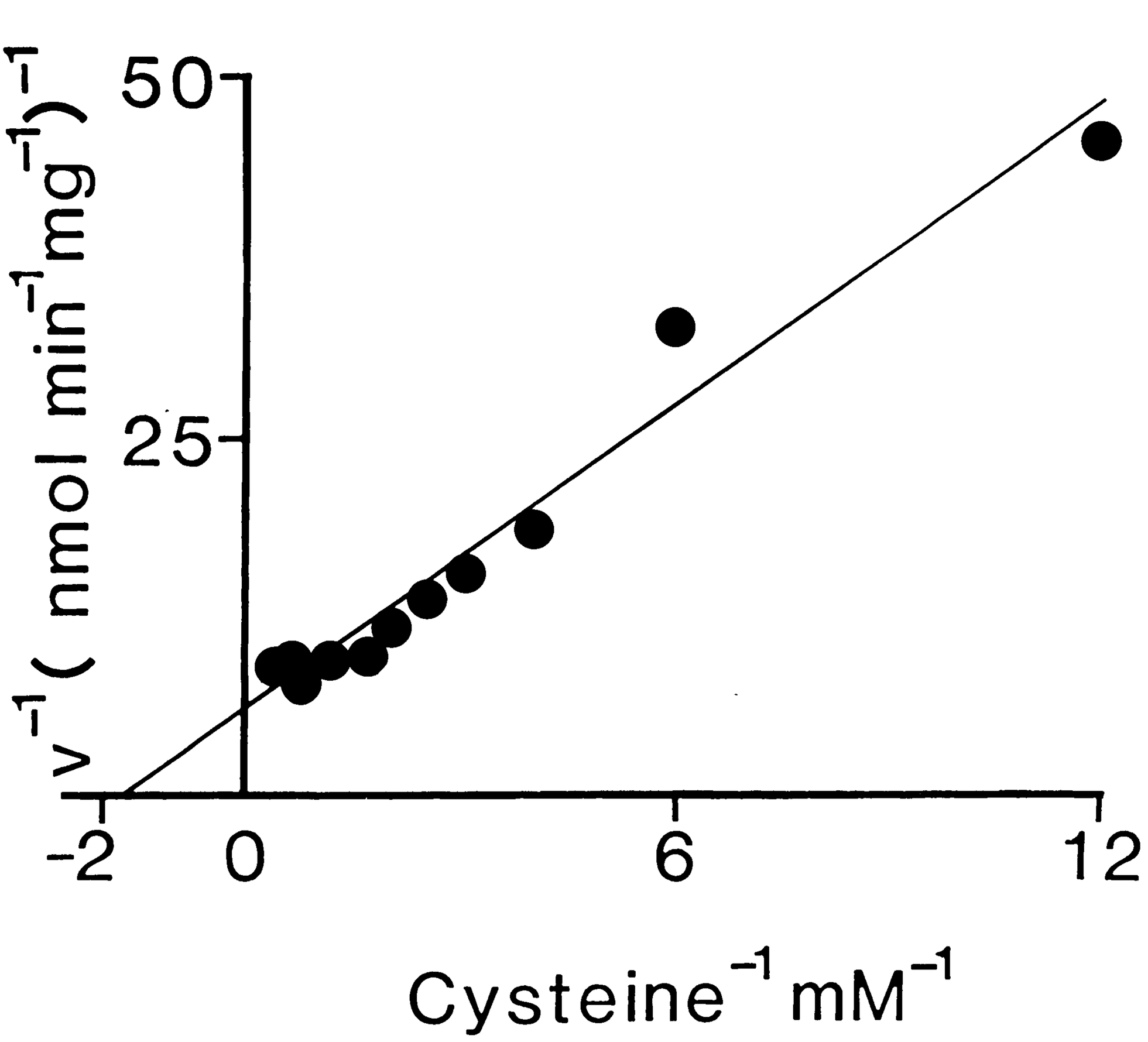


Fig. 19 A Lineweaver-Burk plot of the activity of Trichomonas vaginalis serine sulphydrase at a range of L-cysteine concentrations. The data are from one experiment which is representative of the three experiments carried out.

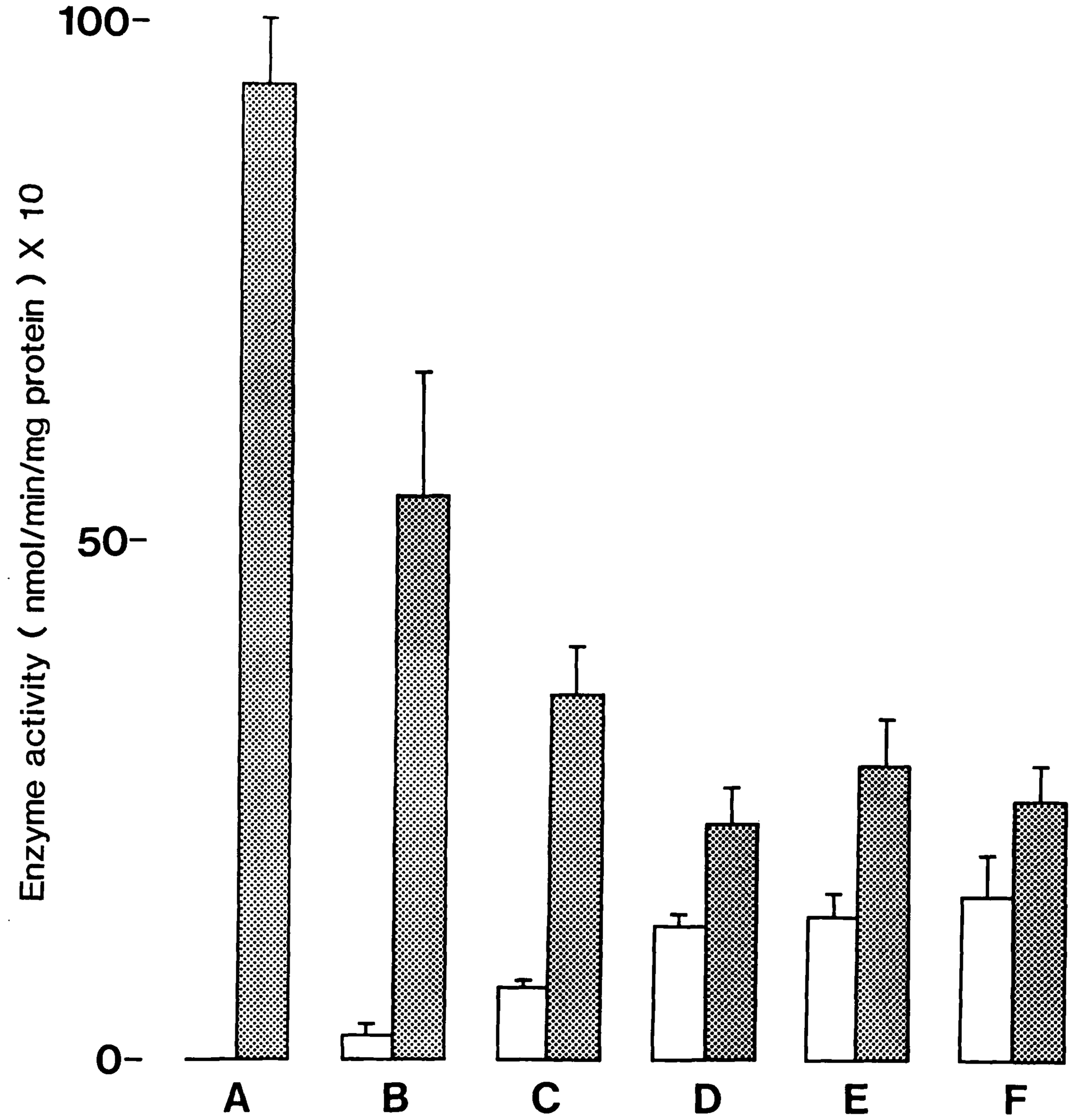


Fig. 20 Trichomonas vaginalis: recovery of homocysteine desulphurase and serine sulphydrase (shaded) activities. Enzyme activities detected in cells grown in the presence of DL-propargylglycine (10^{-5}M) for 24 hr (A); cells at 4, 8, 24 and 28 hr (B, C, D and E, respectively) after removal of the drug (10^{-5} M) from the culture medium; cells grown in the absence of the drug (F). The results are from one experiment (the enzyme assays were done in sextuples).

Fig. 21a Serine sulphydrase isoenzyme patterns. Key to tracks: (1) Human blood lysate; (2)-(6) are Trichomonas vaginalis lines grown in MDM-(2) clone G3; (3) Boston; (4) IR78; (5) Fall River; (6) clone G3; (7)-(9) are T. vaginalis lines grown in MDM in the presence of 10^{-5} M DL-propargylglycine for 24 hr-(7) Boston; (8) IR78; (9) clone G3.

Fig. 21b Serine sulphydrase isoenzyme patterns of three trichomonad species. Key to tracks: (1) Trichomitus batrachorum; (2) Tritrichomonas foetus; (3) Pentatrichomonas hominis

Fig. 22 Homocysteine desulphurase of trichomonads. Key to tracks: (1) Trichomonas vaginalis clone G3; (2) T. vaginalis Boston; (3) T. vaginalis Fall River; (4) Pentatrichomonas hominis; (5) Trichomitus batrachorum; (6) Tritrichomonas foetus.

Fig. 23 Protein profiles of trichomonads. The tracks in (a) were: (1) standards; (2) Trichomonas vaginalis clone G3; (3) Trichomitus batrachorum; (4) Tritrichomonas foetus; (5) Pentatrichomonas hominis; (6) Trichomonas vaginalis Fall River. The tracks in (b) were: (1) standards; (2) T. vaginalis clone G3; (3) T. vaginalis clone G3 grown in the presence of 10^{-5}M DL-propargylglycine for 24 hr; (4) T. vaginalis 69506. The arrows indicate major bands characteristic of T. vaginalis.

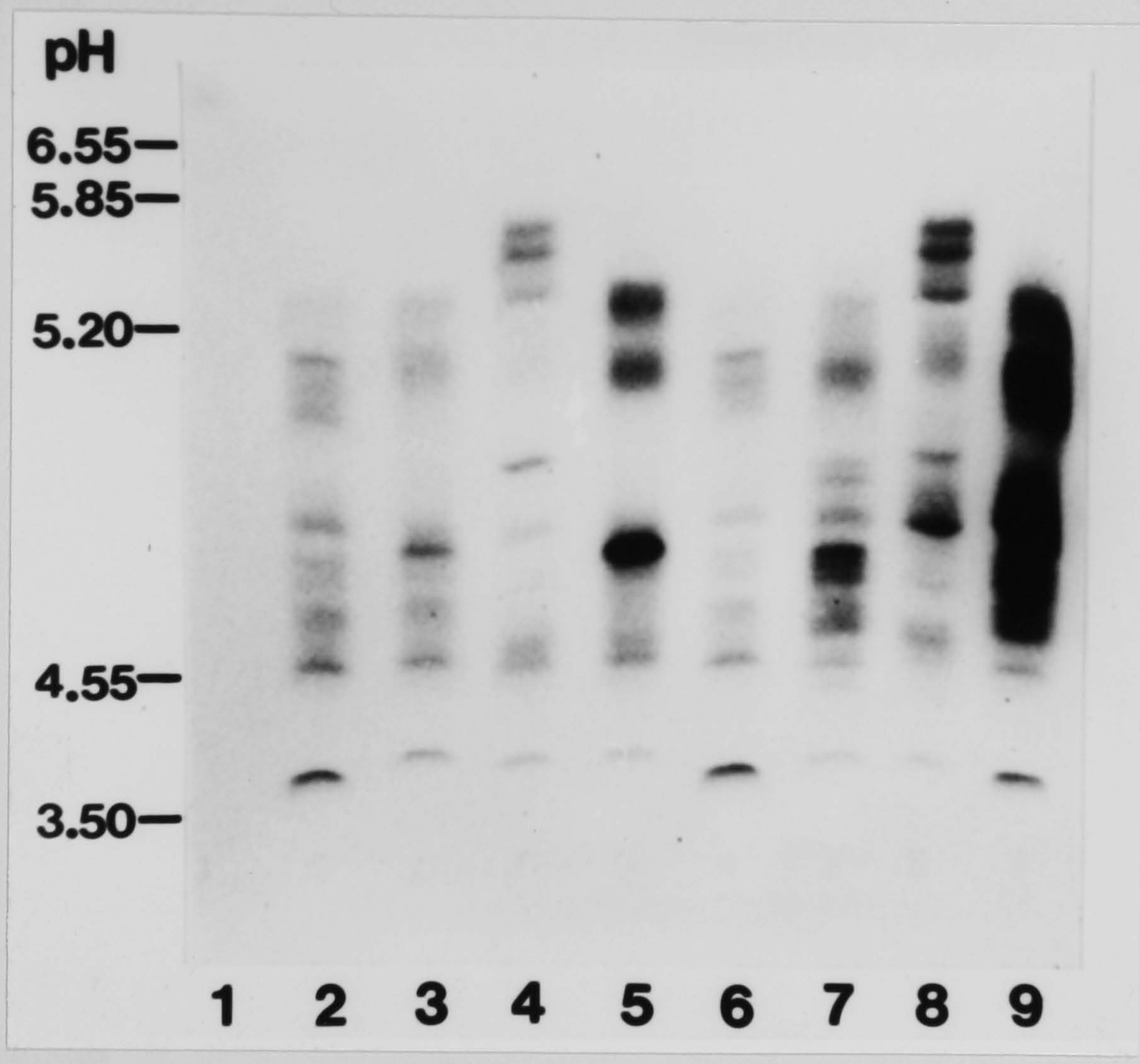


Fig. 21a

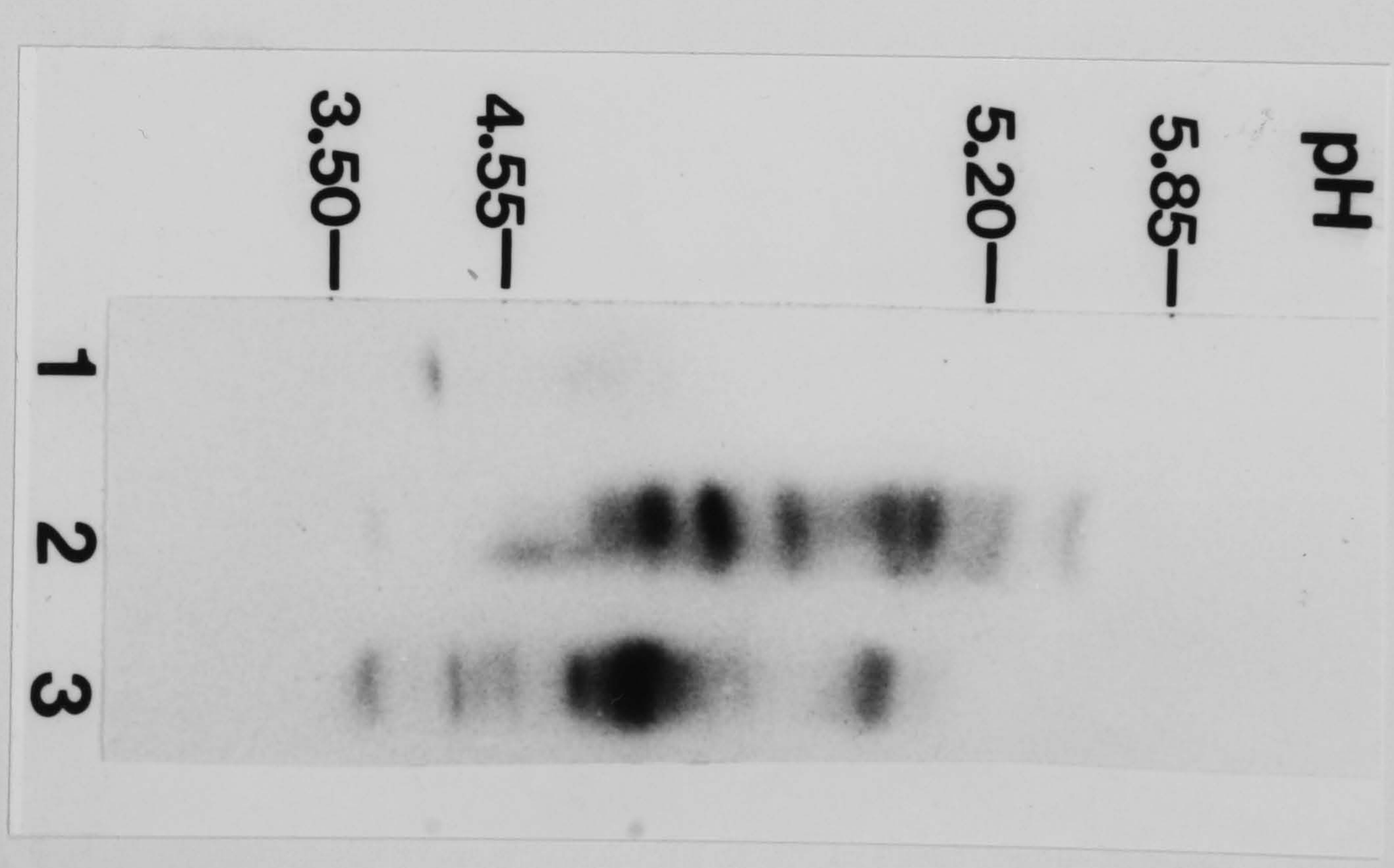


Fig. 21b

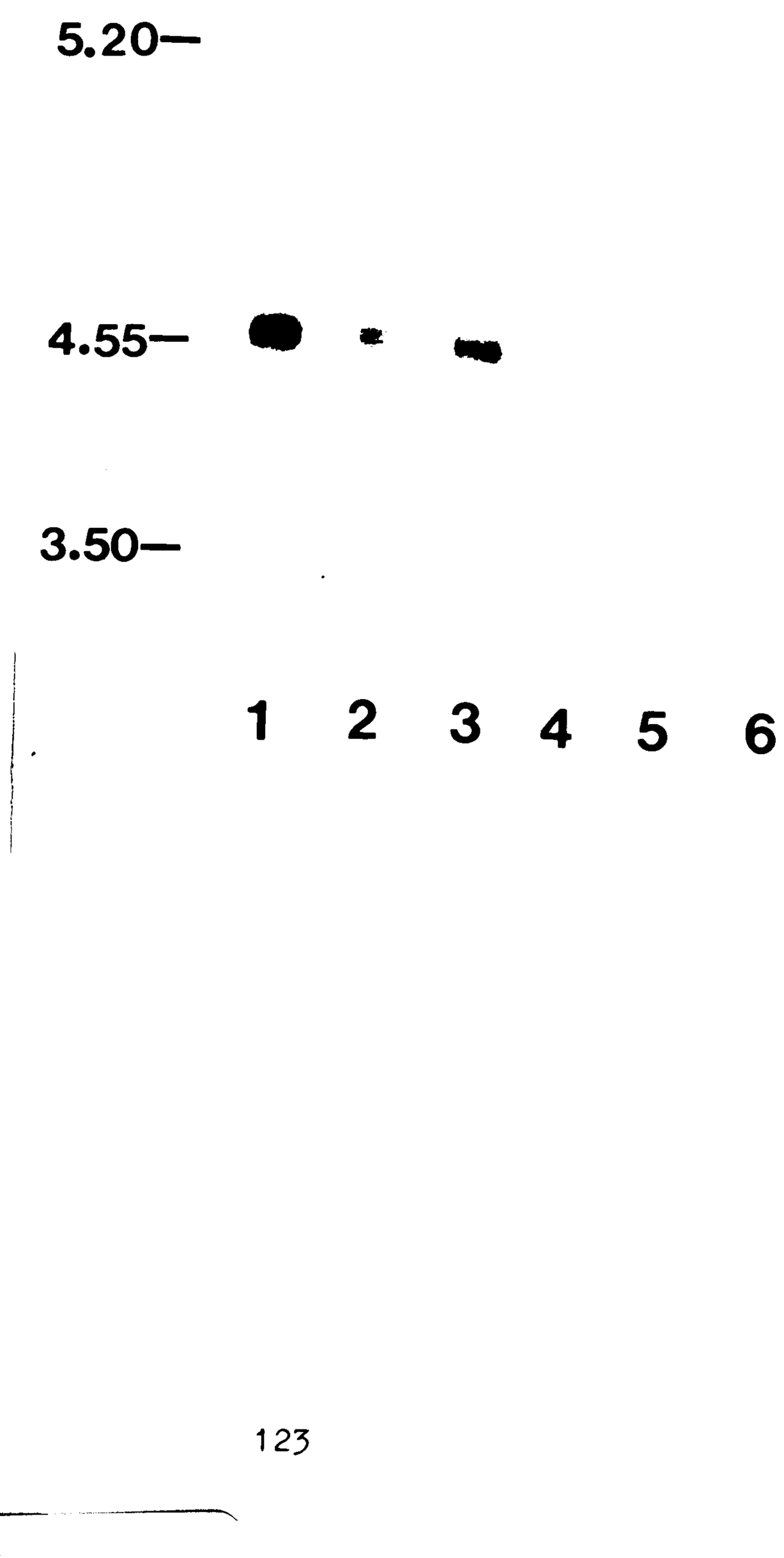


Fig. 22

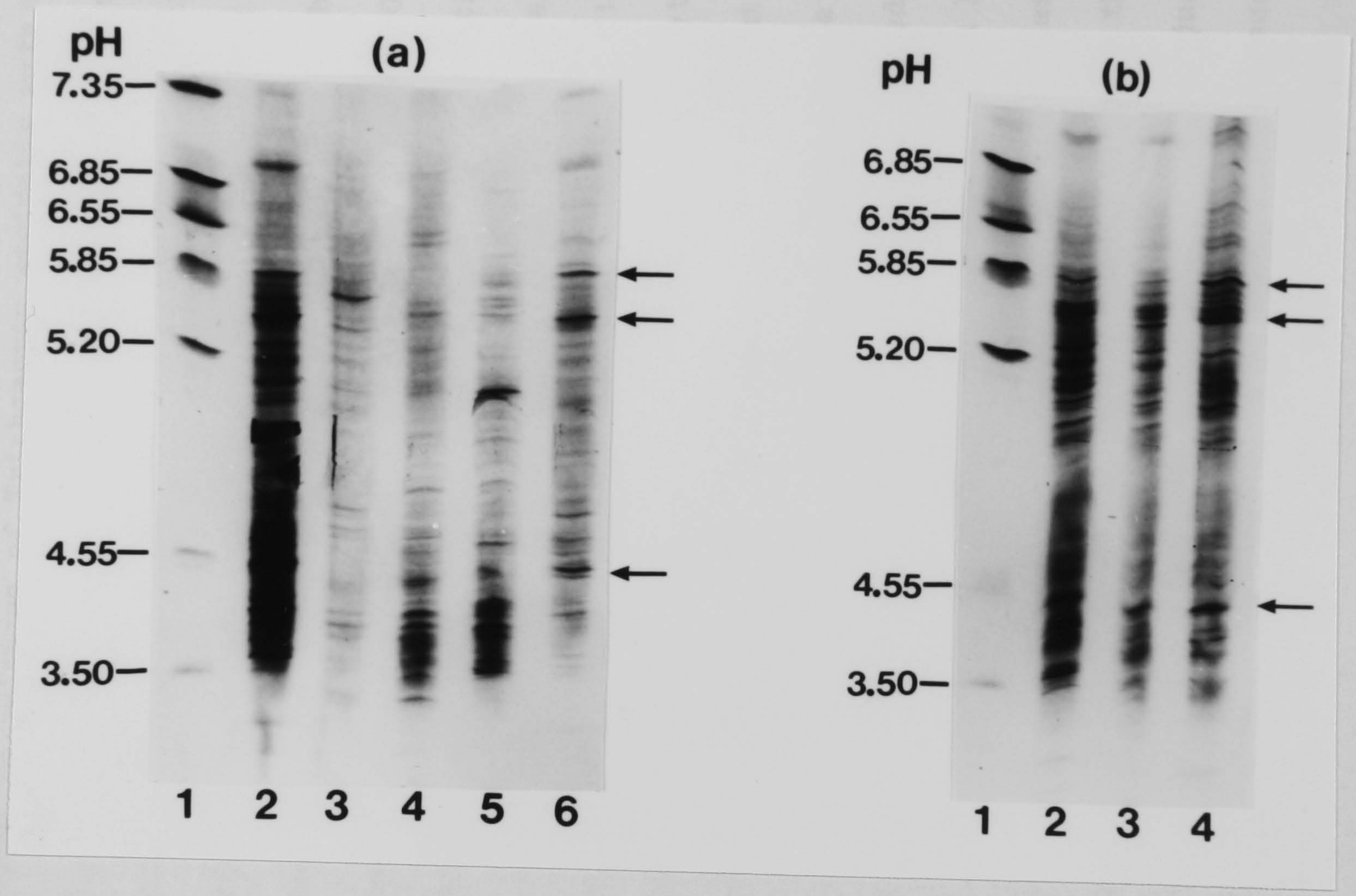

pH
(a)
7.35
6.85
6.55
5.85
5.20
4.55
3.50
1 2 3 4 5 6
pH
(b)
6.85
6.55
5.85
5.20
4.55
3.50
1 2 3 4

Fig. 23

3.3. L-Methionine-catabolising enzyme

L-Methionine catabolism by trichomonads in MDM

Cultures of Trichomonas vaginalis growing in MDM produced volatile thiols (Table 13). Volatile thiol production was not detectable with cultures of Trichomitus batrachorum or Tritrichomonas foetus. None of the three trichomonad cultures produced detectable levels of hydrogen sulphide, nor was hydrogen sulphide production detected using similar cultures of Trichomonas vaginalis with added DL-homocysteine (8 mM) or L-cysteine (16 mM) (limit of detection approximately 0.5 nmol/min/mg protein). Traces of hydrogen sulphide were produced during prolonged incubation of T. vaginalis in MDM with 30 mM DL-homocysteine, but at this concentration cell growth was markedly affected (see Table 21b). Addition of L-methionine (10 mM) to T. vaginalis cultures, however, increased volatile thiol production by 1.7 ± 0.5 (n = 4)-fold, without affecting parasite growth. Interestingly, T. vaginalis grown in the presence of 10^{-4}M or 10^{-5}M DL-propargylglycine for 24 hr (which inhibited growth by about 13% and 22%, respectively) and resuspended in MDM without the inhibitor were found not to produce detectable levels of volatile thiols. An hour preincubation with 10^{-4}M DL-propargylglycine reduced volatile thiol production by 72 ± 2% (n = 2). Similarly, an hour preincubation of the parasite with 3.3×10^{-3}M metronidazole inhibited volatile thiol production by 81 ± 13% (n = 3). Under these conditions, metronidazole was found to kill the parasites (as judged by lack of motility by the end of the preincubation) without causing lysis.

L-Methionine catabolism by trichomonads in PBS

Living T. vaginalis in PBS with 15 mM L-methionine produced volatile thiols (Table 13 and Fig. 24a), α-ketoacid (Fig. 24b) and

ammonia (Fig. 24c). Analysis of the gas phase from these T. vaginalis cultures by mass spectrometry revealed the presence of methanethiol, although this was not quantified. Incubation mixtures containing Tritrichomonas foetus and Trichomitus batrachorum did not produce methanethiol from L-methionine. The production by Trichomonas vaginalis of ammonia, unlike volatile thiol or α-keto acid, occurred in the absence of added L-methionine (Fig. 24). Indeed the addition of L-methionine apparently reduced the rate of ammonia production, although this was not significant ($P > 0.1$). Volatile thiols were released at higher rates than α-ketoacid or ammonia in the presence of exogenous L-methionine. Living Pentatrichomonas hominis, Trichomitus batrachorum and Tritrichomonas foetus did not generate volatile thiols from L-methionine (Table 13). Under the conditions of assay, living Trichomonas vaginalis Trichomitus batrachorum and Tritrichomonas foetus failed to produce hydrogen sulphide from DL-homocysteine (3.3 mM) or L-cysteine (3.3 mM) (limit of detection approximately 0.5 nmol/min.mg protein). The rate of volatile thiol produced was directly proportional to the number of T. vaginalis present, up to 2×10^7 cells/1.5 ml assay volume. Thiol production occurred optimally at pH 7.5 and the K_m for L-methionine was 0.4 ± 0.2 mM ($n = 6$). The parasites catabolised a wide range of substrates, including L-methionine amide and L-ethionine (Table 14). The production of volatile thiols from L-methionine by T. vaginalis was inhibited by various compounds (Table 15). The addition of glucose (up to 333 mM), maltose (up to 187 mM), sodium azide (up to 20 mM), KCl (up to 53 mM), DTNB (up to 0.8 mM) or lactate (up to 73 mM) to the reaction mixture containing living cells of T. vaginalis in PBS with 15 mM L-methionine had no effect on the rate of volatile thiol production. Lactate at 147 mM and 293 mM inhibited the production of volatile thiols by $28 \pm 8\%$ ($n = 4$) and 34

± 14% (n = 4), respectively. At these concentrations, the parasites were found to be immotile but not lysed by the end of the incubation.

L-Methionine catabolism by cell-free extracts of trichomonads

Cell-free extracts of four cell lines of T. vaginalis produced volatile thiols from L-methionine at similar rates (Table 13). Trichomonas vaginalis (clone G3) has been cultivated in vitro for several years and was found to be sensitive to metronidazole under aerobic conditions (MLC, 1 μg/ml). Trichomonas vaginalis Boston, T. vaginalis Fall River and T. vaginalis IR78 were resistant to metronidazole under aerobic conditions when isolated (Thurner and Meingassner 1978; Muller et al. 1980) under our conditions of determination they had MLC values (μg/ml) of 1-2, 50 and 50, respectively. Cell-free extracts of Pentatrichomonas hominis, Trichomitus batrachorum, Tritrichomonas foetus, Leishmania mexicana mexicana (amastigotes and promastigotes), L. terentolae, Crithidia fasciculata, Herpetomonas muscarum muscarum, H. m. ingenoplastis and mouse liver failed to produce detectable levels of thiols from L-methionine (limit of detection approximately 0.7 nmol/min/mg protein). Cell-free extracts of Trichomitus batrachorum, Tritrichomonas foetus and Trichomonas vaginalis did not catabolise L-methionine to hydrogen sulphide (limit of detection approximately 1 nmol/min/mg protein). The enzyme from T. vaginalis catabolised L-methionine to approximately equimolar concentrations of α-ketoacid and volatile thiols; ammonia production, however, was not detectable (Table 16). Experiments were performed to find out if ammonia was consumed by cell-free extracts of T. vaginalis; consumption was not detectable. The parasite L-methionine-catabolizing enzyme was found to be soluble with 90% of the activity recovered in the supernatant fraction. An apparent K_m (L-methionine) of 3.8 ± 1.9 mM (n = 6)

(Fig. 25) and a pH optimum of 7.0 (Fig. 26) were obtained. The rate of volatile thiol production was linear with protein concentration up to approximately 800 μg. Of the range of substrates investigated, the T. vaginalis enzyme catabolised only L-methionine, L-ethionine and S-methyl-L-cysteine to volatile thiols at appreciable rates (Table 14). Notably, α-keto-γ-methiolbutyrate, the first intermediate in the catabolic pathway operating in mammals, was catabolised to thiols only at a very low rate. Addition of pyridoxal-5'-phosphate (up to 0.5 mM) to the assay mixture had no effect on the catabolism of L-methionine. 2-Mercaptoethanol (at 4.2 and 8.4 mM), however, was found to inhibit the production of α-ketoacid (by 54 $\pm$ 4% and 73 $\pm$ 1% (n = 3), respectively) but lead to enhanced rates of volatile thiol excretion (to 180 $\pm$ 66% and 156 $\pm$ 32% (n = 3), respectively). Thiol production catalysed by cell-free extracts of T. vaginalis was inhibited appreciably by DL-propargylglycine, L-cycloserine, hydroxylamine, hexachlorophene and bithionol, whereas very high concentrations of metronidazole had little effect (Table 15).

Effects of inhibitors on the growth in vitro and enzyme content of trichomonads.

It was observed that T. vaginalis (clone G3) grown in the presence of 10^{-5}M DL-propargylglycine for 24 hr had no detectable L-methionine-catabolising enzyme activity. Most interestingly, they also lacked homocysteine desulphurase activity but possessed elevated (3.5 $\pm$ 1.3 (n = 8)-fold) serine sulphydrase activity (see Table 12). These cells were no more or less sensitive to metronidazole than the parent line. The other lines of T. vaginalis (Boston, Fall River and IR78) grown in the presence of 10^{-5}M DL-propargylglycine for 24 hr also had no detectable L-methionine-catabolising enzyme and

homocysteine desulphurase activities. Unlike clone G3, however, the L-serine sulphydrase activities of these lines were similar to those of untreated cells. Similarly, Tritrichomonas foetus grown in the presence of 10^{-5}M DL-propargylglycine for 24 hr possessed normal serine sulphydrase activity. At this concentration, DL-propargylglycine had little effect on the growth in vitro of any of these trichomonad lines. Sublethal concentrations of bithionol (10^{-5} M), hexachlorophene (10^{-5}M) and dichlorophene (10^{-5}M) tested in a similar way had no effect on any of the three enzyme activities of Trichomonas vaginalis (clone G3). At higher concentrations, these three compounds were trichomonacidal (Table 11a).

Table 13 The production of volatile thiols by trichomonads.

Organism	Activity (nmol/min/mg protein)		
	Whole cells[a]	Whole cells[b]	Cell-free extracts
Trichomonas vaginalis			
Clone G3	0.7 ± 0.3 (6)	2.8 ± 0.7 (21)	5.4 ± 1.8 (25)
Fall River	-	-	4.9 ± 0.2 (4)
Boston	-	-	4.5 ± 0.7 (4)
IR78	-	-	3.3 ± 2.3 (4)
Pentatrichomonas hominis	-	n.d. < 0.7 (4)	n.d. < 0.7 (6)
Trichomitus batrachorum	n.d. < 0.1 (3)	n.d. < 0.2 (3)	n.d. < 0.5 (3)
Tritrichomonas foetus	n.d. < 0.1 (3)	n.d. < 0.2 (3)	n.d. < 0.3 (3)

means ± standard deviation, n in parentheses.

n.d., not detectable.

-, not done.

[a], trichomonads in complex medium (MDM).

[b], trichomonads in PBS with 15 mM L-methionine.

Table 14 Rate of thiol production by Trichomonas vaginalis from various substrates.

Substrate	% Activity	
	Cell free extract[a]	Whole Cell[b]
L-methionine	100	100
L-methionine amide	2 ± 3	168 ± 29
L-ethionine	101 ± 32	118 ± 30
L-alanyl-L-methionine	-	87 ± 13
S-methyl-L-cysteine	75 ± 15	83 ± 7
L-methionyl-L-alanine	-	75 ± 1
L-methionine sulphoxide	0	43 ± 13
N-formyl-L-methionine	0	4 ± 3
D-methionine	0	1 ± 1
S-methylthioglycerol	1 ± 2	0
S-adenosylmethionine	0	0
methylthioadenosine	0	-
α-keto-γ-methiolbutyrate	5 ± 3	-

means ± standard deviation from three or more experiments.

-, not done.

[a], assayed as described in section 2.7.10. using 10 mM substrate.

[b], trichomonads in PBS with 15 mM substrate.

Table 15 The effects of inhibitors on volatile thiol production from living *T. vaginalis* and cell-free extracts.

	Volatile thiol production	
	living trichomonads[a]	cell-free extracts
Inhibitor	I_{50}(M)	I_{50}(M)
DL-propargylglycine	$(4.5 \pm 2.5)10^{-5}$ (2)	$(2.3 \pm 1.7)10^{-6}$ (3)
hydroxylamine	$(1.1 \pm 0.3)10^{-4}$ (2)	$(5.8 \pm 2.2)10^{-5}$ (2)
L-cycloserine	$(2.6 \pm 2.4)10^{-4}$ (2)	$(3.3 \pm 0.8)10^{-5}$ (2)
bithionol	-	$(3.4 \pm 1.6)10^{-4}$ (3)
hexachlorophene	-	$(1.1 \pm 0.9)10^{-4}$ (2)
metronidazole	3.10^{-3} (1)	b(3)

means ± range or standard deviation, n in parentheses.

a, trichomonads in PBS with 15 mM L-methionine.

b, 23 ± 14% inhibition of activity at 7×10^{-3}M.

-, not done.

Table 16 Products of L-methionine catabolism by cell-free extracts of *Trichomonas vaginalis*.

Product	Rate of production (nmol/min/mg protein) Clone G3	BOSTON	FALL RIVER
Volatile thiol	5.4 ± 1.8 (25)	4.5 ± 0.7 (4)	4.9 ± 0.2 (4)
α-Ketoacid	4.0 ± 1.1 (15)	5.9 ± 2.0 (2)	4.6 ± 0.1 (2)
Ammonia	n.d. < 1.0 (10)	n.d. < 1.0 (2)	n.d. < 1.0 (2)

means ± range or standard deviation, n in parentheses.

n.d., not detectable.

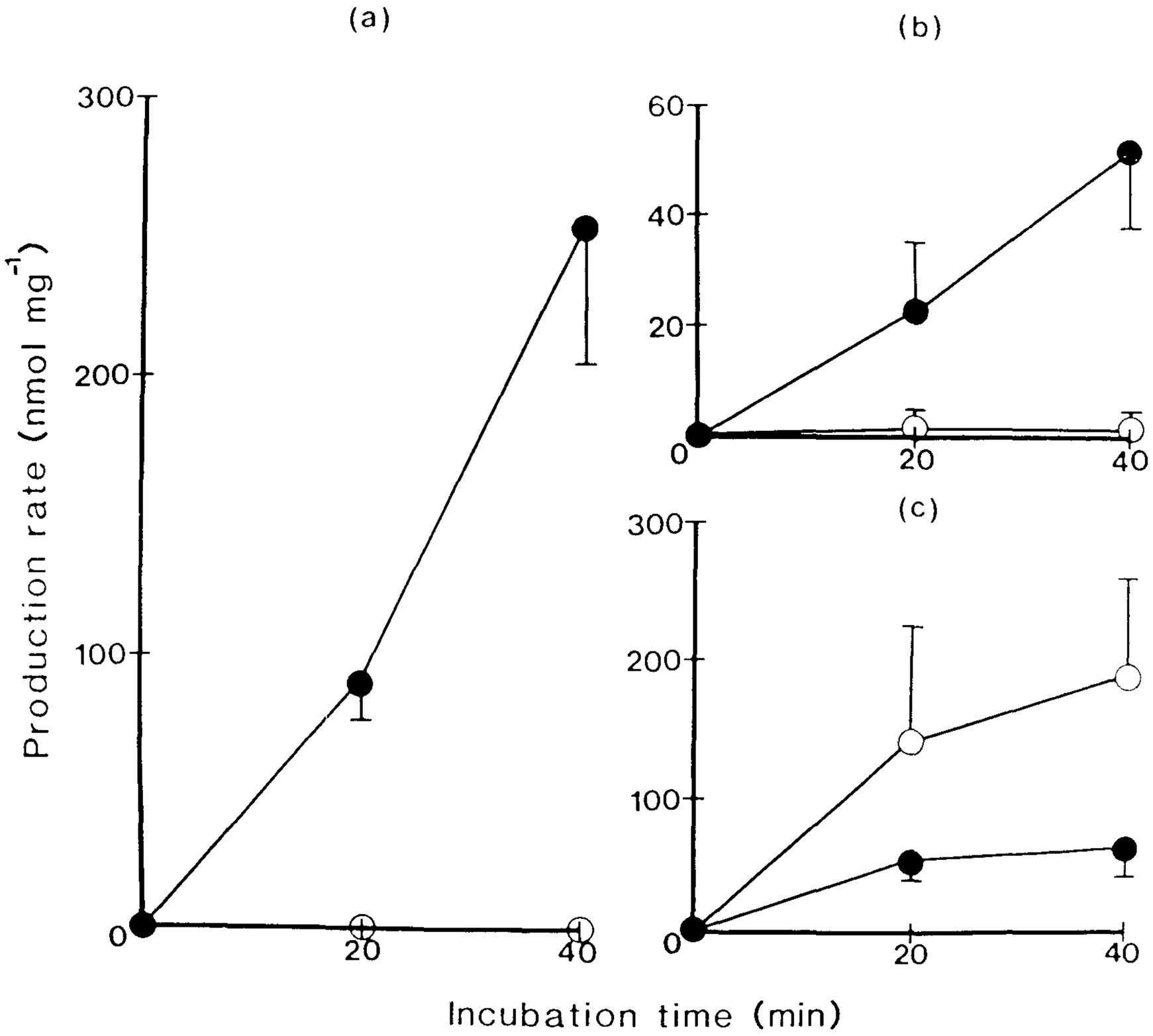


Fig. 24 Rates of production of volatile thiol (a), α-ketoacid (b) and ammonia (c) by Trichomonas vaginalis in PBS with (●) and without (○) 15mM L-methionine. The data presented are the means (± range) from two duplicated experiments.

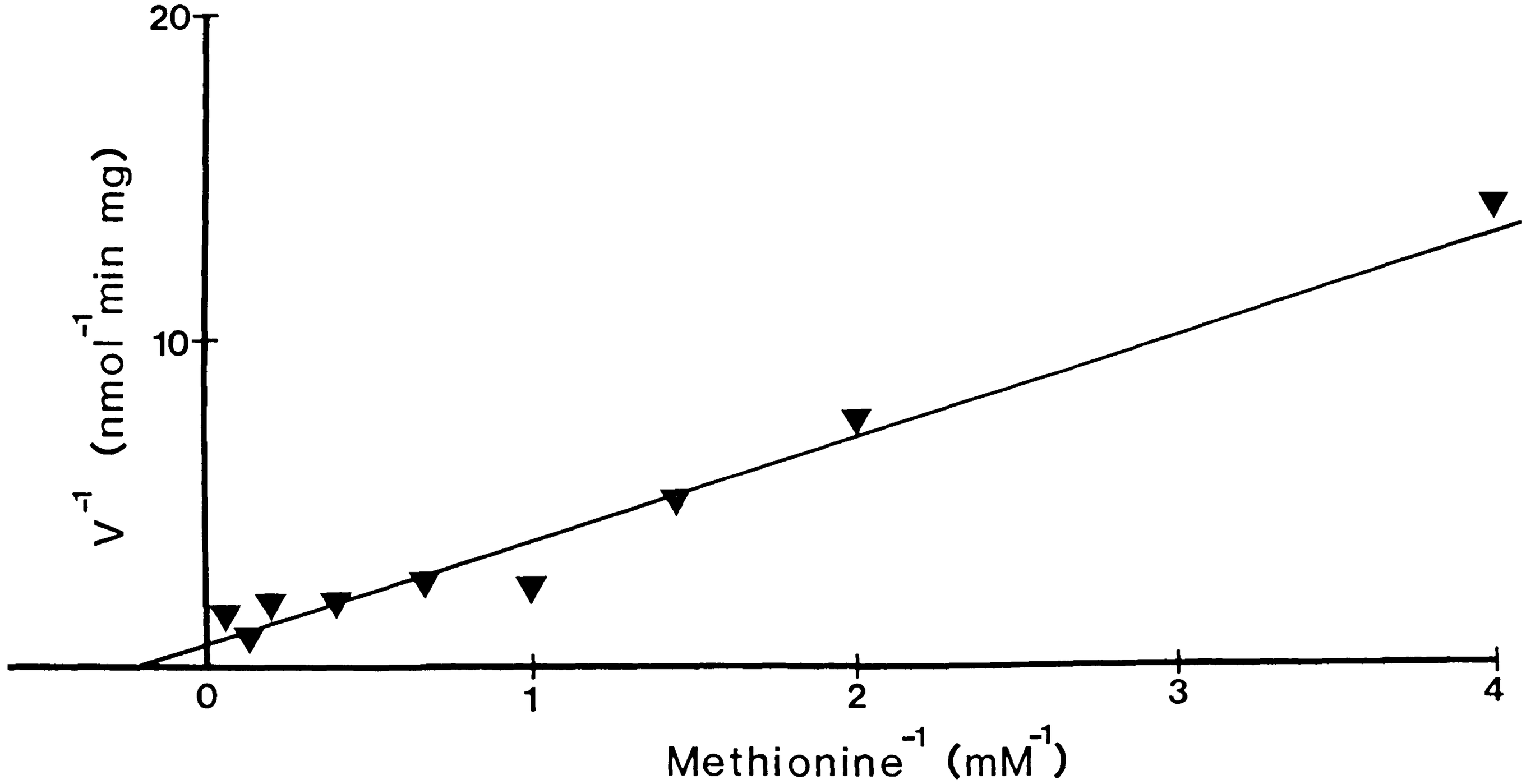


Fig. 25 A Lineweaver-Burk plot of the activity of Trichomonas vaginalis L-methionine-catabolising enzyme at a range of methionine concentrations. The data are from one experiment which is representative of the six carried out. The enzyme was assayed according to the procedure described in section 2.7.10..

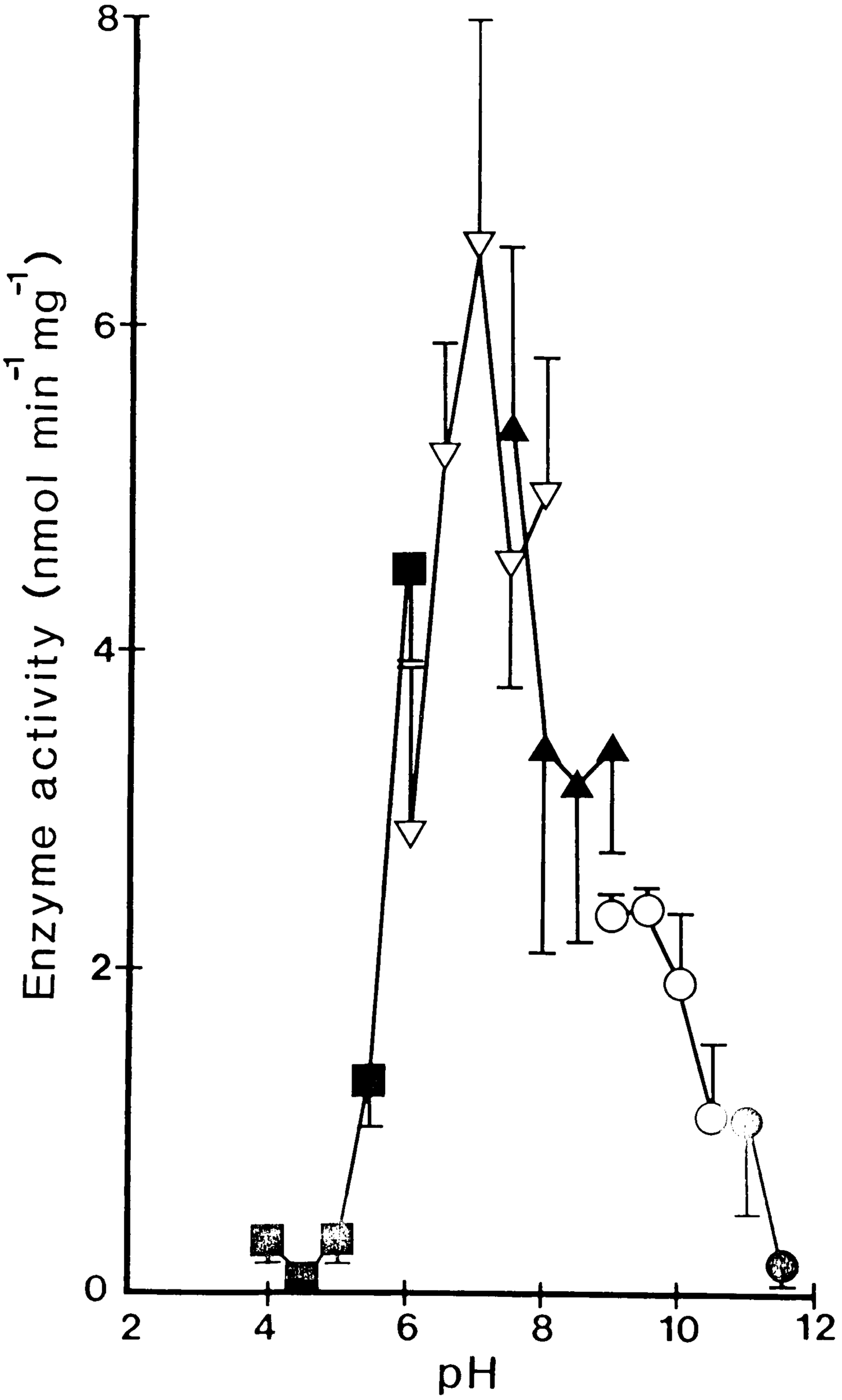


Fig. 26 The pH profile of *Trichomonas vaginalis* L-methionine-catabolising enzyme. The enzyme was assayed under standard conditions as indicated in section 2.7.10.. The buffers (100 mM) were: succinic acid/NaOH (pH 4.0-6.0) (■); sodium phosphate (pH 6.0-8.0) (▽); Tris/HCl (pH 7.5-8.9) (▲); glycine/NaOH (8.9-10.5) (○); sodium phosphate (pH 11.0-11.5) (●). The data presented are the means (± standard deviation) from three experiments.

3.4. SAM synthetase, SAM and SAM-dependent transmethylation reactions

SAM synthetase activity was detected in mouse liver homogenates (0.4 ± 0.2 (n = 9) nmol/min/mg protein) but, when assayed under identical conditions was undetectable in lysates of Trichomonas vaginalis, Trichomitus batrachorum and Tritrichomonas foetus (limit of detection approximately 0.1 nmol/min/mg protein). SAM synthetase activity remained undetectable in Trichomonas vaginalis extracts even with the following modifications to the standard assay system: SAM (0.1 mM); DL-propargylglycine (1 mM); hydroxylamine (1 mM); sinefungin (0.2 mM); homocysteine (10 mM); cysteine (10 mM); dimethylsulphoxide (5-10%, w/v); KCl (50 mM); $MgCl_2$ (50 mM); L-methionine (22.5 μM - 4 mM); ATP (5 mM); buffer pH (7.0 - 8.0); KCN (0.1 mM); T. vaginalis extracts (100,000x g supernatant or particulate fractions); incubation time (up to 60 mins).

The concentrations of material that coeluted with SAM during HPLC that were detected in extracts of a range of protozoa and mouse liver homogenates are given in Table 17. All protozoa contained similar levels except for Herpetomonas muscarum ingenoplastis which possessed about 10-fold more. The apparent SAM concentrations in T. vaginalis incubated in MPBSS declined rapidly (Fig. 27); this loss could be partly alleviated by addition of 10 mM L-methionine to the suspension buffer. Similarly, T. vaginalis resuspended in MPBSS supplemented with 15 mM L-methionine and incubated at 37°C for 40 min was found to contain 3.5 ± 0.9 (n = 3)-fold more SAM than parasites incubated without L-methionine.

The rates of incorporation of exogenous L-methionine into components of T. vaginalis and Tritrichomonas foetus are given in Table 18. The incorporation rates obtained for Trichomitus batrachorum were very variable and inconclusive, and were not included in the Table. Trichomonas vaginalis consumed methionine at

a much higher rate than Tritrichomonas foetus and there was appreciable incorporation of the methyl carbon into the lipid and nucleic acid fractions as well as SAM and proteins. It was found that the use of higher methionine concentrations in the experiments resulted in much greater methionine consumption and incorporation. With T. foetus, however, there was a very low rate of incorporation into nucleic acid, lipid and SAM. The incorporation rates were so low with this parasite that it was not practicable to test higher methionine concentrations involving unlabelled amino acid. The rate of incorporation into each of the components of Trichomonas vaginalis was found to be linear with time, up to 40 min. In addition, the total radioactivity consumed (incorporated and catabolised) by this parasite was inhibited by cycloleucine (15 mM) and norleucine (15 mM) by 52 $\pm$ 8% (n = 3) and 72 $\pm$ 13% (n = 2), respectively. The rates of incorporation of ^{35}S-labelled L-methionine into T. vaginalis components are also given in Table 18. As expected, there was little or no incorporation of the ^{35}S-label into the lipid or nucleic acid fractions. [U - ^{14}C]-L-Leucine and [^{14}C-methyl-]-thymidine were also used to assess the validity of the extraction procedures. The results presented in Table 19 show that most of the radioactivity from these precursors incorporated into macromolecules were recovered in the protein and nucleic acid fractions, respectively. This contrasts with the incorporation of radioactivity from the methyl carbon of methionine into the different macromoleucles of the trichomonads (Table 19).

Table 17 SAM concentrations in trichomonads, a range of trypanosomatids and mouse liver.

Organism	nmole SAM/mg protein
Trichomonas vaginalis	3.8 ± 1.8 (46)
Trichomitus batrachorum	1.5 ± 0.7 (30)
Tritrichomonas foetus	3.2 ± 0.9 (34)
Leishmania mexicana mexicana (promastigotes)	1.3 ± 0.5 (4)
Leishmania mexicana mexicana (amastigotes)	1.2 ± 0.5 (8)
Leishmania tarentolae	1.1 ± 0.9 (6)
Crithidia fasciculata	2.1 ± 0.2 (3)
Herpetomonas muscarum muscarum	2.0 ± 0.3 (3)
Herpetomonas muscarum ingenoplastis	23.5 ± 6.0 (6)
Mouse liver	1.0 ± 0.1 (8)

[a] results are the means (± standard deviation) from the number of determinations given in parentheses.

Table 18 Rates of incorporation of exogenous L-methionine into trichomonad components.

Organism and substrate (concentration)	Total consumed (incorporated and catabolised)	Rate (pmol/hr/mg protein)[a] Incorporation into trichomonad components: intracellular SAM	intracellular methionine	protein	lipid	nucleic acid
Trichomonas vaginalis						
[^{14}C-methyl-]-L-methionine (4 μM)	1,556 ± 312 (4)	6 ± 2 (5)	31 ± 10 (4)	175 ± 6 (4)	40 ± 24 (4)	50 ± 23 (4)
[^{14}C-methyl-]-L-methionine (421 μM)	112,867 ± 21,282 (2)	170 ± 28 (2)	2,223 (1)	1,689 ± 508 (2)	682 ± 11 (2)	576 ± 100 (2)
[^{35}S-]-L-methionine (424 μM)	77,377 ± 18,919 (2)	230 ± 91 (2)	1,434 ± 1,058 (2)	817 ± 496 (2)	20 ± 16 (2)	130 ± 85 (2)
Tritrichomonas foetus						
[^{14}C-methyl-]-L-methionine (4 μM)	140 ± 23 (3)	1 ± 1 (3)	21 ± 20 (3)	26 ± 23 (3)	0.3 ± 0.3 (3)	2 ± 1 (3)

[a] results are the means (± range or standard deviation) from the number of experiments given in parentheses. Incubation conditions were as described in section 2.3..

Table 19 Relative incorporation of radioactivity into trichomonad macromolecules.

Organism and substrate (concentration)	% Incorporation[a] Protein	Lipid	Nucleic acid
Trichomonas vaginalis			
[^{14}C-methyl-]-L-methionine (4 μM)	47 ± 20 (4)	18 ± 8 (4)	18 ± 9 (4)
[U-^{14}C]-L-leucine (0.6 μM)	73 ± 12 (2)	1 ± 1 (2)	6 ± 4 (2)
[^{3}H-methyl-]-thymidine (0.8 μM)	13 ± 1 (2)	0.4 ± 0.1 (2)	109 ± 18 (2)
Trichomitus batrachorum			
[^{14}C-methyl-]-L-methionine (4 μM)	58 ± 5 (3)	9 ± 9 (3)	18 ± 4 (2)
Tritrichomonas foetus			
[^{14}C-methyl-]-L-methionine (4 μM)	101 ± 33 (3)	4 ± 1 (3)	11 ± 7 (3)

[a] results are the means (± range or standard deviation) from the number of experiments given in parentheses and are expressed as the incorporation relative to the total radioactivity recovered in the cold-TCA precipitate. Incubation conditions were as described in section 2.3..

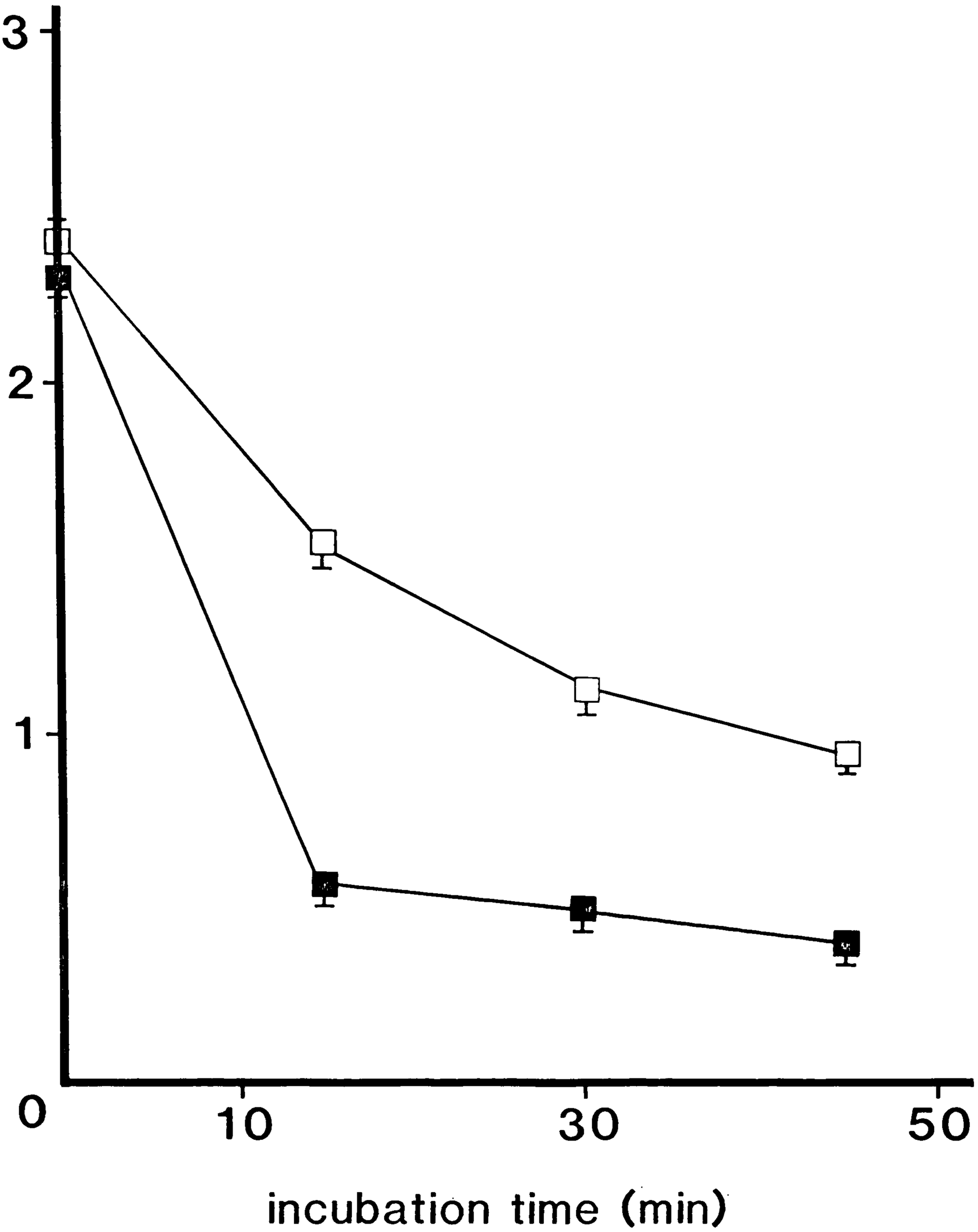


Fig. 27 SAM concentrations in Trichomonas vaginalis resuspended in MPBSS alone (■) or supplemented with 10 mM L-methionine (□) and maintained at 37°C. The conditions of the experiment were as described in section 2.3.1.. Results are the means (± range) from two determinations.

3.5. The antitrichomonal activities of inhibitors of sulphur-containing amino acid metabolism

The inhibitors used have been separated into six groups according to their known effects on enzymes (Table 20). Figures 28 and 29 shows the structures of some of these inhibitors. Their activities against Trichomonas vaginalis in vitro are given in Tables 21a and 21b. Metronidazole was found to be the most potent trichomonacide of those compounds tested. Tubercidin, ara A, sinefungin, 5-azacytidine, bithionol, hexachlorophene and dichlorophene were trichomonacidal at fairly low concentrations, whereas the other inhibitors were either ineffective or had activity only when present at very high concentrations.

I also investigated the effects on T. vaginalis in vitro of combinations of the following compounds: sinefungin (10^{-8}M - 10^{-4}M) and methionine (10 mM, 50 mM); sinefungin (10^{-7}M - 10^{-3}M) and SAM (5 mM); sinefungin (10^{-7}M, 10^{-6}M) and methylglyoxal-bis (guanylhydrazone) (10^{-6}M - 10^{-4}M); sinefungin (10^{-7}, 10^{-6}M) and ara A (10^{-6}M, 10^{-5}M); sinefungin (10^{-7}M, 10^{-6}M) and tubercidin (10^{-6}M, 10^{-5}M); sinefungin (10^{-7}M, 10^{-6}M) and 2'-deoxyadenosine (10^{-5}M, 10^{-4}M); cycloleucine (3 x 10^{-2}M, 10^{-1}M) and methionine (5 mM - 50 mM); cycloleucine (10^{-3}M, 10^{-2}M) and methylglyoxal-bis(guanylhydrazone) (10^{-6}M - 10^{-4}M); tubercidin (10^{-7}M - 10^{-3}M) and methionine (5mM, 50 mM); tubercidin (10^{-6}M, 10^{-5}M)and methylgloxal-bis(guanylhydrazone) (10^{-6}M - 10^{-4}M); tubercidin (10^{-6}M, 10^{-5}M) and 5-azacytidine (10^{-6}M, 10^{-5}M); ara A (3 x 10^{-6}M, 10^{-3}M) and methionine (50 mM); ara A (10^{-5}M) and 5-azacytidine (10^{-6}M, 10^{-5}M); ara A (10^{-6}M, 10^{-5}M) and methylglyoxal-bis(guanylhydrazone) (10^{-6}M - 10^{-4}M); methylglyoxal-bis (guanylhydrazone) (10^{-6}M - 10^{-4}M) and seleno-L-methionine (10^{-5}M, 10^{-4}M); 5-azacytidine (10^{-6}M - 10^{-3}M) and methionine (5mM, 50mM). These combinations exhibited neither synergism nor antagonism to any

extent. Interestingly, however, the activity of monothioglycerol was enhanced when 50 mM methionine was added to the cultures (Fig. 30). At this concentration, methionine itself had no effect on the growth of the parasite *in vitro*.

Substrates and analogues of substrates for several of the enzymes catalysing sulphur-containing amino acid metabolism were also assessed for their effects on the growth of *T. vaginalis in vitro*. These compounds tested (with % inhibition of growth observed and concentration used) were: betaine (20%, 10^{-1}M); choline chloride (55%, 10^{-2}M); N, N-dimethylglycine (100%, 10^{-2}M); L-cysteine sulphinic acid (8%, 10^{-2}M): L-cysteine (10%, 10^{-2}M); S-methyl-L-cysteine (10%, 10^{-2}M); L-methionine (0%, 5 x 10^{-2}M); methionine sulphoximine (38%, 10^{-3}M); norleucine (12%, 10^{-3}M). As can be seen, none were potent trichomonacides. Homocysteine at high concentrations was trichomonacidal (Table 21b); these cultures produced traces of H_2S.

The frog intestinal protozoan *Trichomitus batrachorum* was found to be less sensitive to metronidazole (MLC, 10^{-4}M; LD_{50}, 7.9 x 10^{-7}M) than *Trichomonas vaginalis* (MLC, 10^{-5}M; LD_{50}, 4.1 x 10^{-7}M) and the bovine parasitic protozoan *Tritrichomonas foetus* (MLC, 10^{-5}M; LD_{50}, 1.3 x 10^{-7}M). The sensitivity of these trichomonads to chloramphenicol and DL-propargylglycine was also investigated using microtitre plates. Under anaerobic conditions, *Trichomonas vaginalis* was more sensitive to chloramphenicol (MLC, 3.4 x 10^{-3}M) than *Tritrichomonas foetus* (MLC, 6.8 x 10^{-3}M) and *Trichomitus batrachorum* (MLC, 8.7 x 10^{-3}M). The three species, however, were equally insensitive to DL-propargylglycine (10^{-6}M - 10^{-2}M) under aerobic and anaerobic conditions, with even the highest concentration inhibiting growth by about 20% only.

Table 20 The groups of inhibitors used.

Group	Enzyme(s) Inhibited	Reference
A	SAH hydrolase (EC 3.3.1.1)	Hershfield et al. 1979 Helland and Ueland 1982
B	Pyridoxal phosphate-dependent enzymes such as γ-cystathionase (EC 4.2.1.2), methionine-γ-lyase (EC 4.4.1.11), homocysteine desulphurase (EC 4.4.1.2), serine sulphydrase (EC 4.2.1.22) and cystathionine-β-synthetase (EC 4.2.1.22)	Alexander and Greenberg 1955 Brown et al. 1969 Braunstein et al. 1971 Abeles and Walsh 1973 Kodama et al. 1982 Thong 1986 (this project)
C	Betaine:homocysteine methyltransferase (EC 2.1.1.5)	Awad Jr. et al. 1983
D	SAM synthetase (EC 2.5.1.6)	Coulter et al. 1974a, 1974b, 1974c Hoffman 1977 Sufrin 1979 Raxworthy et al. 1983
E	SAM decarboxylase (EC 4.1.1.50)	Pegg and Jacobs 1983
F	SAM-dependent methyltransferases (EC 2.1.1)	Robert-Gero et al. 1980 Groudine et al. 1981 Houston et al. 1985a, 1985b, 1985c
G	Not known	-

Table 21a Activities of enzyme inhibitors on the growth *in vitro* of *Trichomonas vaginalis*[a].

Inhibitor	Group[b]	LD_{50} (M)[c]	MLC (M)[c]	number of experiments
ara A	A	$(4.4 \pm 1.9)\ 10^{-6}$	$92 \pm 10\%,\ 10^{-3}$	3
tubercidin	A	$(1.9 \pm 0.4)\ 10^{-6}$	3.10^{-5}	4
2'-deoxyadenosine	A	$37 \pm 9\%,\ 10^{-3}$	-	3
L-cycloserine	B	$(3.9 \pm 0.6)\ 10^{-4}$	10^{-3}	2
hydroxylamine	B	$(2.8 \pm 0.8)\ 10^{-3}$	10^{-2}	2
penicillamine	B	$37 \pm 12\%,\ 10^{-2}$	-	2
4'-deoxypyridoxine	B	$20 \pm 7\%,\ 10^{-2}$	-	2
DL-propargylglycine	B	$23 \pm 2\%,\ 10^{-2}$	-	2
bithionol	B	$(8.1 \pm 2.0)\ 10^{-5}$	3.10^{-4}	3
hexachlorophene	B	$(1.9 \pm 1.2)\ 10^{-5}$	3.10^{-4}	3
dichlorophene	B	$(8.7 \pm 2.4)\ 10^{-5}$	3.10^{-4}	2
3,3-dimethylbutyric acid	C	$(3.9 \pm 1.0)\ 10^{-3}$	3.10^{-2}	3
isovaleric acid	C	$(5.8 \pm 1.8)\ 10^{-3}$	3.10^{-2}	3

[a] results are the means (± range or standard deviation).

[b] see Table 20 for information about the enzyme targets of these inhibitors.

[c] for some compounds, the % inhibition at the highest concentration used is given.

Table 21b Activities of enzyme inhibitors on the growth *in vitro* of *Trichomonas vaginalis*[a].

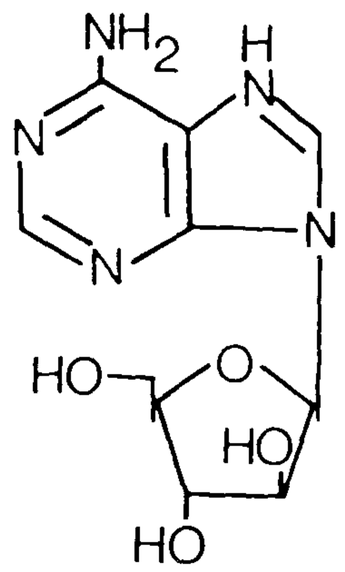
Inhibitor	Group[b]	LD_{50} (M)[c]	MLC (M)[c]	number of experiments
cycloleucine	D	$(2.2 \pm 0.3)\ 10^{-2}$	$88 \pm 2\%,\ 10^{-1}$	3
3-deazauracil	D	$4 \pm 4\%,\ 10^{-2}$	-	2
seleno-L-methionine	D	$(1.2 \pm 0.2)\ 10^{-4}$	$-\ 10^{-3}$	2
methylglyoxal-bis(guanylhydrazone)	E	$4.\ 10^{-4}$	3.10^{-3}	3
sinefungin	F	$(1.6 \pm 0.5)\ 10^{-6}$	3.10^{-2}	3
SAH	F	$8 \pm 8\%,\ 10^{-3}$	-	2
5-azacytidine	F	$(5.4 \pm 2.2)\ 10^{-6}$	$98 \pm 3\%,\ 10^{-3}$	4
monothioglycerol	G	$(3.9 \pm 0.1)\ 10^{-2}$	8.10^{-2}	3
S-methylthioglycerol	G	$(7.1 \pm 0.8)\ 10^{-2}$	$71 \pm 2\%,\ 10^{-1}$	2
metronidazole	G	$(4.1 \pm 0.7)\ 10^{-7}$	10^{-5}	5
homocysteine	G	$(3.0 \pm 1.6)\ 10^{-2}$	$68 \pm 11\%, 5.10^{-2}$	4

[a] results are the means (± range or standard deviation).

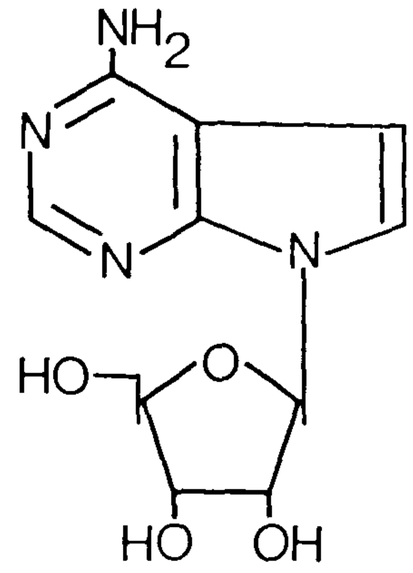
[b] see Table 20 for information about the enzyme targets of these inhibitors.

[c] for some compounds, the % inhibition at the highest concentration used is given.

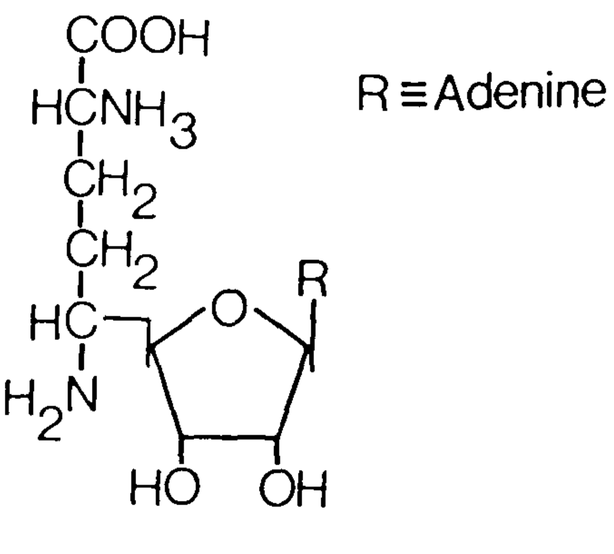
Ara A



Tubercidin

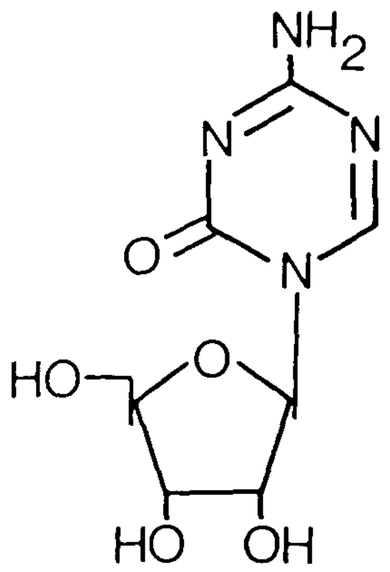


Sinefungin



R ≡ Adenine

5-Azacytidine



Metronidazole

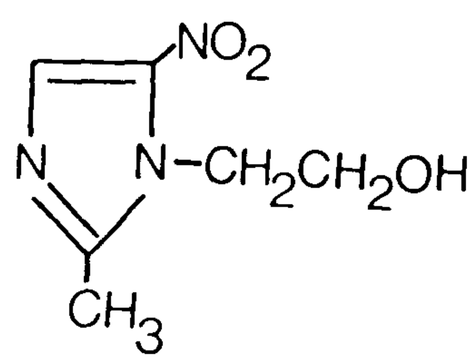
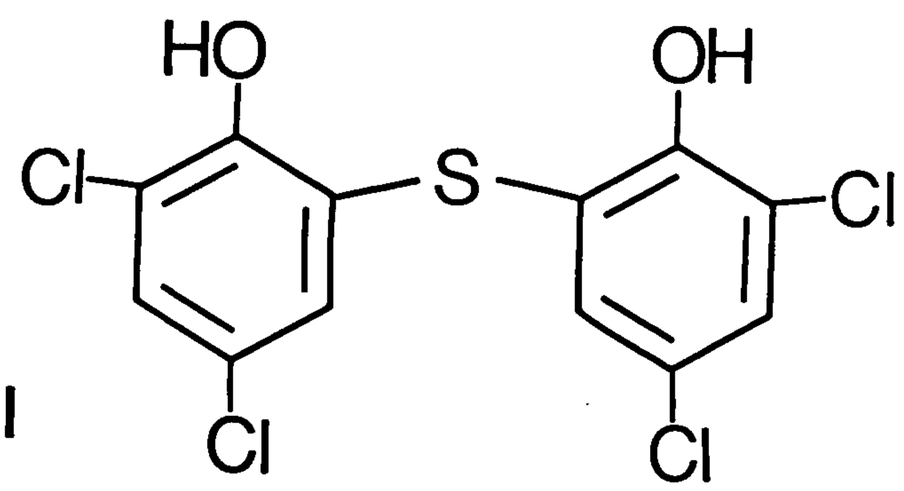
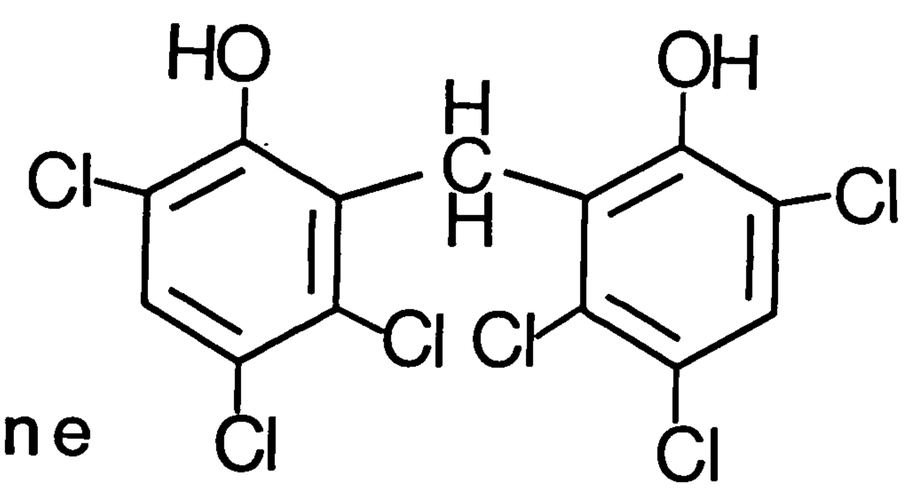


Fig. 28 Structures of inhibitors.

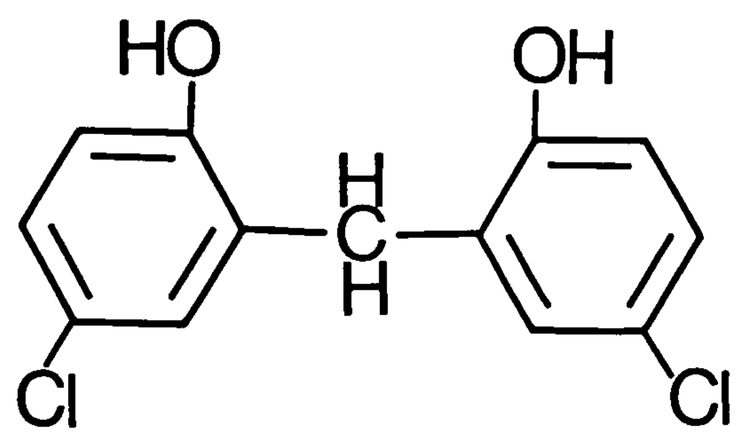
Bithionol



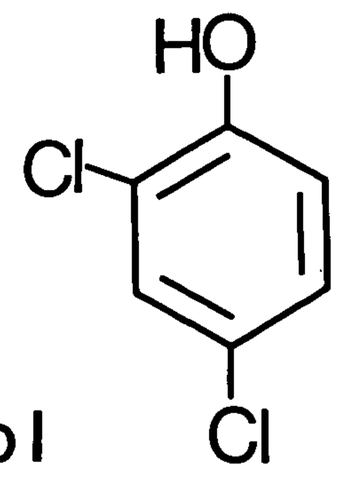
Hexachlorophene



Dichlorophene



2,4-Dichlorophenol



2,2′-Thiodiethanol

$HOCH_2CH_2SCH_2CH_2OH$

Fig. 29 Structures of inhibitors.

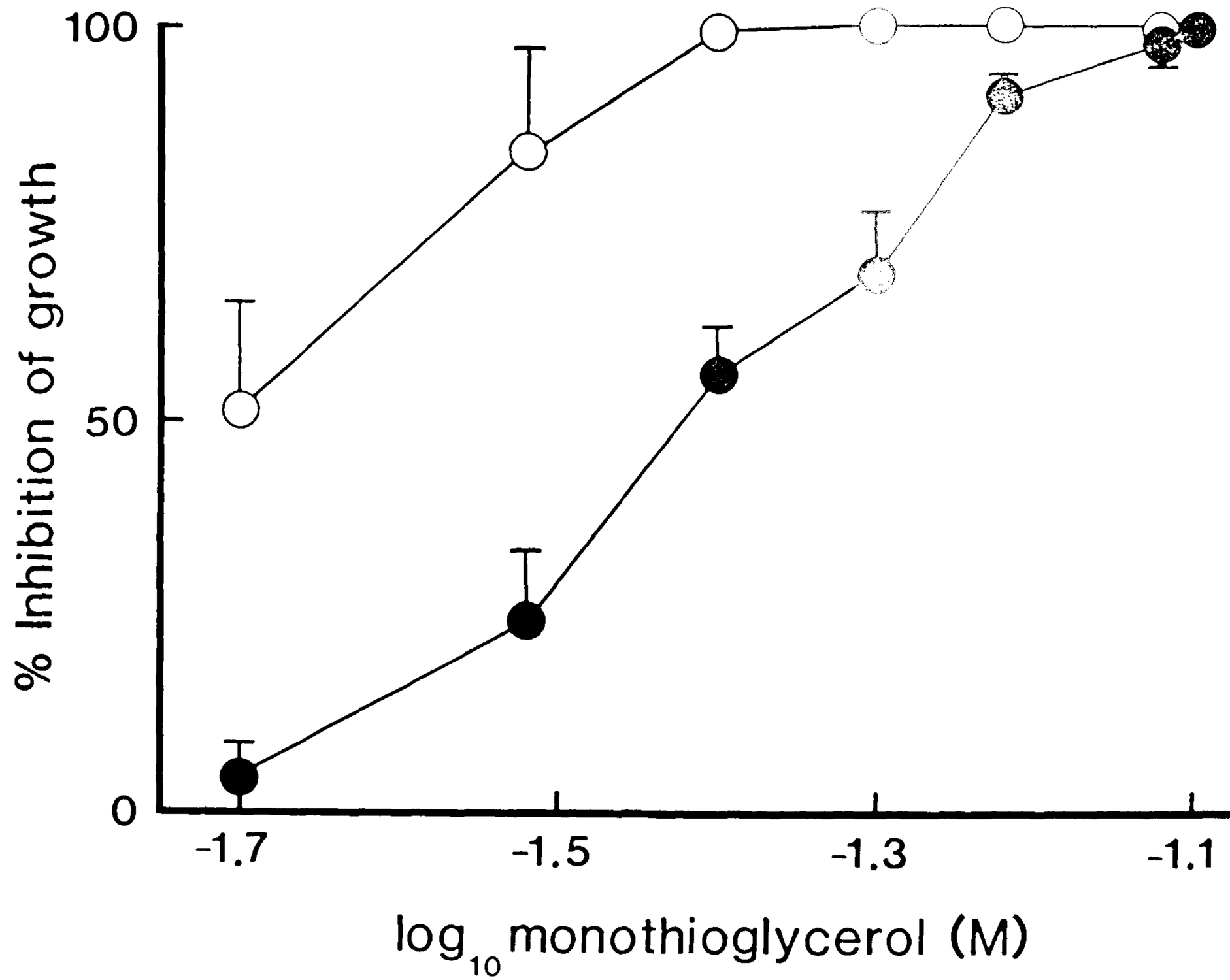


Fig. 30 The effects of monothioglycerol (●) and a combination of monothioglycerol and 50 mM L-methionine (○) on the growth of Trichomonas vaginalis in vitro. Results are the means (± standard deviation) from three experiments. Methionine by itself had no effect on growth.

3.6. The activities of ferredoxin-linked and oxygen-metabolising enzymes.

The activities of NADH oxidase and NADPH oxidase detected in trichomonads are given in Table 22. The three trichomonads possessed similar levels of NADPH oxidase but markedly different activities of NADH oxidase. Using methyl viologen as a substitute for ferredoxin, the natural electron acceptor in the reactions, pyruvate: methyl viologen oxidoreductase and hydrogenase activities in the three trichomonads were found to be similar, whereas the activities of NADPH: methyl viologen oxidoreductase and especially NADH: methyl viologen oxidoreductase differed considerably (Table 23). Trichomitus batrachorum contained the highest activities of the latter two enzymes, with Tritrichomonas foetus possessing the lowest. Interestingly, the apparent pyruvate: methyl viologen oxidoreductase activities of Trichomonas vaginalis and Trichomitus batrachorum were antagonised by NAD^+, whereas that of Tritrichomonas foetus was affected little if at all (Fig. 31). The effects of metronidazole and NADH on the apparent pyruvate: methyl viologen oxidoreductase activity of Trichomonas vaginalis are also shown in Fig. 31. $NADP^+$ (up to 4 mM) tested in the same way had no detectable affect on the apparent pyruvate: methyl viologen oxidoreductase activity of T. vaginalis. Similarly, neither GSSG (up to 5 mM) nor GSH (up to 5 mM) had any effect. On the other hand, these compounds added just before the start of the enzyme assays were found to reduce the apparent T. vaginalis pyruvate: methyl viologen oxidoreductase activity by the following extents (concentration used, % inhibition of enzyme activity): NAD^+ (2 mM, 92%); NADH (2 mM, 99%); $NADP^+$ (2 mM, 34%): NADPH (2 mM, 9%); GSSG (2 mM, 62%); GSH (2 mM, 51%); metronidazole (0.25 mM, 100%).

Table 22 Activities of NADH oxidase and NADPH oxidase in trichomonads.

Organism	Enzyme activity (nmol/min/mg protein)[a]	
	NADH oxidase	NADPH oxidase
Trichomonas vaginalis	794 ± 205 (25)	32 ± 24 (12)
Tritrichomonas foetus	260 ± 16 (9)	53 ± 32 (7)
Trichomitus batrachorum	108 ± 44 (6)	25 ± 13 (7)

[a] results are the means (± standard deviation) from the number of experiments given in parentheses.

Table 23 Activities of ferredoxin-linked enzymes in trichomonads.

Enzyme	Enzyme activity (nmol/min/mg protein)[a]		
	Trichomonas vaginalis	Tritrichomonas foetus	Trichomitus batrachorum
Pyruvate: methyl viologen oxidoreductase	574 ± 141 (10)	647 ± 61 (11)	643 ± 144 (11)
Hydrogenase	1314 ± 3 (3)	-	1492 ± 251 (3)
NADH: methyl viologen oxidoreductase	555 ± 215 (6)	57 ± 27 (4)	1629 ± 332 (5)
NADPH: methyl viologen oxidoreductase	47 ± 23 (2)	28 ± 6 (2)	81 ± 18 (3)

[a] results are the means (± range or standard deviation) from the number of experiments given in parentheses.

-, not done.

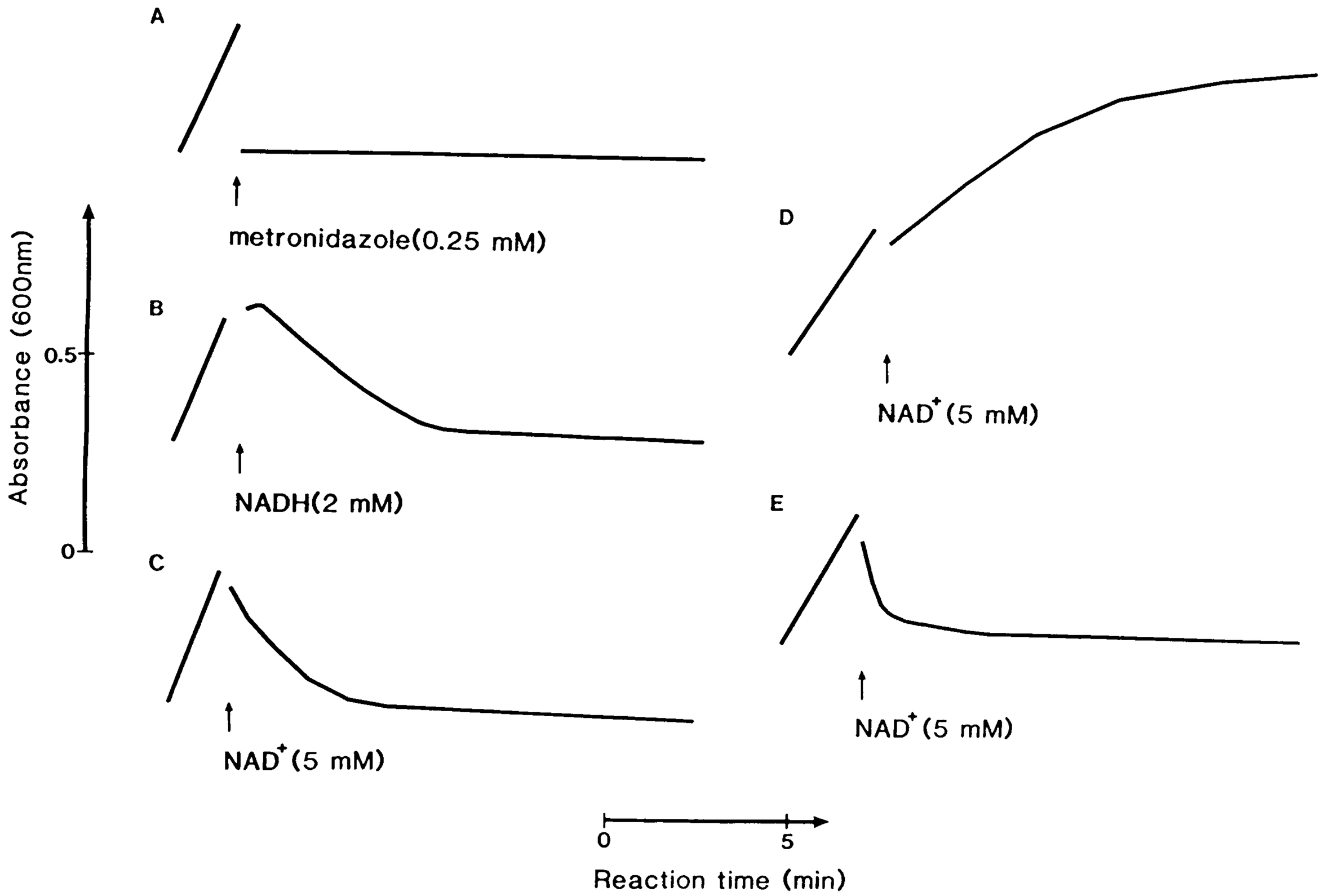


Fig. 31 The effects of metronidazole (A), NADH (B) and NAD^+ (C) on *Trichomonas vaginalis* pyruvate: methyl viologen oxidoreductase activity, and NAD^+ on *Tritrichomonas foetus* (D) and *Trichomitus batrachorum* (E) pyruvate: methyl viologen oxidoreductase activity. The enzyme was assayed as described in section 2.7.3.. The compounds were added approximately 1-1.5 min after the start of the reaction (indicated by arrows, with concentrations in parentheses). The results shown are representative of the several experiments carried out.

4.0. DISCUSSION

4.1. SAH hydrolase

The results have revealed both similarities and differences between the properties of Trichomonas vaginalis and mammalian SAH hydrolases. The pH optimum of the parasite enzyme (pH 8.0) is greater than those reported for the enzymes from rat liver (pH 7.0 - 7.5) (Kajander et al. 1976) and rat brain (pH 6.5) (Schatz et al. 1979). My finding, however, that the mouse liver enzyme activity did not vary significantly in the pH range 7-8 suggests that this difference between parasite and host enzymes may be of only minor importance. The apparent K_m of adenosine for the parasite enzyme (110 μM) falls within the range (0.2 μM - 1.5 mM) reported for various mammalian enzymes, including those from rat liver (Walker and Duerre 1975; Kajander and Raina 1981), mouse liver (Ueland and Saebo 1979), beef liver (Richards et al. 1978), murine lymphoid cells (Kamantani et al. 1983) and human placenta (Hershfield and Kredich 1978). The apparent K_m of homocysteine for the T. vaginalis enzyme (155 μM) also is within the range (60 μM - 4.5 mM) found with mammalian enzymes (Finkelstein and Harris 1973; Walker and Duerre 1975; Kajander and Raina 1981; Kamantani et al. 1983). Differences were discovered, however, with respect to substrate inhibition. I found that homocysteine (even at 6 mM) did not inhibit the parasite enzyme, which agrees with the findings of Kajander and Raina (1981) with rat liver enzyme but contrasts to the earlier work of Finkelstein and Harris (1973) and Walker and Duerre (1975) who demonstrated marked inhibition by homocysteine of the enzyme from the rat liver. The inhibition by adenosine (>450 μM) of the parasite enzyme is similar to that found for the rat liver enzyme by two groups (Finkelstein and Harris 1973; Walker and Duerre 1975); interestingly, Kajander et al. (1976) subsequently reported that the rat liver SAH hydrolase was unaffected by adenosine (up to 5 mM).

The large differences in apparent K_ms and substrate inhibition observed for mammalian enzymes by different workers may have been due to species or isoenzyme variation. It has been suggested by Ueland and Saebo (1979), however, that differences in the incubation conditions may have been one contributing factor. Comparison of my results for T. vaginalis with those obtained for mammalian cells using very similar methods shows that the apparent K_m (adenosine) for the parasite enzyme (110 μM) is about 250-fold higher than that for murine lymphoid cell line SAH hydrolase (0.4 μM) (Kamantani et al. 1983). On the other hand, the apparent K_ms of homocysteine found for T. vaginalis (155 μM) and the murine lymphoid cell line (156-163 μM) are very similar.

The T. vaginalis enzyme differs from the mouse liver enzyme in other ways. The appreciable inhibition by EDTA could have been due to the chelation of an essential divalent cation; strangely, however, all of those tested either inhibited the enzyme activity or were without effect. The slight stimulatory effect of Mg^{2+} on mouse liver SAH hydrolase (Ueland and Saebo 1979) is the only report on the effects of divalent cations on a mammalian enzyme, whereas nothing has been published concerning EDTA. The greater sensitivity of the mouse liver enzyme to ara A compared with the trichomonal enzyme is another difference between the enzymes. In contrast, the inhibitions by tubercidin, 2'-deoxyadenosine and sinefungin of the mouse liver and parasite enzyme were rather similar and relatively minor compared with the effect of ara A (Figs. 14 and 15). Ara A, tubercidin and sinefungin are potently trichomonicidal in vitro (see Tables 21a and 21b). It is yet to be elucidated, however, whether the inhibition of SAH hydrolase plays any part in these actions which may be due to the incorporation of the adenosine analogues into nucleotides by the

action of T. vaginalis adenosine kinase.

The SAH hydrolase activity detected in T. vaginalis was found to be similar to those reported for Tetrahymena pyriformis (Dang and Cook 1977) and T. thermophila (Murphy and Fall 1985). My results also indicate that in possessing SAH hydrolase trichomonads are similar to other eukaryotic cells, but differ from the prokaryotes which are known to lack this enzyme (Walker and Duerre 1975). Prokaryotes, however, possess SAH nucleosidase and S-ribosyl-homocysteinecleavage enzyme (Walker and Duerre 1975). These enzyme reactions have been described in section 1.5.5.(i). The former enzyme is present at very high activities in many bacteria indicating that SAH probably plays no bioregulation function in these prokaryotes (Walker and Duerre 1975) in contrast to its role in the control of biological transmethylations in eukaryotes (Cantoni and Scarano 1954; Kamantani et al. 1983).

The finding that SAH hydrolase was present in the three trichomonads studied but at different levels of activity shows both that the enzyme has a role in these parasites and also that the species differ significantly in this as well as in many other ways. The difference in SAH hydrolase activity between Trichomonas vaginalis and Tritrichomonas foetus could be related to the ways in which the two species synthesize AMP. Trichomonas foetus depends mainly on purine phosphoribosyltransferases (Wang et al. 1983b) whereas Trichomonas vaginalis relies primarily on adenosine kinase (Heyworth et al. 1982; Miller and Linstead 1983). Trichomonas vaginalis SAH hydrolase may play a part in supplying intracellular adenosine for this purpose. An additional explanation of the species variation in SAH hydrolase activity is that the homocysteine produced may be metabolised by homocysteine desulphurase, which is highly active in T. vaginalis, present at low activity in Trichomitus

batrachorum and apparently absent from Tritrichomonas foetus (see Table 6). It would be interesting to elucidate the precise roles of SAH hydrolase activity in transmethylation and transsulphuration reactions in trichomonads. This could be achieved by following the fate of SAH in living cells, and by testing the effect of SAH on the enzymes catalysing transmethylation reactions.

4.2. Homocysteine desulphurase and serine sulphydrase

My results have shown that Trichomonas vaginalis, unlike Tritrichomonas foetus, Trichomitus batrachorum or Pentatrichomonas hominis assayed under the same conditions, possesses high activities of homocysteine desulphurase and serine sulphydrase. The finding that all the other microorganisms investigated with the exception of the ciliate Tetrahymena pyriformis and mouse liver possessed serine sulphydrase whereas none of them had detectable homocysteine desulphurase suggests that homocysteine desulphurase may be of key importance in trichomonads and Trichomonas vaginalis in particular.

The products of the catabolism of homocysteine by T. vaginalis are hydrogen sulphide, ammonia and an α-ketoacid. The latter is probably α-ketobutyrate although this has to be confirmed. The α-ketoacid and ammonia produced could be further metabolised to aminobutyrate, as occurs in rumen ciliate protozoa (Onodera and Migita 1985). The effect of 2-mercaptoethanol on the rates of production of hydrogen sulphide, α-ketoacid and ammonia by T. vaginalis homocysteine desulphurase is indicative of an exchange reaction occurring between homocysteine and 2-mercaptoethanol; some bacterial methionine-γ-lyases also catalyse such an exchange reaction (Ito et al. 1975; Tanaka et al. 1977). Interestingly, the effect of 2-mercaptoethanol on the homocysteine desulphurase of T.

vaginalis closely resembles its effect on T. vaginalis methionine-catabolising enzyme (see section 3.3.). This, together with the other similarities in their properties such as sensitivity to inhibition, pH optimum and solubility suggests that T. vaginalis homocysteine desulphurase and methionine-catabolising activities are probably different expressions of the same enzyme. In addition, the relatively low γ-cystathionase activity detected in T. vaginalis and lack of inhibitory action of cystathionine on T. vaginalis homocysteine desulphurase suggest that they are distinct enzymes. Trichomonas vaginalis extracts did not catalyse the production of ammonia or α-ketoacid from cysteine confirming the absence of cysteine desulphurase from this parasite.

Bithionol, hexachlorophene and dichlorophene are anthelmintics which inhibit mitochondrial oxidative phosphorylation and fumarate reductase (Murakoshi and Moriya 1968; Murakoshi et al. 1969). The antitrichomonal activity of these compounds (Table 21a; Takeuchi et al. 1985) cannot be explained in this way, however, as trichomonads lack mitochondria and cytochromes (Lloyd et al. 1979). The finding that these compounds inhibited both homocysteine desulphurase and serine sulphydrase at concentrations similar to those which affected parasite growth suggests that their antitrichomonal activities may be exerted through inhibition of these enzymes. These compounds also inhibit the growth in vitro of Entamoeba histolytica (Takeuchi et al. 1984), which possesses serine sulphydrase activity (see Table 7) but no homocysteine desulphurase activity (see section 3.2.). These findings suggest that inhibition of serine sulphydrase could be the key feature. This conclusion is supported by the lack of antitrichomonal activity of DL-propargylglycine, a potent inhibitor of homocysteine desulphurase but which affects serine sulphydrase only if at high concentrations. The ineffectiveness of 2,2'-

thiodiethanol and 2,4-dichlorophenol against the enzymes gives some information on the structural requirements for their inhibition.

Trichomonas vaginalis homocysteine desulphurase and serine sulphydrase activities were affected little by α-ketobutyrate and serine, respectively, suggesting that the enzymes are not tightly regulated by product-inhibition. The ineffectiveness of a range of analogues of homocysteine and methionine (M & B compounds, see sections 3.2. and 2.2.3.) on T. vaginalis homocysteine desulphurase provides further information on the structural requirements for the inhibition of this enzyme.

Interestingly, the isoenzyme patterns of trichomonal serine sulphydrase were found to differ considerably both between species and also between metronidazole-resistant and-sensitive lines of T. vaginalis. Resistance to metronidazole is exhibited only in the presence of oxygen and the resistant lines appear to have an impaired ability to metabolise oxygen (Muller and Gorrell 1983). It is tempting to speculate that the differences in isoenzyme patterns relate to sensitivity to the drug, and the line's ability to metabolise oxygen. The differences between the line's response to DL-propargylglycine is similarly intriguing and could be interpreted in terms of a deficiency of the drug-resistant lines to modulate serine sulphydrase activity. It was disappointing therefore to find that these lines were no more sensitive to DL-propargylglycine alone or together with metronidazole, and had similar homocysteine desulphurase and serine sulphydrase activities to drug-sensitive lines (see section 3.2.). Nevertheless, the results provide strong evidence that in T. vaginalis homocysteine desulphurase and serine sulphydrase have complementary functions and that these are of particular importance to T. vaginalis rather than other trichomonads.

Although certain features of these enzymes are known, their exact physiological role in trichomonads remains uncertain. Interestingly, preliminary studies carried out in our laboratory found that T. vaginalis grown in the presence of DL-propargylglycine (10^{-5}M) for 24 hr (these cells lacked detectable homocysteine desulphurase activity but possessed elevated serine sulphydrase activity) were less able to establish intravaginal infections in mice than normal cells (A.F. Bremner, personal communication). This finding indicates the involvement of the enzymes in the host-parasite interaction.

In addition, the results obtained from IEF and enzyme kinetic studies suggest that T. vaginalis serine sulphydrase is quite distinct from homocysteine desulphurase but is similar to serine sulphydrase of other sources (see section 1.5.5. (iv)).

4.3. L-methionine catabolism

It has been reported that T. vaginalis growing in complex medium (CPLM) produced methane, ethane, propane, ethylene, carbon monoxide, carbon dioxide and other unidentified gases, probably from the catabolism of amino acids (Ishiguro 1985). I have shown in my studies that cultures of T. vaginalis, but not Tritrichomonas foetus, Trichomitus batrachorum or Pentatrichomonas hominis, growing in complex medium (MDM) and in methionine-supplemented PBS also produce volatile thiols. The lack of detectable hydrogen sulphide production suggested that the volatile thiols produced were likely to be methanethiol. The use of mass spectrometry confirmed that this was a product, although I do not at present know if it is the only thiol produced. The other established end-product of L-methionine catabolism in T. vaginalis is an α-ketoacid (probably α-ketobutyrate). The differences in the rates of production of α-ketoacid and volatile thiol by cell-free extracts and living

parasites in PBS with added methionine indicate that in the living parasite α-ketoacid is probably channeled into other reactions; the synthesis of aminobutyrate or propionate are possibilities. Interestingly, rumen ciliate protozoa are known to catabolise L-methionine or L-threonine to α-ketobutyrate, propionate and aminobutyrate (Onodera and Migita 1985). Trichomonas vaginalis in PBS also produced ammonia suggesting that the parasite is similar to most protozoa in being ammonotelic (Kidder 1967).

The difference in apparent K_m for L-methionine between thiol production by living T. vaginalis and cell-free extracts (0.4 and 3.8 mM, respectively) suggests that the parasite has a high affinity transporter for the amino acid. The findings that metronidazole-killed parasites produce volatile thiols at a very much reduced rate supports such an idea. The results, however, do not resolve whether L-methionine is an essential amino acid for the growth of this parasite as it is for Tetrahymena pyriformis (Kidder and Dewey 1945) and the intraerythrocytic form of Plasmodium knowlesi (Fulton and Grant 1955).

The pH optima obtained for thiol production by living and cell-free extracts of T. vaginalis were similar to each other and that reported for Pseudomonas fluorescens (Laakso and Nurmikko 1976). Parasite cell-free extracts, however, differed considerably from living cells in the catabolism of substrates other than L-methionine. The situation for living T. vaginalis is very much like that for Brevibacterium linens (Ferchichi et al. 1985) in that both catabolised a wide range of substrates. The higher rate of thiol production from L-methionine amide as compared to L-methionine probably reflects the easier entry of L-methionine amide into living T. vaginalis. Presumably it is then hydrolysed by amidase and

consequently yields a high intracellular concentration of L-methionine, as described also for Brevibacterium linens (Ferchichi et al. 1985) and Streptococcus lactis (Rice et al. 1978). The inability of the parasite cell-free extracts to catabolise L-methionine amide and also L-methionine sulphoxide may be explained by either the inactivation of the enzymes responsible for the generation of free L-methionine or the loss by dilution, during extraction, of some essential cofactor of the enzymes. The low rate of catabolism of α-keto-γ-methiolbutyrate, an important intermediate in the mammalian catabolism of L-methionine, by cell-free extracts of T. vaginalis provides strong evidence that this is not intermediate in the trichomonad degradative pathway.

The T. vaginalis L-methionine-catabolising enzyme is similar in several ways to bacterial L-methionine-γ-lyase (Ito et al. 1975; Tanaka et al. 1977). Both enzymes catalyse γ-elimination (e.g. with methionine or ethionine) and β-elimination (e.g. with S-methyl-L-cysteine) reactions. The parasite enzyme also catalyses an exchange reaction between L-methionine and 2-mercaptoethanol, as indicated by the inhibition and potentiation by the latter of the production of α-ketoacid and volatile thiol, respectively. Such exchange reactions are known to occur with Pseudomonas putida and P. ovalis L-methionine-γ-lyases (Ito et al. 1975; Tanaka et al. 1977). The enzyme from T. vaginalis also is strongly inhibited by known antagonists of pyridoxal phosphate-dependent enzymes such as DL-propargylglycine. This implies that the parasite enzyme is probably pyridoxal phosphate-dependent, as is bacterial L-methionine-γ-lyase (Tanaka et al. 1977). The main difference between the trichomonal and bacterial L-methionine-catabolising enzymes is the lack of detectable α-elimination of ammonia with the parasite enzyme. The explanation for this lack of ammonia production by the T. vaginalis

enzyme, and indeed the fate of the amino group in the reaction, is, at present, unknown. Further studies using a purified enzyme preparation may help to solve this mystery.

Another interesting aspect of the parasite L-methionine-catabolising enzyme is its similarity to T. vaginalis homocysteine desulphurase. The two activities are soluble and have similar pH optima. They are equally affected by inhibitors of pyridoxal phosphate-dependent enzymes (including DL-propargylglycine) and other compounds such as bithionol and hexachlorophene. In addition, the activities of homocysteine desulphurase and L-methionine-catabolising enzyme are found at relatively high levels in T. vaginalis but are either at low activity or undetectable in Pentatrichomonas hominis, Trichomitus batrachorum and Tritrichomonas foetus. Most interestingly, both enzymes are related to Trichomonas vaginalis serine sulphydrase in function. Although the precise nature of this relationship is still to be elucidated, it is already apparent that the two enzymes are not essential for growth of the parasite axenically in vitro, as shown by the experiment with DL-propargylglycine. Based upon these findings, it seems very likely that L-methionine-catabolising enzyme and homocysteine desulphurase activities in T. vaginalis are different expressions of the same enzyme. This suggestion could be confirmed by IEF; unfortunately at present there is no methods suitable for detecting the former enzyme activity, whereas the latter activity was found to be due to a single protein (see Fig. 22).

Bithionol, hexachlorophene and dichlorophene have been reported to be inhibitors of the growth in vitro of T. vaginalis (Takeuchi et al. 1985), Giardia lamblia (Takeuchi et al. 1985) and Entamoeba histolytica (Takeuchi et al. 1984). I also found that these

compounds were effective against T. vaginalis growth in vitro. My results show that bithionol and hexachlorophene are good inhibitors of T. vaginalis L-methionine-catabolising enzyme, homocysteine desulphurase and serine sulphydrase. The similarity between the I_{50} and LD_{50} of these compounds against the trichomonad enzymes and the parasite's growth in vitro (see Tables 11a and 15) suggests that the two events may not be unrelated.

It is most intriguing that the L-methionine-catabolising enzyme is present at relatively high activity in only T. vaginalis, being absent in other trichomonad species, the range of trypanosomatids tested and mouse liver. These findings suggest that it performs a function peculiar to T. vaginalis. It seems unlikely, however, that the enzyme is associated with the metabolism or scavenging of oxygen as our results revealed no apparent differences in enzyme activities between metronidazole-resistant and -sensitive lines of T. vaginalis and DL-propargylglycine-treated parasites with no L-methionine-catabolising enzyme activity were no less sensitive to metronidazole than untreated cells. The role of the parasite L-methionine-catabolising enzyme may be anticipated from the properties of methanethiol, one of the end-products. The toxicity of methanethiol to mammals (Shults et al. 1970; Zieve et al. 1974) and pathogenic fungi (Walker et al. 1937), its ability to inhibit mitochondrial respiration (Waller 1977) and catalase activity (Finkelstein and Benevenga 1986), as well as its effectiveness as a repelling agent (see Kadota and Ishida 1972), all suggest that T. vaginalis L-methionine-catabolising enzyme may be intimately linked to the survival of the parasite in the host and the pathogenicity of the parasite; methanethiol being one toxin released. Indeed, there is preliminary evidence that the parasite enzyme may be involved in establishing intravaginal infections in mice (A.F. Bremner, personal

communication). Methanethiol may also be responsible, in part, for the foul-smelling discharge produced by trichomoniasis patients. The T. vaginalis L-methionine-catabolising enzyme/homocysteine desulphurase, therefore, may be a highly promising target for chemotherapeutic attack. If the suggested role of the enzyme is true, inhibitors of the enzyme should have profound effect on the establishment of intravaginal trichomonad infections, they may also reduce the pathogenicity of the parasites and could be curative. Another approach would be to exploit the high enzyme activity of the parasite by designing non-toxic prodrugs that will be activated by the parasite enzyme.

4.4. SAM synthetase, SAM and SAM-dependent transmethylation reactions

The results of this study suggest that trichomonads and a range of trypanosomatids possess SAM. Intracellular SAM has been previously found in only one protozoan species, the ciliate Tetrahymena pyriformis (Dang and Cook 1977). The finding of SAM in trichomonads is suggestive of both the presence of polyamine metabolism and transmethylation reactions in these parasites. Indeed, Trichomonas vaginalis is known to possess an active polyamine metabolism as reflected by the secretion of large amounts of putrescine (White et al. 1983) and the presence of SAM decarboxylase (North et al. 1986) and ornithine decarboxylase activities (Linstead and Cranshaw 1983; North et al. 1986). The very high levels of SAM in the protozoan Herpetomonas muscarum ingenoplastis is most fascinating, although the explanation for and significance of this are at present unknown. Nevertheless, the presence of SAM in all protozoa tested suggests that this compound has a role in these cells

similar to those proposed for other cell types.

It has previously been postulated that T. vaginalis uses L-methionine to methylate phospholipids (Linstead and Bradley 1985). My experiments have revealed that T. vaginalis is able to synthesize SAM from exogenous L-methionine and SAM is subsequently used as a methyl donor in the methylation of lipids, nucleic acids and possibly proteins. The extraction procedures employed for separating these macromolecule fractions from trichomonads were confirmed as being efficient by the use of radiolabelled leucine and thymidine as markers for protein and nucleic acid, respectively. These findings suggest the presence of SAM-dependent methyltransferases. My results also provide evidence, albeit indirect, that T. vaginalis possesses SAM synthetase. So far I have been unable to detect SAM synthetase activity in cell-free extracts of T. vaginalis using methods effective for the mouse liver enzyme (see section 2.7.6.). SAM synthetase has been found in Tetrahymena extracts (Dang and Cook 1977; Murphy and Fall 1985), but using the conditions described by Murphy and Fall (1985) I was still unable to detect the enzyme activity in Trichomonas vaginalis extracts. The findings indicate that either the mouse liver and trichomonal SAM synthetases differ significantly in their requirements for activity or that the parasite enzyme is present at a level below detectability or is usually labile.

The high rate of consumption of exogenous methionine by T. vaginalis is partly explained by the presence in this trichomonad of a methionine catabolizing enzyme activity which generates the volatile methanethiol (see section 3.3.). The apparent absence of this activity from Tritrichomonas foetus helps to account for the large difference in methionine consumption between the two species. My results show, however, that methionine was also incorporated into

macromolecules more rapidly by Trichomonas vaginalis than Tritrichomonas foetus. A similar observation has recently been reported by Torian and Kenny (1986). Our finding of the large inhibition by cycloleucine and norleucine, both analogues of methionine, of methionine consumption (incorporated and catabolised) of T. vaginalis suggests that methionine uptake and possibly also SAM synthetase of the parasite is affected by these compounds. The higher rates of incorporation obtained for T. vaginalis compared with Tritrichomonas foetus may be related to the parasite's higher SAH hydrolase (see section 3.1.) and homocysteine desulphurase (see Table 6) activities. Another possible explanation for the observed species differences is that T. foetus may be able to recycle methionine to a greater extent than Trichomonas vaginalis. As yet the extent and mechanism of methionine synthesis from homocysteine in trichomonads is unknown, it will be interesting to see if there is a similar species variation in this pathway. Nevertheless, my results have shown that these two important parasites differ significantly in this area of metabolism and it is tempting to speculate that such species differences in the utilisation of exogenous methionine and in transmethylations may be related to the ways in which these parasites are adapted for growth in their respective hosts.

4.5. The effects of inhibitors of sulphur-containing amino acid metabolism

Sinefungin, a potent inhibitor of biological methylations, has been reported to be a good inhibitor of the growth of Leishmania species (Paolantonacci et al. 1985), Plasmodium falciparum (Trager et al. 1980), Entamoeba histolytica (Ferrante et al. 1984) and Trypanosoma species (Dube et al. 1983). I have now found it to be

potently trichomonacidal. Interestingly, 5-azacytidine, a cytosine analogue and inhibitor of DNA methylation in retrovirus (Groudine et al. 1981) was also observed to be a good antitrichomonal agent. These results suggest that methylations, particularly DNA methylation, are important to Trichomonas vaginalis. I have confirmed by direct observation that methylation reactions do occur in the parasite (see section 3.4.).

The observation that ara A and tubercidin, both known inhibitors of SAH hydrolase, inhibited the growth of T. vaginalis in vitro is in agreement with the report of Wang et al. (1984b). Differences between the effectiveness of these compounds against the parasite SAH hydrolase (I_{50}s 1 x 10^{-4}M and > 1 x 10^{-3}M, respectively, see section 3.1.) and parasite growth in vitro suggests that their antitrichomonal activity is due to interference with other cellular functions; the metabolism of purines is a possibility (Wang et al. 1984b).

The trichomonacidal activities of bithionol, hexachlorophene and dichlorophene, all antihelminthic agents, may be due to their effects on homocysteine desulphurase, serine sulphydrase and methionine-catabolizing enzyme activities, as described in sections 4.2. and 4.3.

The toxicity of seleno-L-methionine to the parasite is probably related to its inhibition of SAM synthetase, which has been detected in in vivo in T. vaginalis (see section 3.4.). Similarly, methylglyoxal-bis(guanylhydrazone), a specific inhibitor of SAM decarboxylase, probably exerts its antitrichomonal activity through inhibition of this enzyme which also has been reported to be present in T. vaginalis and Tritrichomonas foetus (North et al. 1986). These findings indicate the importance of SAM as a precursor to the biosynthesis of polyamines in trichomonads.

The antibacterial action of monothioglycerol has been partly

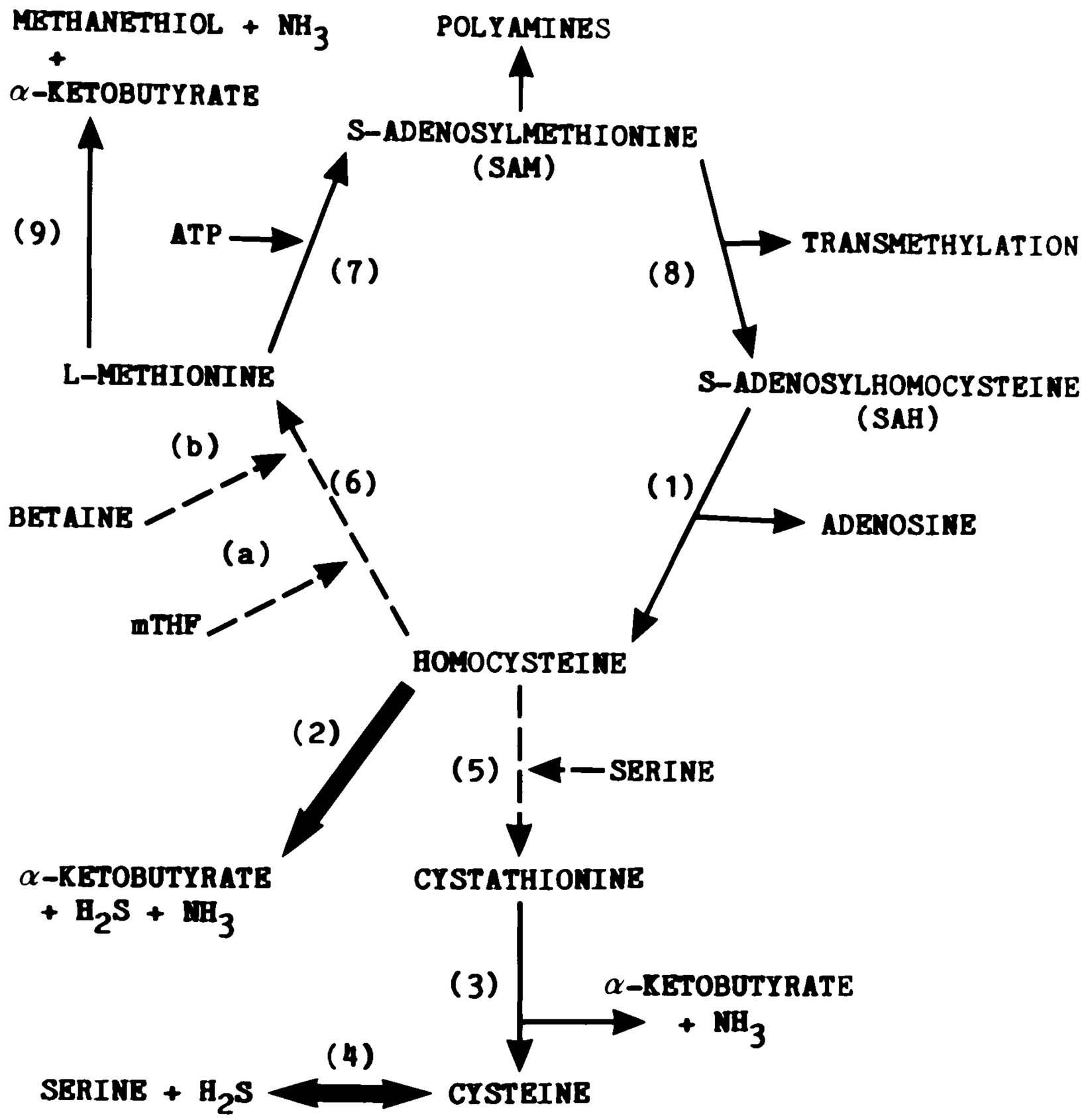
attributed to the depression of cellular SAM concentrations (Javor 1983a, 1983b). This is thought to be a consequence of the compound being used to methylate monothiolglycerol (Javor 1983a); the methylated product is S-methylthioglycerol. It is tempting to speculate that the trichomonacidal action of monothioglycerol is mediated in a similar way, as this would be in agreement with my earlier suggestions of the importance of SAM to T. vagainalis. It was, therefore, both surprising and interesting to find that the addition of methionine to the trichomonal cultures enhanced the activity of monothioglycerol rather than antagonising its effect, as it did with Escherichia coli (Javor 1983a). The explanation for this effect is yet to be elucidated. The parasite also differed from E. coli in its sensitivity to S-methylthioglycerol; the growth of the latter organism being unaffected by the compound at a concentration of 0.1M (Javor 1983a). The mode of action of S-methylthioglycerol against T. vaginalis also is unknown.

The combinations of compounds tested in this study were selected with the aim of assessing the importance of substrate availability on the efficacy of inhibitors and the possibilities of two compounds interfering with the same area of metabolism being synergistic. The lack of marked antagonism of the antitrichomonal activities of the compounds by addition of methionine suggests that it was already available to the parasite at saturating concentrations. There are several possible explanations for the lack of synergism between the inhibitors tested, but clearly these combinations have little potential as antitrichomonal agents.

The lower sensitivity of Trichomitus batrachorum to metronidazole than Trichomonas vaginalis and Tritrichomitus foetus may be due to the lower oxygen scavenging capacity of this organism.

I have shown that Trichomitus batrachorum contains lower NADH oxidase activity than Tritrichomonas foetus and Trichomonas vaginalis (see Table 22). It is possible, however, that the lower growth rate and temperature also contribute to the differences observed.

Overall, the results have provided further information on the occurrence and importance of sulphur-containing amino acid metabolism in trichomonads. Based upon my findings, a metabolic map of the reactions that apparently occur in T. vaginalis has been constructed (see Fig. 32). More details, however, are required of the precise effects of the inhibitors used, and of the physiological importance of sulphur-containing amino acid metabolism in trichomonads, before a full assessment of the potential of this area of the parasite's metabolism for chemotherapeutic attack can be made. Few if any of the compounds tested in this study appear to have high potential as antitrichomonal agents, certainly none was as potent as the 5'-nitroimidazole, metronidazole. Nevertheless, some, for instance sinefungin, exhibited levels of activity that give hope that other compounds that act in a similar way could be promising. The characterisation of the key enzymes in more detail should provide the information required to allow specific inhibitors to be designed.



ENZYMES: (1) SAH HYDROLASE
(2) HOMOCYSTEINE DESULPHURASE
(3) γ-CYSTATHIONASE
(4) L-SERINE SULPHYDRASE (or L-CYSTEINE SYNTHETASE)
(5) CYSTATHIONINE-β-SYNTHETASE
(6) (a) mTHF: HOMOCYSTEINE METHYLTRANSFERASE
(6) (b) BETAINE: HOMOCYSTEINE METHYLTRANSFERASE
(7) SAM SYNTHETASE
(8) TRANSMETHYLASES
(9) L-METHIONINE-γ-LYASE

KEY: - - → NOT KNOWN
——→ PRESENT AND GRADED ACCORDING TO ENZYME ACTIVITY

Fig. 32 The metabolism of sulphur-containing amino acids in *Trichomonas vaginalis*.

4.6. Comparative study of trichomonal ferredoxin-linked and oxygen-metabolising enzymes

The NADH oxidase and NADPH oxidase activities detected for T. vaginalis and Tritrichomonas foetus (see Table 22) are similar to those previously reported (Muller 1973; Muller and Gorrell 1983). It is noteworthy that in this study the NADPH oxidase activity was assayed in the absence of FMN, a known stimulator of the enzyme (Linstead 1978). The very high NADH oxidase activity in Trichomonas vaginalis indicates a major importance of the enzyme for this parasite in particular. The low NADH oxidase activity detected in Trichomitus batrachorum is intriguing and could reflect the lower oxygen concentration of its natural environment compared to that of Trichomonas vaginalis.

The hydrogenosomal enzyme pyruvate: ferredoxin oxidoreductase is thought to be central to the metabolism of the organelle (Muller 1976). My studies using methyl viologen as a substitute for ferredoxin showed that the enzyme activity in Trichomitus batrachorum is similar to those detected for the two parasitic trichomonads in both this study and others (Lindmark and Muller 1974a; Lindmark et al. 1975). The observation that NAD^+ greatly antagonised the apparent activities of Trichomonas vaginalis and Trichomitus batrachorum, whereas it had little or no effect on that of Tritrichomonas foetus, indicated that in the former two organisms NAD^+ was able to accept electrons from the reduced methyl viologen at an appreciable rate. This finding was suggestive of a NAD^+: ferredoxin (methyl viologen) oxidoreductase activity in Trichomonas vaginalis and Trichomitus batrachorum. It has been known for some time that a similar activity exists in some bacteria and although it had been suggested earlier (see Gutteridge and Coombs 1977) that NAD(P)H: ferredoxin oxidoreductase must be present in trichomonads,

the enzyme had not been reported in detail until very recently (Steinbuchel and Muller 1986b). These investigators measured the enzyme activity of Trichomonas vaginalis and Tritrichomonas foetus in the direction of NAD^+ reduction using isolated hydrogenosomes and ferredoxin reduced by either hydrogenase or pyruvate: ferredoxin oxidoreductase. Using this assay system they detected similar activities in the two trichomonads. My results (Fig . 31), however, suggested that there are significant differences between the two parasites and this was confirmed by the observed NADH: methyl viologen oxidoreductase activities (Table 23). Interestingly, using similar methodology I was also able to measure NADPH: methyl viologen oxidoreductase, an activity Steinbuchel and Muller (1986b) were unable to detect using their assay. My results confirm, however, that the activities in Trichomonas vaginalis and Trichomitus batrachorum towards NADPH are much less than those towards NADH. This may explain the lack of a marked effect of $NADP^+$ on the apparent pyruvate: methyl viologen oxidoreductase activity of Trichomonas vaginalis. Thus, my study has shown clearly that both Trichomitus batrachorum and Trichomonas vaginalis possess very high NADH: methyl viologen oxidoreductase activity which make them good organisms to use for further investigations concerning the nature of the enzyme. The high enzyme activity in the two species suggest that it plays some key role. One presumed function of this enzyme is that it recycles NADH which is used by malate dehydrogenase (decarboxylating) (see Fig. 5), it will be interesting to see if there is similar species variation in the activity of this enzyme to that we have found for NADH: methyl viologen oxidoreductase.

I found that metronidazole and NAD^+ caused the rapid oxidation of methyl viologen, but it was most surprising to find that NADH

produced a similar effect (see Fig. 31). The explanation for the effect of the latter compound is not known. Similarly, the antagonistic effects of NADH, NADPH and GSH, when added to the reaction mixture just before the start of the reaction, on T. vaginalis pyruvate: methyl viologen oxidoreductase activity are at present not understood. These compounds, in contrast to metronidazole, NAD^+ or $NADP^+$, are not likely to accept electrons from methyl viologen or be reduced directly by the parasite pyruvate: methyl viologen oxidoreductase. An inhibitory effect on the enzyme itself is one possible explanation. The results also revealed that GSSG was not reduced by methyl viologen at significant rate, although this compound also inhibited T. vaginalis pyruvate: methyl viologen oxidoreductase activity. Most interestingly, the parasite also lacks detectable GSSG reductase activity, the enzyme responsible for the production of GSH from GSSG, despite the presence of cellular GSH (Clackson 1984). These findings suggest that T. vaginalis may differ significantly from typical mammalian cells with respect to glutathione metabolism.

In addition to the enzyme differences found in my studies, including the work on sulphur-containing amino acid metabolism, T. vaginalis, Trichomitus batrachorum and Tritrichomonas foetus also differ considerably in polyamine metabolism (North et al. 1986), proteolytic activity (Lockwood et al. 1984) and total protein profile on IEF (see Fig. 23). Trichomonas vaginalis and Tritrichomonas foetus also are significantly different in several other aspects of biochemistry (see Steinbuchel and Muller 1986a, 1986b; Wang et al. 1984b), including their major end-products of energy metabolism. Interestingly, Trichomitus batrachorum resembles Tritrichomonas foetus in this respect, producing succinate but no detectable lactate. Taken together, these results clearly show that

trichomonads differ biochemically in a variety of ways, although it is a little premature to speculate on the implications of these differences with respect to the phylogenetic relationships of trichomonads. Nevertheless, these findings provide further evidence that not only are the environments in which the protozoa survive and multiply in their respective hosts significantly different but also that these differences are reflected in the biochemistry of the protozoa themselves. This highlights the danger of using inappropriate models in the search for new therapies of the diseases caused by trichomonads.

5.0. REFERENCES

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