Studies on the drug sensitivity and metabolism of *Trichomonas vaginalis*

by

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I N D E X

SUMMARY

S U M M A R Y

Three lines of Trichomonas vaginalis, one metronidazole-sensitive (clone G3) and several resistant lines, in particular line IR78 and line LRS (2.0), have been studied with a view to elucidating the mode of metronidazole resistance exhibited by the latter lines. Drug resistance was found to be dependant upon aerobic conditions in all cases except in line LRS (2.0), suggesting that molecular oxygen plays a key role, possibly the inhibition of drug activation. Parasite cytoplasmic NADH and NADPH oxidases are thought to reduce the oxygen entering T. vaginalis, therefore maintaining the anaerobic nature of the hydrogenosomes, in living cells. It is within these organelles that metronidazole is thought to be reduced and so activated. Parasite homogenates of clone G3, line IR78 and line LRS (2.0) had NADH and NADPH oxidase activities of 570 ± 87, 413 ± 55, 507 ± 153 and 389 ± 101, 127 ± 42, 559 ± 123 (n moles / min / mg protein) respectively, suggesting that drug resistance in IR78 is related to the parasites poor ability to handle molecular oxygen.

The medium used to culture T. vaginalis contained high levels of cysteine and ascorbate (12.7 mM and 21.0 mM respectively). In an attempt to confirm the poor ability of line IR78 to handle molecular oxygen, clone G3 and line IR78 were grown under aerobic and anaerobic conditions in medium containing lowered levels of cysteine and ascorbate. The adverse effects of such conditions upon growth of all lines of T. vaginalis in the presence of oxygen support the suggestion that T. vaginalis is an anaerobe. Under

anaerobic conditions, in contrast, growth of the parasite occurred in media lacking both cysteine and ascorbate, although at a reduced rate. Using other reducing agents as cysteine substitutes enhanced growth, but it was still less than when cysteine was present. The effect of lowering the cysteine and ascorbate concentrations upon growth under aerobic conditions was greater with line IR78 than clone G3, and line IR78 was also more sensitive to oxygen than clone G3. These results correlate well with line IR78's lower NADH and NADPH oxidase activities. The results suggest that drug resistance in line IR78 may be due to significant amounts of the oxygen entering the parasite permeating unmetabolised to the hydrogenosomes where it would interfere with the reductive activation of the drug.

The lower metronidazole sensitivity of line LRS (2.0) was found to be independant of oxygen. The rate of reduction of the nitro-group of metronidazole by homogenates of clone G3, line IR78 and line LRS (2.0) were 65 ± 9, 91 ± 18 and 19 ± 3 units (nmoles/min/mg protein) respectively. The low rate of metronidazole reduction exhibited by line LRS (2.0) could explain why this line is less sensitive to metronidazole than clone G3 under both aerobic and anaerobic conditions.

The reduction of nitro-compounds, such as 5-nitroimidazoles, by T. vaginalis is well established. An attempt was made to assess the parasites' ability to metabolise nitrobenzene and

azobenzene, compounds that are metabolised by aerobic cells through pathways involving oxygen. The results indicate that metabolism is mainly reductive, reduction of the nitrogroup of nitrobenzene and the azo-group of azobenzene probably takes place using the same ferredoxin-linked system that reduces 5-nitroimidazoles. High performance liquid chromatography (HPLC) identified hydrazobenzene as a major reductive metabolite of azobenzene in T. vaginalis. Oxidative metabolites such as nitrophenol were not detectable.

Lactate and acetate at 5 mM were found to be antagonistic to the killing action of metronidazole on T. vaginalis under aerobic conditions, raising the aerobic MLC eightfold. This was not a pH effect. Lactate and acetate had no effect on the growth rate of the parasites, or on the rate of metronidazole reduction by, and the activities of NADH and NADPH oxidases in, parasite homogenates. However, lactate dehydrogenase activity was enhanced on the addition of lactate to the growth medium, although this did not result in greater amounts of lactate produced as an endproduct. These phenomena could have some bearing on the effectiveness of metronidazole in the treatment of trichomoniasis.

There have been suggestions that DNA is the primary target of 5-nitroimidazoles, but no reports on the ability of T. vaginalis to repair damaged DNA. Aminobenzomides have been shown to inhibit poly (ADP-ribose) polymerase, an enzyme implicated with DNA repair, and to potentiate the alkylating agent dimethyl sulphate (DMS)

against mammalian cells. The trichomonacidal activity of DMS was increased one hundred-fold by co-administration with benzamide or aminobenzamides. The trichomonacidal activity of metronidazole was not increased by these compounds. These results suggest that DNA repair is of some importance in T. vaginalis, and that metronidazole does not effect DNA in a similar way to DMS.

A rapid method has been developed for testing the in vitro trichomonacidal activity of compounds under aerobic and anaerobic conditions. A range of compounds have been tested using this system.

Thiosemicarbazones containing disulphide bonds and able to form chelates with transition metals were found to be highly toxic to T. vaginalis. This trichomonacidal activity was antagonised by compounds containing thiol groups. Chelates were obtained by the addition of manganese, zinc and copper. The thiosemicarbazone bound to equimolar concentrations of these metal ions. A purified copper chelate was as effective against T. vaginalis in vitro as the parent compound, and its activity was also antagonised by thiol groups. Since thiosemicarbazones react with thiol groups, the possibility exists that they may inhibit enzymes with essential thiol groups. A thiosemicarbazone (TSC1) and its copper chelate (TSC2) were tested for inhibitory activity against parasite proteinases and glycolytic kinases. Antiproteinase activity was

detected with the chelate, but at concentrations significantly greater than those required to kill the parasite. TSC1 and TSC2 were tested for inhibitory activity against glycolytic kinases from T. vaginalis, mouse liver and yeast. The major effects were the inhibition of T. vaginalis hexokinase (55%) using 100 µM TSC1 and T. vaginalis pyruvate kinase (90%) using 100 µM TSC2. The additions of TSC1 and TSC2 to cultures of T. vaginalis were found to cause a drop in the thiol content of the parasite cultures, however this loss was transient, and did not correlate with parasite death. Although the primary target has not been elucidated, these studies suggest that binding to thiol groups plays a part in the activity of thiosemicarbozones.

1. INTRODUCTION

1.1. THE TRICHOMONADS

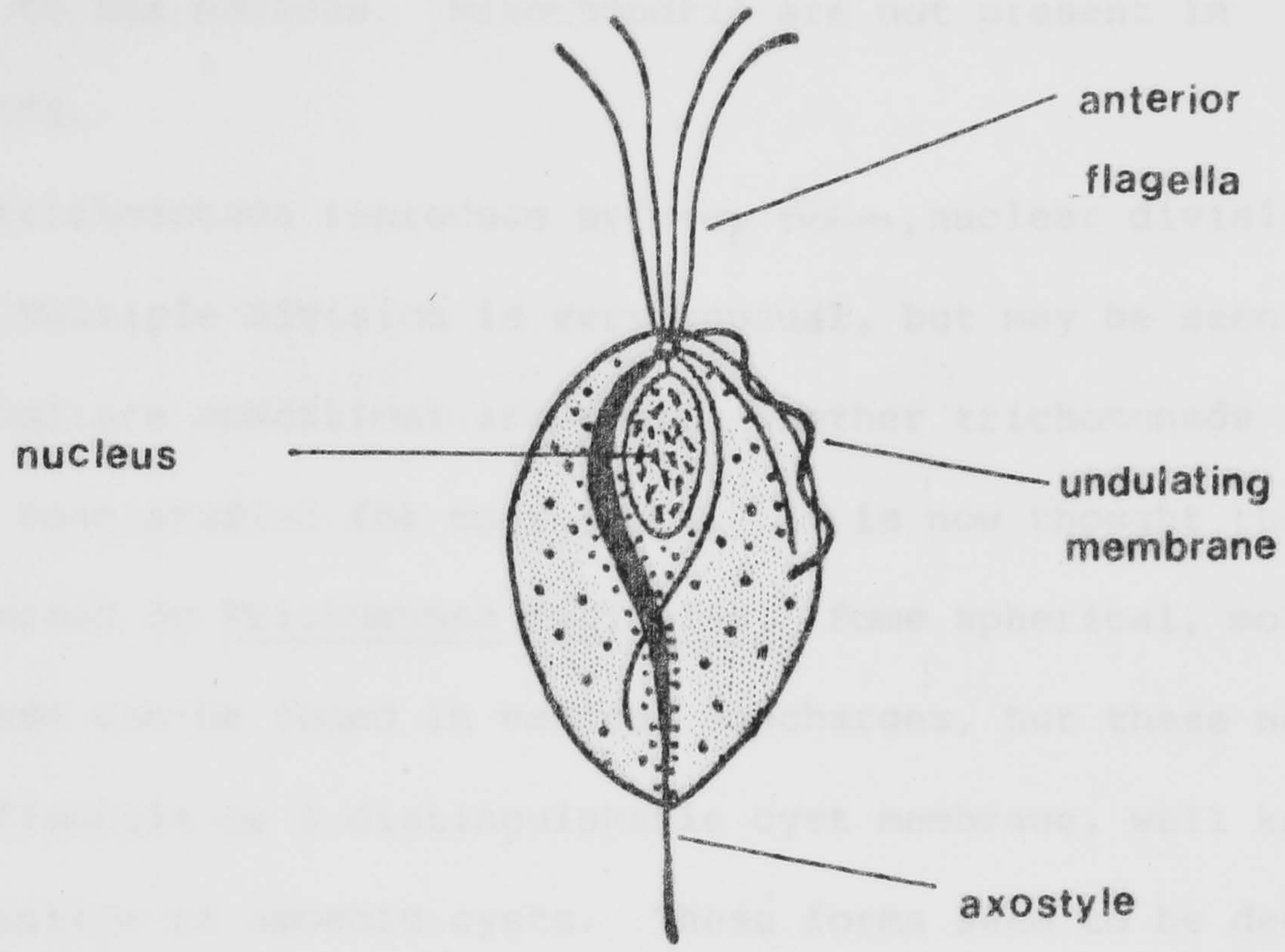
1.1.1. General Biology

Trichomonads are members of the order Trichomonadida, family Trichomonadidae. They are flagellate protozoa. They are classified into three genera (Honigberg, 1963): genus Trichomonas, genus Pentatrichomonas and genus Tritrichomonas. The classification is based mainly on the number of anterior flagella, members of the genera Trichomonas, Pentatrichomonas and Tritrichomonas having 4, 5 and 3 anterior flagella respectively. Trichomonads are motile, ovoid or pear-shaped organisms with 3 to 5 anterior flagella and an undulating membrane with an accessory fibril along its outer margin (See Figure 1), giving it a double appearance. All flagella arise from basal bodies grouped at the anterior end, just in front of the single ovoid nucleus (Honigberg and King, 1964). Trichomonads also have a prominent skeletal axostyle which may protrude from the hind end of the body.

Ultrastructural studies described an ordinary, three-layered nuclear membrane. The nucleus contains several large electron-dense granules, which either represent nucleoli or are simply clusters of chromatic material. The parabasal body lies in the anterior part of the cell, mostly dorsal to the nucleus, and is morphologically a Golgi zone, being composed of flattened cisternae. Vesicles have been seen which seem to have arisen from cisternae by a process of constriction or budding (Jirovec and Petru, 1968). The endoplasmic reticulum is found as a corona around the nucleus. Free ribosomes are distributed throughout the cytoplasm. Vesicles and vacuoles,

Figure 1

Diagram of Trichomonas vaginalis



some containing electron-dense material, are situated at the caudal end of the body. Most of them are food vacuoles. Paraxostylar bodies (hydrogenosomes) are found grouped round the axostyle posterior to the nucleus. Mitochondria are not present in trichomonads.

All trichomonads reproduce by binary fission, nuclear division is mitotic. Multiple division is very unusual, but may be seen when in vitro culture conditions are poor. Whether trichomonads form cysts has been studied for many years. It is now thought that cysts are not formed by Trichomonas vaginalis. Some spherical, motionless trichomonads can be found in vaginal discharges, but these never have any flagella or a distinguishable cyst membrane, well known characteristics of amoebic cysts. These forms seem to be degenerate trichomonads which are dying. Cysts were reported by Holz (1953), but this was never confirmed, and he may have observed degenerate host cells. Other trichomonads develop into "pseudocysts" (Mattern and Wendell, 1980), forms lacking a cyst wall but which appear to have internalised their locomotor organelles, including anterior flagella and the undulating membrane with its recurrent flagellum. These "pseudocysts" are viable upon incubation in culture medium and "excyst" and become fully mobile. However, it is yet to be shown whether they play a role in transmission or are simply degenerate forms.

Many species (e.g. T. vaginalis) lack a cytostome, but many can apparently take in solid food at the posterior region, whole leucocytes or bacteria being ingested.

All the members of the order are parasitic, in vertebrates or invertebrates. They parasitize the intestines of mammals, including man (Pentatrichomonas hominis), birds (Trichomonas gallinae), reptiles, amphibia, slugs and termites. Species are also found in the mouth of man and monkeys (T. tenax) and in the urogenital tract of man and cattle (T. vaginalis and Tritrichomonas foetus). Some trichomonads are wide ranging about their hosts. Trichomonas gallinae can be found in pigeons, chickens, turkeys and hawks, and Pentatrichomonas hominis in monkeys, rats and cats, as well as in man.

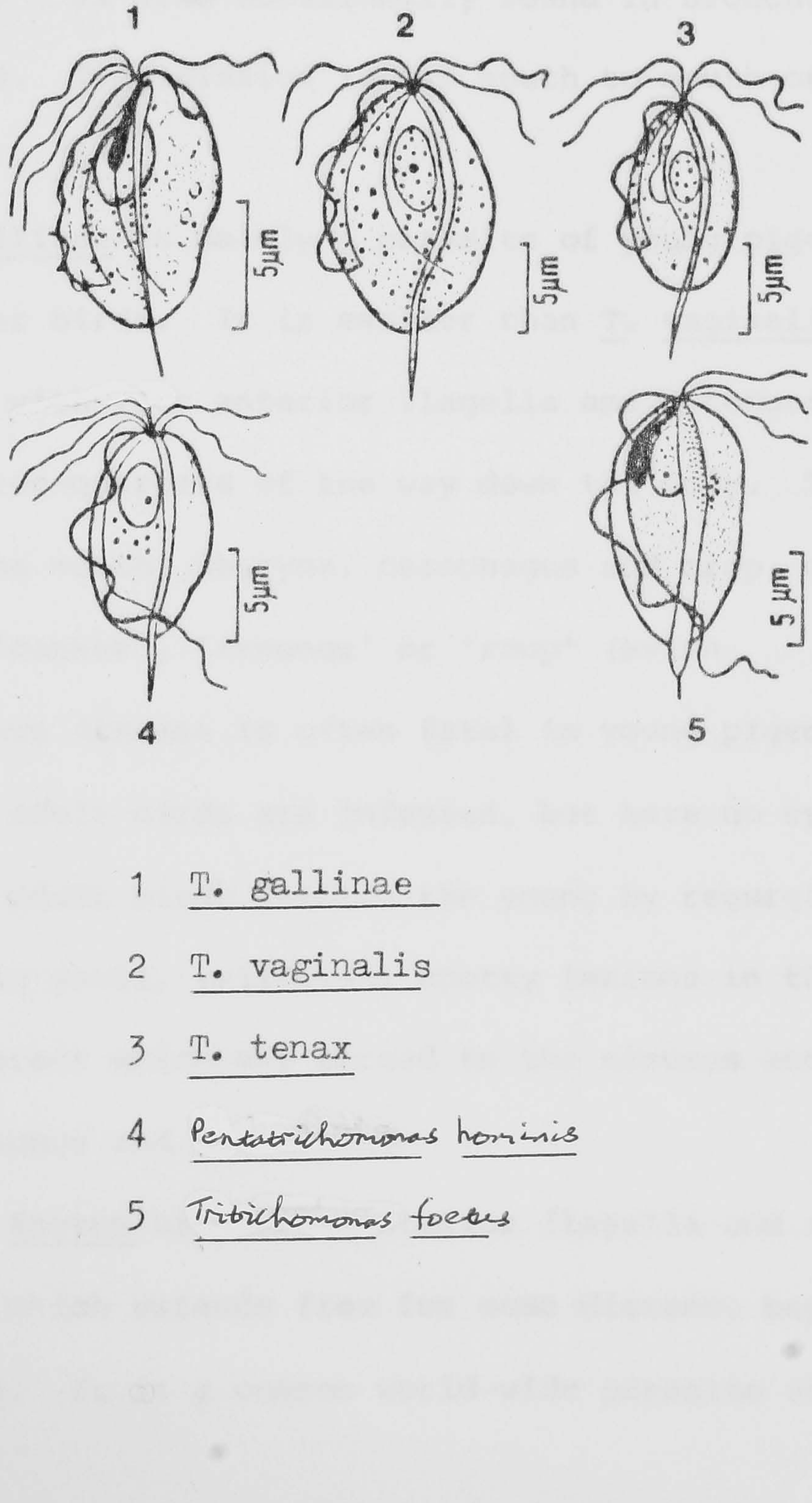
1.1.2. Important parasitic species

There are several species of trichomonads of medical or commercial importance to man, as they infect either man himself or his domestic animals. A diagram of trichomonads of medical or veterinary importance is given in Figure 2.

Pentatrichomonas hominis is a parasite of the lower alimentary canal which infests a small percentage of people in temperate climates, but may affect more than 10% of children in the tropics. This species has been associated with persistant diarrhoea, especially in children. This parasite is easily separable from Trichomonas vaginalis since it has five anterior flagella and an undulating membrane which extends the full length of the body, the flagellum along its margin continuing free at the posterior end (see Figure 2). Transmission of the parasite is by the faecal-oral route.

Figure 2

Trichomonads of medical or veterinary importance



1 T. gallinae

2 T. vaginalis

3 T. tenax

4 Pentatrichomonas hominis

5 Tritrichomonas foetus

Trichomonas tenax resembles T. vaginalis in most respects, but is smaller (6-10 μM in length). This parasite inhabits the gums and roots of the teeth and was initially regarded as uncommon, but is now thought to infest 10-25% of adults (Smyth, 1976). The parasite is often found associated with advanced inflammatory pyorrhea, although its pathogenicity has not been proven. However, it may play some role, along with Entamoeba gingivalis in this disease. T. tenax is also occasionally found in bronchial and pulmonary infections. Transmission is via mouth to mouth or hand to mouth contact.

Trichomonas gallinae is mainly a parasite of young pigeons, but can also infest other birds. It is smaller than T. vaginalis (6-18 μm in length) with four anterior flagella and a recurrent flagellum ending three-quarters of the way down the body. The parasite inhabits the mouth, pharynx, oesophagus and crop, causing a disease known as 'canker', 'frounce' or 'roup' (avian trichomoniasis). This disease is often fatal in young pigeons, although nearly all adult birds are infested, but have no symptoms. Infection occurs by adult birds feeding the young by regurgitation. The organisms produce small, yellowish, spotty lesions in the anterior digestive tract which may spread to the sinuses and organs such as the heart, lungs and liver.

Tritrichomonas foetus has three anterior flagella and a recurrent flagellum which extends free for some distance beyond the hind end of the body. It is a common world-wide parasite of the

genital tract of cattle, which infests the vagina and uterus of the female and is found beneath the prepuce of the male. This is a venereal disease and infection is transmitted during coitus. In the female, the parasite attacks the mucous membrane of the vagina and invades the uterus, causing abortions and stillbirths. The infection is usually self-limiting as all the parasites are expelled with the aborted foetus and placenta. Bulls are infected for life and cannot be used for breeding unless successfully treated.

Trichomonas vaginalis is the most important of the trichomonads as it is a world-wide, common human parasite. It was first described by Alfred Francis Donné in 1836 when he noted motile microorganisms in the leukorrhea of women with vaginal discharge and genital irritation. T. vaginalis is an ovoid organism, ranging from 10-30 μM in length, and 5-15 μM in breadth. It has four anterior flagella and a recurrent flagellum and undulating membrane which ends about half-way down the body. T. vaginalis mainly infests the female vagina (Kean, 1955), but also invades skenes glands in the urethra and is occasionally found in other parts of the female urogenital system (e.g. the cervical canal). In males it occurs in the urethra and prostate. The parasite is often non-pathogenic and indeed its pathogenicity was only slowly accepted. The parasite is almost always non-pathogenic in males, though mild urethral inflammation may result. In women the parasite is responsible for the condition known as 'trichomonal vaginitis'.

1.2. TRICHOMONAL VAGINITIS

1.2.1. Transmission, symptoms and pathology

Trichomonas vaginalis is the causative agent of trichomonal vaginitis. Infection rates of 20% of unselected women have been recorded; the incidence in women with a history of vaginal upsets is higher, up to 70%. Infection was at first considered rare in males, but is now thought to be as frequent as in women.

The parasite is normally transmitted during coitus. In the laboratory a single inoculation of T. vaginalis into the vaginal cavity of Golden Hamsters has given 20-30% infection; and repeated inocula 80-90% infection (Schnitzer and Kelly, 1954), suggesting that could transmission occur easily during coitus. This is indirect evidence that transmission between humans may be as easy. Transmission by methods other than coitus have been postulated to explain how young children become infected. Infection may occur during childbirth. Transmission may also perhaps occur via damp towels and toilet seats. This may be possible as T. vaginalis is capable of surviving for 24 hrs on damp cloth.

Trichomonal vaginitis may take one of two forms:- acute and chronic. The acute form of the disease is characterised by an acid, creamy-white, foul-smelling, frothy discharge from the vagina. This discharge may be very abundant. The vulva becomes red and chafed, and the mucosa of the vagina and cervix is congested, with a deep red mottling. Some patients complain of a severe itching or

irritation in the genital region. Inflammation of the vagina of experimentally infected guinea-pigs was expressed by a dilation and hyperaemia of the vaginal blood vessels, especially under the epithelium, and by an accumulation of masses of leucocytes, trichomonads and shed epithelial cells in the discharge. This is relevant to the human condition, as the patients inflammatory response and the discharge are similar, suggesting that a similar mechanism is responsible for symptoms in both humans and guinea-pigs. In the chronic form of the disease, women have few symptoms except for an atypical discharge.

If T. vaginalis is cultured in vitro with other cells, they have adverse effects upon the other cells. T. vaginalis caused degenerative changes in HeLa cells and inhibited cell division in fibroblasts (Honigberg and King, 1964) in culture. It is not known why, but it may be due to toxins produced by T. vaginalis.

1.2.2. Diagnosis

Diagnosis of trichomonal vaginitis was originally based on the presence of trichomonads in a wet smear made of the vaginal discharge, but there has been dissatisfaction with the sensitivity of this procedure (Burch, Rees and Rearden, 1959). It has recently been shown by Fouts and Kraus (1980) that if this is the only diagnostic criterion, then over 80% of women infected with T. vaginalis, as diagnosed by the cultivation of trichomonads from a vaginal smear, will not be indetified. Diagnosis is usually confirmed by culturing

trichomonads from a vaginal smear, although even this method is not 100% effective. The staining of epithelial smears of the cervix,'Papnicolou's method' is also useful for diagnosis. The chronic form of the disease however, can be diagnosed only by culture in the majority of cases.

Serological identification using the complement fixation reaction have been attempted (Trussell, Wilson, Langwell and Laughlin, 1942) but was found to be less sensitive than the culture method. An intradermal test using prepared T. vaginalis antigen was also unsatisfactory as it gave false positive results (Adler and Sadowsky, 1947). More recently Ackers, Lumsden, Catterall and Coyle (1975) reported on antibodies to T. vaginalis, but these were not a practical diagnostic tool. Honigberg (1978) reported that a significant correlation between positive complement fixation reaction and T. vaginalis could be demonstrated, however false positive results occurred. It was concluded that this test could serve as a useful tool in epidemiological investigations, provided that the major antigenic types of T. vaginalis prevalent in the area being tested were employed as antigens. T. vaginalis has also been shown to attract polymorphonuclear leucocytes (Mason and Forman, 1980) and Gillin and Sher (1981) demonstrated that T. vaginalis activates the alternative complement pathway.

These findings show that there is further scope for Sero - logical identification systems, but as yet culturing of trichomonads gives the most reliable diagnosis in individual cases.

1.2.3. Changes in the vaginal environment

The vaginal fluid from normal, healthy women is at pH 4-4.5 (Stamey and Kaufman, 1975; Parsons, Lofland and Mullholland, 1977). The acid pH of the vagina may be due to the presence of pyruvate, glutamate (Hunter and Nicholls, 1959) and lactate (Stamey, Fair, Timothy and Chung, 1968) in the vaginal fluid. Lactate is a major metabolic end product of the main bacterial species in the vagina (Doderlein's bacilli).

The normal bacterial flora of the introitus, vagina and urethra of healthy women consists mainly of lactobacilli and staphylococci (Elkins and Cox, 1974; Pfau and Sacks, 1977). The former are also known as Doderlein's bacilli and are the major bacterial flora of the vagina. Gram-negative bacteria are also found in approximately 25% of cases (Elkins and Cox, 1974) and Candida albicans is also present in many women (Goldacre, Watt, Milne, Louden and Vessey, 1979).

Infestations of Trichomonas vaginalis are associated with a reduced vaginal acidity and a change in the bacterial flora of the vagina. The vaginal pH of women with trichomonal vaginitis is pH 5-6 (Parsons, Lofland and Mullholland, 1977) and the predominant microbial flora are gram-negative enteric bacteria, mainly Escherichia coli, with a heavier introital colonization by colonic organisms, such as Streptococcus faecalis, than normal women (Parsons, Lofland and Mullholland, 1977; Pfau and Sacks, 1981). Lactobacilli were rarely found (Thadepalli, Gorbach and Kieth, 1973).

Strang and Timothy (1975) suggested that the low vaginal pH in normal wo men (approximately pH 4) may be a significant defense mechanism. The change in vaginal pH is presumably correlated with the change in bacterial flora. Vaginal fluid from normal women was bactericidal to Escherichia coli, but when the pH was increased with NaOH, the same fluid supported bacterial growth. Also, Yeaw (1940) found that most bacteria grew well in urine at pH 5-7, but were killed below pH 4.4. The acidification of urine as a means of treating urinary infections was used for many years. It was suggested that the presence of Trichomonas vaginalis in the vagina, and the concomitant decrease in acidity, may act as a trigger for the establishment of a predominantly gram-negative colonization of the vagina. It has also been suggested that the bactericidal activity of vaginal fluid may be due to an antimicrobial factor, as has been found in prostatic fluid (Stamey, Fair, Timothy and Chung, 1968), but it has never been identified.

Although T. vaginalis is referred to as an anaerobic organism, it is likely that the tissues where it lives are not completely anaerobic. For instance, the epithelial cells will be well provided with oxygen in the blood. However, there is no detailed information available about the amount of oxygen present in the different parts of the urogenital tract.

1.3. PARASITE CULTIVATION

Since the introduction of penicillin and streptomycin to inhibit bacterial growth, the cultivation *in vitro* of *Trichomonas vaginalis* (Adler and Pulvertaft, 1944; Johnson, Trussell and Jahn, 1945; Magara, Amino and Yokouti, 1953) has become relatively easy. Until recently all culture media have contained serum of one type or another. However, Linstead (1981) developed a serum-free defined medium which supported continuous growth. Most media are used at relatively low oxygen tensions and the redox potential of many culture media are decreased by adding cysteine HCl. The pH is usually adjusted to pH 5.5-6.0 and glucose or maltose used as a source of energy. Most media contain protein digests of some type. The media used most frequently are modifications of Johnsons CPLM (Johnson, 1947), Diamonds medium (Diamond, 1957) and Bushby's medium (Bushby and Copp, 1955). Other anaerobic protozoa can also be cultivated *in vitro* using similar media and conditions. *Entamoeba histolytica* is cultivated in the presence of low oxygen tensions (Diamond, 1968; Wittner, 1968) as is *Giardia lamblia* (Gillin and Diamond, 1980b).

Although the axenic culturing of trichomonads is now routine, it has been reported that *Trichomonas vaginalis* and related organisms may become less virulent after prolonged *in vitro* cultivation (Lindgren and Ivey, 1964; Stabler, Honigberg and King, 1964; Honigberg, Stabler, Livingston and Kulda, 1970; Dwyer and Honigberg, 1970). However, loss of virulence can be prevented by

storage of the organisms in liquid nitrogen, thus making possible long-term studies using strains of constant characteristics. T. vaginalis has been shown to retain its original virulence after two years storage in liquid nitrogen (Diamond et al., 1965) using a strain of parasites isolated several years previously, and Ivey (1965) has demonstrated this using a freshly isolated strain.

The growth of T. vaginalis in vivo has been attempted using a variety of hosts and locations within the host. Schnitzer and Kelly (1954) succeeded in infecting the vagina of Golden Hamsters. The infection persisted for over one year and was transmissable. The growth and transmission of T. vaginalis from the vagina of rats has also been attempted, the infections persisting and the number of parasites multiplying. Subcutaneous innoculation of trichomonads produced lesions containing large numbers of flagellates (Honigberg, 1961), although infections of this type only persist for a few weeks. However, this type of culture has been used in many studies, including drug activity and parasite virulence. Intraperitoneal innoculation of trichomonads produced flagellate containing lesions, but again this type of infection only persists for a few weeks.

In vivo methods of cultivation are neither as quick or as reliable as in vitro cultivation methods and are mainly used today for the testing of compounds for in vivo trichomonacidal activity.

1.4. BIOCHEMISTRY OF TRICHOMONADS

The biochemistry of trichomonads is not yet fully elucidated, although our knowledge has increased markedly over the last forty years. The first axenic cultures of Trichomonas vaginalis were isolated in 1944 by Adler and Pulvertaft. The use of the axenic culture method was followed by an upsurge in the interest shown about trichomonal biochemistry.

1.4.1. Energy Metabolism

1.4.1.1. Substrates

The utilization of the sugars glucose, maltose and dextrin by Trichomas vaginalis was first reported by Trussell and Johnson (1941), despite the difficulty of the interpretation of data in a culture which was not axenic. However, this work was confirmed and expanded by Ninomiya and Suzuoki (1952) who demonstrated the rapid uptake of glucose and maltose, and a slower pyruvate uptake. These workers also reported a low level of lactate uptake. This has never been confirmed by other workers however, and it is now generally agreed that it does not occur. Succinate, citrate, fumarate, acetate and several amino acids were shown not to be suitable substrates (Ninomiya and Suzuoki, 1952). Read (1957) confirmed the uptake of glucose and galactose in T. vaginalis and Doran (1958) reported the uptake of these sugars and of mannose, fructose, sucrose, trehalose, glycogen, starch and pyruvate by four strains of Tritrichomonas foetus.

It appears however, that the main substrates of Trichomonas vaginalis are glucose, maltose and glycogen and of Tritrichomonas foetus are glucose and glycogen (Muller, 1976). The substrates used by Trichomonas vaginalis correlate well with the parasites environment, as vaginal fluids have been shown to contain glucose, maltose, glycogen, lactose, pyruvate and a range of amino acids (Hunter and Nicholas, 1959).

1.4.1.2. Intermediary Metabolism

The uptake of glucose and glycogen, and the large reserves of glycogen in the cell (Manners and Ryley, 1955; Ryley, 1955; Wellerson and Kupferberg, 1962) suggested the existence of an Embden-Meyerhof system in trichomonads. Wellerson and Kupferberg (1962) were unable to detect phosphorylase (glycogen $\rightleftharpoons$ glucose-1-phosphate) in T. vaginalis, although it is now known to be present, and phosphoglucomutose, the enzyme mediating the conversion of glucose-1-phosphate to glucose-6-phosphate, has been demonstrated in both T. vaginalis (Wirtschafter and Jahn, 1956) and Tritrichomonas foetus (Lindblom, 1961). Hexokinase, glucose-phosphate-isomerase and phosphofructo kinase have been found in both Trichomonas vaginalis (Wirtschafter, 1954; Wirtschafter and Jahn, 1956) and in Tritrichomonas foetus (Ryley, 1955; Lindblom, 1961). Aldolase was found in Trichomonas vaginalis in 1954, and this was confirmed by Baernstein (1955) who reported that this enzyme was soluble, and metal activated, ferrous or cobalt being the most effective. Enzyme

activity was enhanced by cysteine or thioglycolate and inhibited by EDTA. Aldolase in Tritrichomonas foetus and triose phosphate isomerase in both species have also been detected (Ryley, 1955; Lindblom, 1961; Wellerson and Kupferberg, 1962). Glyceraldehyde-3-phosphate dehydrogenase, 3-phosphoglycerate kinase, 3 phospho-glyceromutase, enolase and pyruvate kinase, the enzymes responsible for completing the glycolytic pathway up to the production of pyruvate, have all been demonstrated in Trichomonas vaginalis (Arese and Cappuccinelli, 1974). These enzymes are not attached to any membrane structure and are localised in the cytosol.

Arese and Coppuccinelli (1974) also reported the presence of glucose-6-phosphate dehydrogenase (G6PDH), 6-phosphogluconate dehydrogenase (6PGDH) and transaldolase, three of the enzymes of the pentose phosphate cycle, in T. vaginalis, and suggested that this cycle is functional in this organism. Although the low activities of G6PDH and 6PGDH and hence the capacity of the oxidative portion of the cycle were shown to be very low, the high activity of transaldolase points to the importance of the anaerobic portion, which provides the cell with pentoses and deoxypentoses for nucleic acid synthesis.

All attempts to demonstrate the presence of a functional Krebs cycle have failed and only Kuntake, Stitt and Saltman (1962) indicated a possibility of the tricarboxylic acid cycle being operative. The absence of mitochondria (Smith and Stewart, 1966) and of detectable cytochromes (Inoki, Nakanishi and Nakabayashi, 1959; Ryley, 1955;

Loyd, Lindmark and Müller, 1979) correlates well with the anaerobic nature of the parasite.

In cultures of Tritrichomonas foetus the fermentation of sugars in the culture medium, or of endogenous glycogen if there are no sugars present, under aerobic conditions, leads to the production of succinate, acetate, carbon dioxide, ethanol and small amounts of pyruvate (Ryley, 1955; Lindblom, 1961). Under anaerobic conditions the same end products were excreted, and in addition there is hydrogen production (Suzuoki and Suzuoki, 1951; Lindblom, 1961).

The organic end products of Trichomonas vaginalis carbohydrate metabolism are lactate (Kupferberg et al, 1953; Read and Rothman, 1955; Wellerson, Doscher and Kupferberg, 1960), acetate (Mack and Muller, 1980), small amounts of malate (Wellerson, Doscher and Kupferberg, 1959) and pyruvate (Kupferberg et al, 1953) under both aerobic and anaerobic conditions. Under anaerobic conditions H_2 and CO_2 are produced and under aerobic conditions O_2 was consumed and CO_2 produced (Kupferberg et al, 1953; Read and Rothman, 1955; Mack and Muller, 1980).

1.4.1.3. Pyruvate catabolism in the hydrogenosome

In the hydrogenosomes, pyruvate is catabolised to acetate, ATP, CO_2 and H_2. The mechanism of hydrogen formation in the hydrogenosomes of trichomoads is of considerable interest as in fermentative prokaryotes there are two routes by which this can occur.

In the Enterobacteriace, formate is the proximal substrate of the 'formate hydrogenlyase' system, which produces hydrogen.

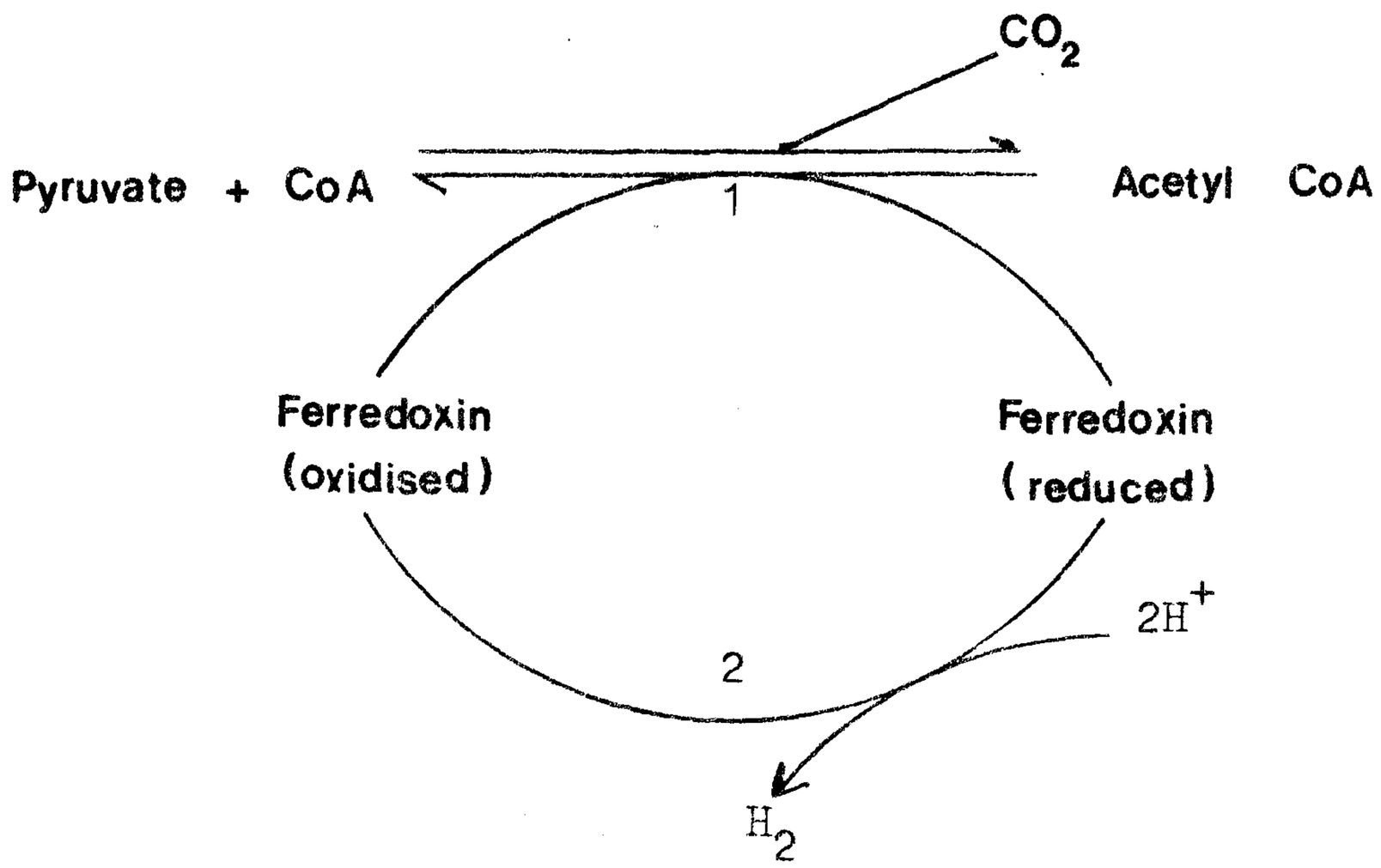
$HCOOH + fumarate \longrightarrow CO_2 + Succinate + H_2$ (After Gest, 1954).

This system has been elucidated using cell-free preparations (Gest, 1952; Barkulis and Gest, 1953) and in intact cells (Stephenson and Stickland, 1932).

In Clostridia and many other strict anaerobes, the oxidation of pyruvate produces reducing equivalents leading to hydrogen production, a process that used to be known as 'phosphoroclastic' pyruvate metabolism. The term 'phosphoroclastic' is actually a misnomer in this case, as acetyl phosphate is not a primary product, but the result of a subsequent reaction of acetyl CoA. The term 'phosphoroclastic' was coined to describe the cleavage of pyruvate to acetyl phosphate and formate by colon-aerogenes bacteria. In Clostridia, however, the pathway involves the oxidative decarboxylation of pyruvate resulting in the formation of acetyl CoA (Koepsell and Johnson, 1942; Gest, 1954), (Figure 3). This results in the reduction of an electron carrier protein with a low redox potential (Mortenson, Valentine and Carnaham, 1962). This was found to contain iron, but no detectable haeme or flavin, and was named ferredoxin (Mortenson, Valentine and Carnaham, 1962). The role of ferredoxin was found to be the transfer of electrons from pyruvate:ferredoxin oxidoreductase to hydrogenase in the formation of hydrogen from pyruvate (Mortenson, Valentine and Carnaham, 1962, 1963; Valentine and

FIGURE 3

THE ROLE OF FERREDOXIN IN CLOSTRIDIUM sp.



1. Pyruvate: ferredoxin oxidoreductase

2. Hydrogenase

Wolfe, 1963). Ferredoxin is the most electronegative electron carrier yet found in the oxidation-reduction chain in bacteria (Valentine, 1964). A diagramatic representation of the role of ferredoxin is given in Figure 3.

Methyl viologen was found to enhance the rate of activity of the reaction catalysed by cell-free extracts of Clostridium butyricum and to reactivate preparations that had lost activity (Mortlock, Valentine and Wolfe, 1959). It was suggested that it can substitute for ferredoxin in the catabolism of pyruvate as described. This pathway can also operate in the reverse direction, with the production of pyruvate. The enzyme catalysing this reaction was called pyruvate synthase, but probably is the same protein as pyruvate:ferredoxin oxidoreductase.

The reaction catalysed by pyruvate:ferredoxin oxidoreductase requires coenzyme A, but not phosphate. Hydrogenase could be replaced by oxygen, but under these conditions, of course, pyruvate oxidation did not result in hydrogen production. Carbon monoxide inhibited the formation of H_2 and the oxidation of reduced ferredoxin by hydrogenase, but did not inhibit the oxidation of reduced ferredoxin by oxygen. Both pyruvate:ferredoxin oxidoreductase and hydrogenase were found to be sensitive to oxygen, although ferredoxin protected pyruvate:ferredoxin oxidoreductase when present (Mortenson, Valentine and Carnaham, 1963).

The above pathways differ from the oxidative decarboxylation of pyruvate in mitochondria and in many microorganisms. In these cases

NAD^+ is the final electron acceptor and the enzyme cannot donate electrons to ferredoxin or to some other similar electron transport protein. The reoxidation of NADH usually occurs in the electron transport chain with O_2 as the terminal electron acceptor.

The nature of the hydrogen producing system in trichomonads has been under investigation for some time. The formate hydrogenlyase system has not been detected in Trichomonas vaginalis (Ninomiya and Suzuoki, 1952; Edwards and Mathison, 1970) or in Tritrichomonas foetus (Bauchop, 1971). However, a hydrogenosomal "phosphoroclastic-type" system, similar to that observed in Clostridia and utilizing hydrogenase has been observed (Edwards and Mathison, 1970; Bauchop, 1971; Lindmark and Muller, 1973a). Pyruvate:ferredoxin oxidoreductase and hydrogenase have been found in all the species of trichomonads studied to date; in Trichomonas vaginalis (Lindmark, Muller and Shio, 1975), in Tritrichomonas foetus (Lindmark and Muller, 1973a) and also in Monocercomonads (Lindmark and Muller, 1974). Lindmark, Muller and Shio (1975) noted that both enzymes function with exogenous clostridial ferredoxin or methyl viologen as electron acceptors, but not NAD^+ or $NADP^+$, and that these enzymes were sensitive to oxygen and required thiol compounds for their activity. The electron transport protein linked to this system has not yet been identified, however, the ability of the enzymes to function with ferredoxin, and the overall similarity of the pathway to that found in Clostridia, suggests that a ferredoxin-like protein participates in this process in trichomonads (Cerkasova, 1970; Lindmark and Müller, 1973b). In addition, the presence of iron-sulphur proteins and flavoproteins have been suggested in Tritrichomonas foetus (Müller, 1980).

The enzyme associated with the formation of acetyl CoA from pyruvate in trichomonads has been named pyruvate:ferredoxin oxidoreductase in the expectation that the electron transport molecule involved will be found to be a ferredoxin.

1.4.1.4. Hydrogenosomes and carbohydrate metabolism in other anaerobic protozoa

The biochemical characteristics of hydrogenosomes suggested that if they are present in organisms other than trichomonads, they will be most likely to occur in protozoa from anaerobic habitats. One such group is the Holotrich ciliates of the rumen (Anderson and Dumont, 1966). The absence of mitochondria and presence of microbody-like organelles have also been reported in ciliates from the bottom sediments of oceans (Fenchel, Perry and Thane, 1977). In neither of these cases, however, is there biochemical evidence for the identification of organelles as hydrogenosomes. However, it was recently reported that hydrogenosomes had been identified in Dasytricha (Yarlett, Hann, Loyd and Williams, 1981; Yarlett, Loyd and Williams, 1982).

In some protozoa both mitochondria and hydrogenosome-like structures are lacking. Entamoeba histolytica is the causative organism of amoebic dysentery and is an aerotolerant anaerobe (Band and Cirrito, 1979) lacking cytochromes. The end products of the catabolism of glucose by E. histolytica are CO_2, acetate and ethanol (Montalvo, Reeves and Warren, 1971). There is evidence that

this organism utilizes the glycolytic pathway to metabolise glucose to pyruvate (Reeves, 1972) and metabolises pyruvate to acetate in a process involving an electron transfer protein (Weinbach, Diamond and Claggett, 1976). The proposed enzyme system is similar to the pyruvate:ferredoxin oxidoreductase system present in Trichomonas. However, this system is localised in the cytoplasm (Reeves, Warren, Susskind and Lo, 1977; Lindmark, 1976b) and there is no evidence of hydrogenosomes.

Giardia lamblia is a flagellate parasite of the order Diplomonadida, which inhabits the human intestinal tract and causes 'traveller's diarrhoea' (Giardiasis). G. lamblia is another aerotolerant anaerobe which lacks mitochondria (Friend, 1966). The end products of glucose metabolism by G. lamblia are CO_2, acetate and ethanol (Lindmark, 1980). The initial steps of carbohydrate metabolism in this organism are those of classical glycolysis (Lindmark, 1980). Pyruvate is formed from phosphoenol pyruvate (PEP) by two pathways; pyruvate kinase forms pyruvate from PEP with the phosphorylation of ADP; or the oxaloacetate formed from PEP by PEP carboxy kinase can be reduced to malate and then to pyruvate by the sequential action of malate dehydrogenase and a decarboxylating malate dehydrogenase. The pyruvate formed is oxidised to acetyl CoA, a step involving a ferredoxin-like protein (Lindmark, 1980; Weinbach, Claggett Keister and Diamond, 1980) and acetate is formed from acetyl CoA by acetyl CoA synthetase (Lindmark, 1980). This system is similar to that proposed for Entamoeba histolytica, and is

localised in the cytoplasm, again there being no evidence of hydrogenosomes.

Thus, in trichomonads, Entamoeba spp. and Giardia lamblia, the production of acetate is accomplished by similar pathways, however it is only in Trichomonas spp. that the production of acetate from pyruvate occurs in hydrogenosomes.

1.4.1.5. Endproducts

The endproducts of the energy metabolism of Trichomonas vaginalis and Tritrichomonas foetus have been extensively investigated and have been found to differ, although in both species the carbon originating from the utilized carbohydrates are excreted in the form of organic acids (Honigberg, 1978).

1. Acetate production

In prokaryotes such as Clostridia, acetyl phosphate is an intermediate in acetate production (See Figure 4). However, acetyl phosphate and this pathway have not been found in Tritrichomonas foetus (Lindmark, 1976a), the most studied trichomonad to date.

In eukaryotes it has been postulated that acetate production is by way of acetyl-CoA synthetase working in the reverse direction, that is towards acetate production (Saz, 1970), but this enzyme has not been detected in trichomonads.

Lindmark (1976a) reported two methods of acetate formation in T. foetus. Firstly the direct production of acetate from acetyl

CoA by acetate thiokinase, involving a substrate level phosphorylation of ADP or GDP, and requiring Mg^{2+}. Secondly, the sequential action of two enzymes; acetyl CoA:succinate transferase, producing succinyl CoA and acetate, and succinate thiokinase, converting succinyl CoA to succinate with the simultaneous phosphorylation of ADP or GDP (See Figure 5).

2. Malate production

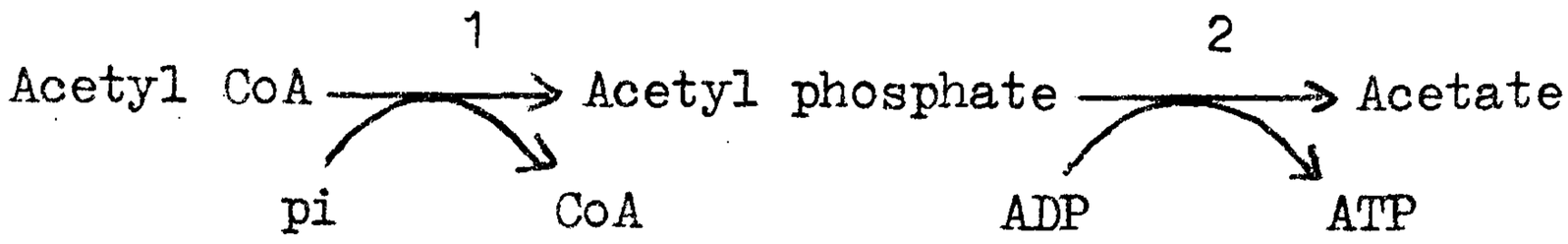
The production of malate by Trichomonas vaginalis does not involve the "phosphoroclastic" system. In this case, oxaloacetate is formed from PEP by CO_2 fixation involving PEP carboxy kinase, and the phosphorylation of GDP (Arese and Cappuccinelli, 1974). Oxaloacetate is then reduced to malate by malate dehydrogenase. In T. vaginalis the malate produced by this pathway is either excreted, or transported into the hydrogenosomes and converted to pyruvate by NAD(P) linked decarboxylating malate dehydrogenase (Brugerolle and Metenier, 1973).

3. Succinate production

The production of succinate by Tritrichomonas foetus involves the production of malate by an identical pathway to that reported for Trichomonas vaginalis. Succinate is formed from malate by fumarate hydratase and fumarate reductase (Muller and Lindmark, 1974). This takes place in the cytosol. T. vaginalis does not produce succinate.

Figure 4

Acetate production in Clostridia sp.

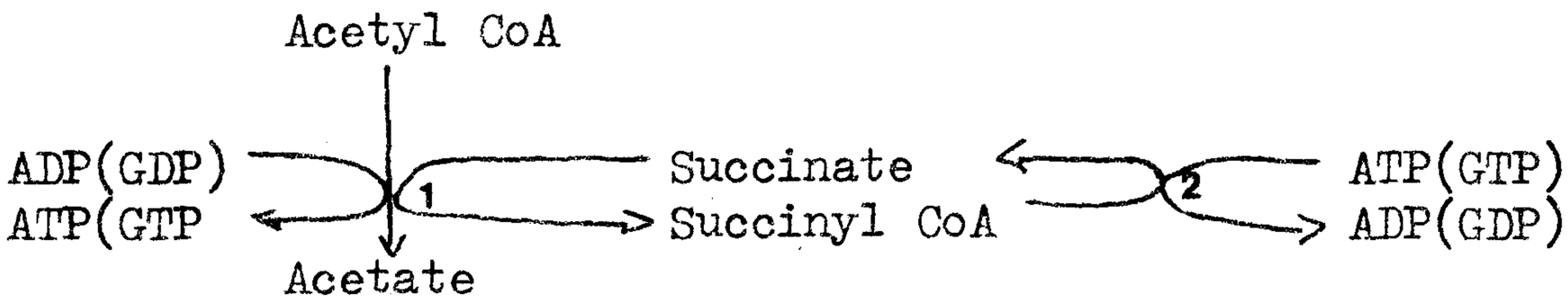


1. Phosphotransacetylase

2. Acetate kinase

Figure 5

The formation of acetate in Trichomonads



1. Acetyl CoA: Succinate transferase

2. Succinate thiokinase

4. Lactate production

Lactate is produced from pyruvate by T. vaginalis in a reaction catalysed by Lactate dehydrogenase (Wirtschafter and Jahn, 1956; Wellerson and Kupferberg, 1962). Lactate is not produced by Tritrichomonas foetus.

5. Ethanol production

The production of ethanol by T. foetus involves the decarboxylation of pyruvate to acetaldehyde and a subsequent reduction catalysed by alcohol dehydrogenase. Trichomonas vaginalis does not produce ethanol.

6. H_2 and CO_2 production

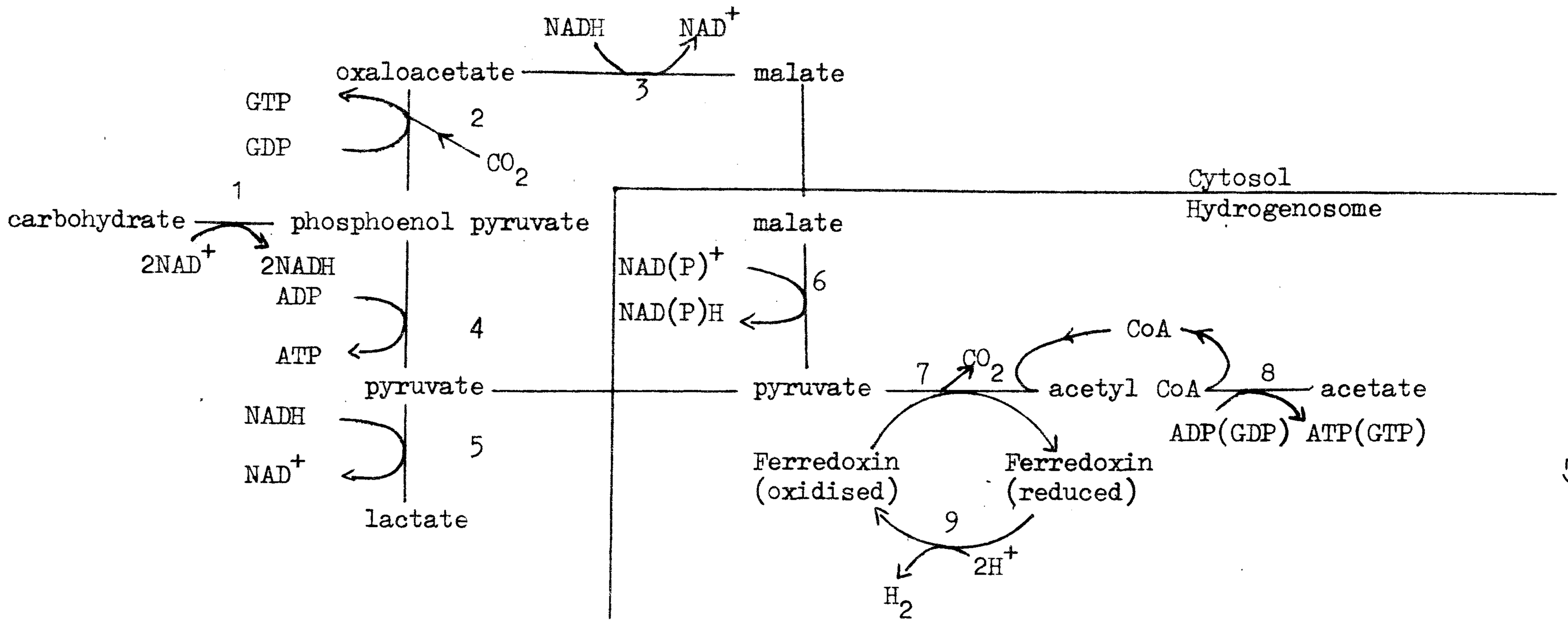
The pathways involved in the production of these gases are dealt with in detail in section 1.4.1.3.

A summary diagram of the energy metabolism of Trichomonas vaginalis is given in Figure 6.

1.4.1.6. Aerobic metabolism

It has been shown that under aerobic conditions trichomonads show intense respiration (Kupferberg et al, 1953; Read and Rothman, 1955; Müller and Lindmark, 1978) and that exogenous carbohydrates stimulate respiration (Čerkasov, Čerkasovová, Kulda and Vilhelmova, 1978). Cell respiration is insensitive to cyanide, amytal, rotenone, iodoacetate and several other inhibitors (Ryley, 1955). It was shown by Ryley (1955) that under anaerobic conditions all of the carbon

Figure 6. Metabolic map of Trichomonas vaginalis



1. Glycolytic enzymes; 2. Phosphoenol pyruvate carboxykinase; 3. Malate dehydrogenase; 4. Pyruvatekinase; 5. Lactate dehydrogenase; 6. Malate dehydrogenase (decarboxylating); 7. Pyruvate:ferredoxin oxidoreductase; 8. Deacetylation of CoA; 9. hydrogenase.

Modified from Muller (1980).

of utilised carbohydrate can be accounted for in the acetate and succinate which is produced in equimolar quantities by Tritrichomonas foetus. However, under aerobic conditions, Ryley (1955) found that there was a greater release of carbon as acetate and a decrease in succinate production, and that hydrogen production ceased. It was suggested by Müller (1976) that this would occur if O_2 served as an electron acceptor, diverting the flow of reducing equivalents, suppressing the reduction of oxaloacetate to malate and stimulating the conversion of pyruvate to acetate. Since electrons from reduced ferredoxin are transferred to oxygen instead of to protons, hydrogen production would not take place. As acetate formation from PEP yields two moles of ATP and succinate formation yields only one, the shift in metabolism under aerobic conditions is advantageous in terms of energy yield (Čerkasová, 1970). However, the parasite does not benefit from this higher energy yield as one might expect, that is, by growing faster. Under aerobic conditions trichomonads grow more slowly than under anaerobic conditions. This suggests that growth may be limited by something other than energy availability, and that the detoxification of oxygen metabolites may require energy. Indeed, oxygen detoxification may require more energy than the extra produced under aerobic conditions, and may explain why the cells grow more slowly under aerobic conditions.

The hydrogenosomal membrane has been shown to be impermeable to NADH (Čerkasovová and Čerkasov, 1976) and although hydrogenosomes are able to oxidise NADH at a low rate (Čerkasov, Čerkasovová, Kulda and Vilhelmova, 1978), the predominant NADH oxidase is localised

in the cytoplasm (Brugerolle and Metenier, 1973; Čerkasovová and Čerkasov, 1974; Tanabe, Asami and Kobayashi, 1977; Tanabe, 1979). It was reported by Tanabe (1979) that one reaction product of NADH oxidase was NAD^+, and that ratio of oxygen uptake to NADH oxidation was approximately 1:2. The enzyme was substrate specific for NADH and would not accept NADPH. Catalase effected neither NAD^+ formation nor NADH oxidation and hydrogen peroxide was not produced during the reaction. Tanabe (1979) suggested that the following reaction takes place (See Figure 7).

Figure 7. The action of NADH oxidase

$$NADH + H^+ + \tfrac{1}{2} O_2 \xrightarrow{1} NAD^+ + H_2O \qquad \text{1.NADH oxidase}$$

That NADH oxidase has as one of its products H_2O is of interest. As Trichomonas vaginalis has no catalase, peroxidase or NADH peroxidase (Tanabe, Asami and Kobayashi, 1977), it is necessary for this parasite to produce H_2O and not H_2O_2 as a product of oxygen metabolism. NADPH oxidase has also been found in trichomonads and is thought to catalyse a reaction similar to that catalysed by NADH oxidase, but probably producing H_2O_2.

It has been postulated that NADH oxidase and NADPH oxidase are involved in the removal of oxygen from the cytoplasm of the parasite (Müller, 1982). Under aerobic conditions oxygen entering the cell may be reduced by NADH oxidase and NADPH oxidase in the cytoplasm, therefore maintaining the anaerobic integrity of the hydrogenosomes.

These enzymes probably account for the major part of the trichomonal O_2 consumption under aerobic conditions. Their operation would lead to a lowering in the NAD(P)H concentration in the cell and hence tend to push the metabolism of PEP away from malate production to acetate production.

It is not unusual for a supposedly anaerobic organism to tolerate and even utilise oxygen when it is present. Other anaerobic protozoa have been shown to be tolerant to oxygen. Entamoeba histolytica and related species are aerotolerant, and often infect aerobic tissues (e.g liver). It has been reported that E. histolytica has a limited ability to detoxify products of oxygen (Band and Cirrito, 1979) and that it is capable of growth in vitro under low oxygen tensions (Gillin and Diamond, 1980a). Giardia lamblia is another protozoan which is supposedly anaerobic, but it is known to consume oxygen if present (Weinbach, Claggett, Keister and Diamond, 1980).

1.4.2. Protein metabolism

The mechanisms of mammalian and bacterial protein synthesis have been studied extensively. The mechanisms are similar in the two groups, differing only in detail. However, these differences constitute the basis of activity of many antibacterial drugs. Protein synthesis in parasitic protozoa has been investigated very little despite its potential importance. Available evidence suggests it is more similar to the eukaryotic process than that of prokaryotes. Little has been reported on Trichomonads.

Protein breakdown is carried out, in part, by enzymes most broadly referred to as 'proteases' or 'proteinases', the two terms often being considered interchangeable. Proteinases are grouped into four classes: Serine, Thiol, Aspartic and Metallic (Hartley, 1960). The activity of the thiol or 'cysteine' proteinases is dependant upon the thiol group of a cysteine residue at the active site (Light, Frater, Kimmel and Smith, 1964). There are few, if any drugs of proven value that undoubtably act by proteinase inhibition, however there are clear indications that proteinases mediate a range of processes that may be potential targets for rational chemotherapy (Barrett, 1980).

A number of pathogenic organisms have been shown to make use of proteinases in entering the host body or living there (Barrett, 1980), and thiol proteinases are numbered amongst the proteinases so used. Thiol proteinases may be involved in the processing of the precursors of proteolytic proteins in Rhinoviruses (Korant, 1975; Dittmar and Moelling, 1978), and have been reported in adult schistosomes (Timms and Bueding, 1959; Dresden, Rutledge and Chappell, 1981). Thiol proteinases have been reported to be present in a range of protozoan parasites. In Leishmania mexicana mexicana amastigotes and promastigotes (Coombs, 1982), in the promastigotes of L. donovani and L. braziliensis (Camargo, Itow and Alfieri, 1978; Steiger, Van Hoof, Bontemps, Nyssens-Jadin and Druetz, 1979), in Trypanosoma brucei (Sieger et al, 1979), Trypanosoma cruzi (Bongeretz and Hungerer, 1978), in Entamoeba histolytica (McLaughlin and Faubert,

1977) and from the free-living ciliate Tetrahymena pyriformis (Viswanatha and Liener, 1956; Dickie and Liener, 1962; Levy, Sisskin and McConkey, 1976). The presence of more than one thiol proteinase has been suggested for this organism (Dickie and Liener, 1962). In malaria parasites, only acid (Levy and Chou, 1974) and EDTA-sensitive alkaline proteinases (Cook, Grant and Kermack, 1961) have been reported, however, and therefore thiol proteinases may not be present in all protozoan species.

Thiol proteinase activity has been reported for Tritrichomonas foetus (McLaughlin and Müller, 1979) and Trichomonas vaginalis (Coombs, 1982). The proteinase isolated from Tritrichomonas foetus resembled the mammalian intracellular thiol proteinase cathepsin B. However, the T. foetus enzyme had a pH optimum at pH 7.0, while the optimum for cathepsin B is in the range of pH 3.5 to pH 5.0 (Otto, 1971). In addition, the T. foetus enzyme was not labile on exposure to pH values above pH 7.0, unlike mammalian cathepsin B (de lumen and Tappel, 1972). Cell fractionation studies demonstrated that T. foetus proteinase was associated with a population of large subcellular particles, containing other neutral hydrolases (McLaughlin and Müller, 1979), that are located posteriorly in the parasite. These structures are closely related to lysosomes, differing only in the neutral pH optima of their hydrolases. Thiol proteinases activity has also been located in a similar fraction of Trichomonas vaginalis (Wink, unpublished data), suggesting a similar location to that found in Tritrichomonas foetus. The part played by the trichomonal

thiol proteinases are yet to be fully elucidated.

1.4.3. Xenobiotic metabolism

The ability of trichomonads to metabolise potentially toxic compounds other than nitro-compounds has been little studied. Since the most successful antitrichomonal compound to date (metronidazole) is a nitroimidazole, the metabolism of nitro-compounds has been studied extensively (Stambaugh, Feo and Manthei, 1967; Ings and McFadzean, 1975; Perez-Reyes, Kalyanaraman and Mason, 1980). The metabolism of metronidazole is reductive in trichomonads. Trichomonads are anaerobes that grow optimally in the absence of oxygen and so it was of interest to investigate the metabolic capabilities of trichomonads with respect to compounds whose metabolism in the mammal involves oxidative steps. Any inability of the parasite to utilise such oxidative pathways could perhaps be exploited in the design and development of new anti-tritrichomonal agents.

Two compounds whose metabolism in the mammal involves hydroxylation and reductive steps are nitrobenzene and azobenzene. Both compounds have been extensively studied in the mammal, but have not been previously investigated with Trichomonas vaginalis.

1.4.3.1. Nitrobenzene

Nitrobenzene was originally used under the name 'oil of mirbane' or 'artificial oil of bitter almonds' for flavouring and perfuming

purposes, although its use today is in the industrial manufacture of aniline and benzidine.

The older literature suggests that the main metabolite of nitrobenzene found in mammalian urine (Crowdle and Sherwin, 1923) is p-aminophenol and its conjugates. A more recent investigation of the fate of nitrobenzene in the mammal was undertaken by Robinson, Smith and Williams (1951a) who reported that nitrobenzene undergoes both reductive and oxidative steps in vivo, and that both amino- and nitro-compounds are excreted as metabolites, although the authors could only account for approximately 55% of the nitrobenzene in the urine. They reported that nitrobenzene and o-nitrophenol occurred in small quantitites in the urine, and that m- and p-nitrophenol, and p-nitrocatechol occurred in relatively large amounts. They also showed that the production of p-nitrocatechol resulted from the oxidation of nitrophenols in the mammal (Robinson, Smith and Williams, 1951b). Nitroquinol could be similarly formed, however they were unable to detect the presence of nitroquinol as a metabolite of nitrobenzene. Small amounts of aniline, and o- and m-aminophenol were detected (Robinson et al, 1951a) but p-aminophenol was the main metabolite. It was suggested that as the urine from nitrobenzene-fed animals was not highly reducing, unlike the urine produced by aniline-fed animals (Smith and Williams, 1949), and aniline was not produced in large amounts, that the formation of p-aminophenol did not arise via aniline or p-nitrophenol. This was refuted by Parke (1956) who accounted for over 85% of the nitrobenzene

taken by the mammal. 58% of this was found in the urine, the rest being in the faeces (9%) and in the tissues (15-20%) of the animal. Parke (1956) confirmed the presence of the metabolites previously reported and also noted two additional urinary metabolites, nitroquinol and p-nitrophenolmercaptic acid, as well as a yield of CO_2 (See Table 1). The denitration products, phenol and catechol were not detected in the urine, neither were nitrosobenzene, azoxybenzene, azobenzene and benzidine, suggesting that nitroso-benzene and phenylhydroxylamine were not intermediates in the formation of the p-aminophenol produced. A diagram of the main steps in the mammalian metabolism of nitrobenzene is given in Figure 8.

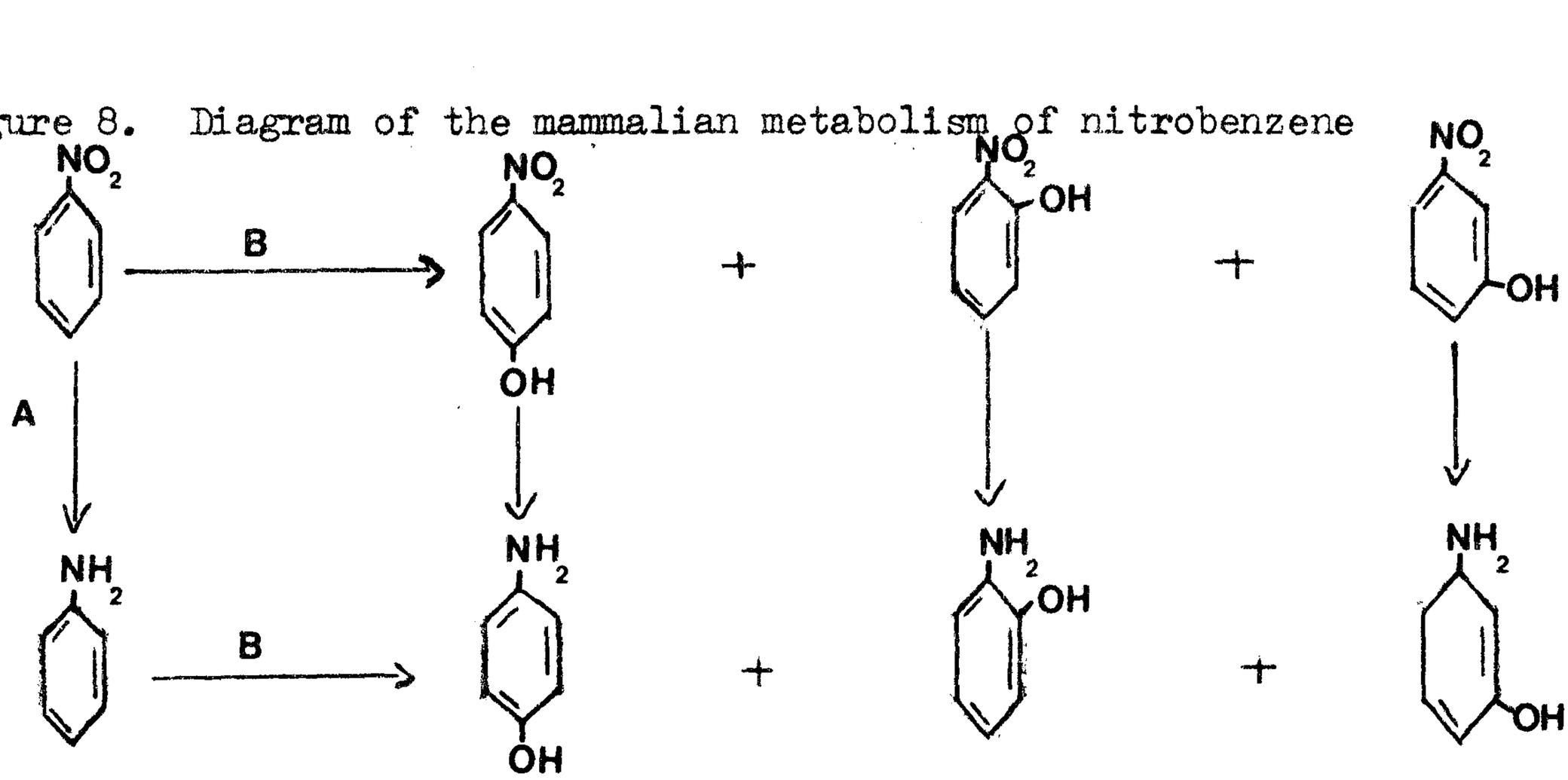
The metabolism of nitrobenzene and related nitrocompounds has been investigated in organisms other than mammals. Douch and Blair (1975) demonstrated nitroreductase activity in the cestode Moniezia expansa and the nematode Ascaris lumbricoides var suum. Both species readily reduced a variety of compounds to their corresponding amines. Oxidative products were not found. Douch also reported on the effect of various inhibitors and activators, and gave the pH optima and cofactor requirements, for the nitro-reductases of both species (Douch, 1975a; 1975b; 1975c; 1976). The results suggested that the worms were able only to reductively metabolise the nitro-compounds and that the enzymes involved were similar to those in mammals.

TABLE 1. The metabolic fate of an oral dose of ^{14}C-nitrobenzene in the rabbit 4-5 days after dosing.

Metabolite	% of dose	Metabolite	% of dose
Respiratory CO_2	1.0	O-aminophenol	3.0
Nitrobenzene	0.6	M-aminophenol	4.0
Aniline	0.4	P-aminophenol	31.0
O-nitrophenol	0.1	Nitrocatechol	0.7
M-nitrophenol	9.0	Nitroquinol	0.1
P-nitrophenol	9.0	P-nitrophenylnogstic acid	0.3

Modified after Parke (1956)

Figure 8. Diagram of the mammalian metabolism of nitrobenzene



Aniline P-aminophenol O-aminophenol M-aminophenol

A. Reductive step

B. Oxidative step

Only the main metabolic products are given.

Modified from Williams (1959a).

1.4.3.2. Azobenzene

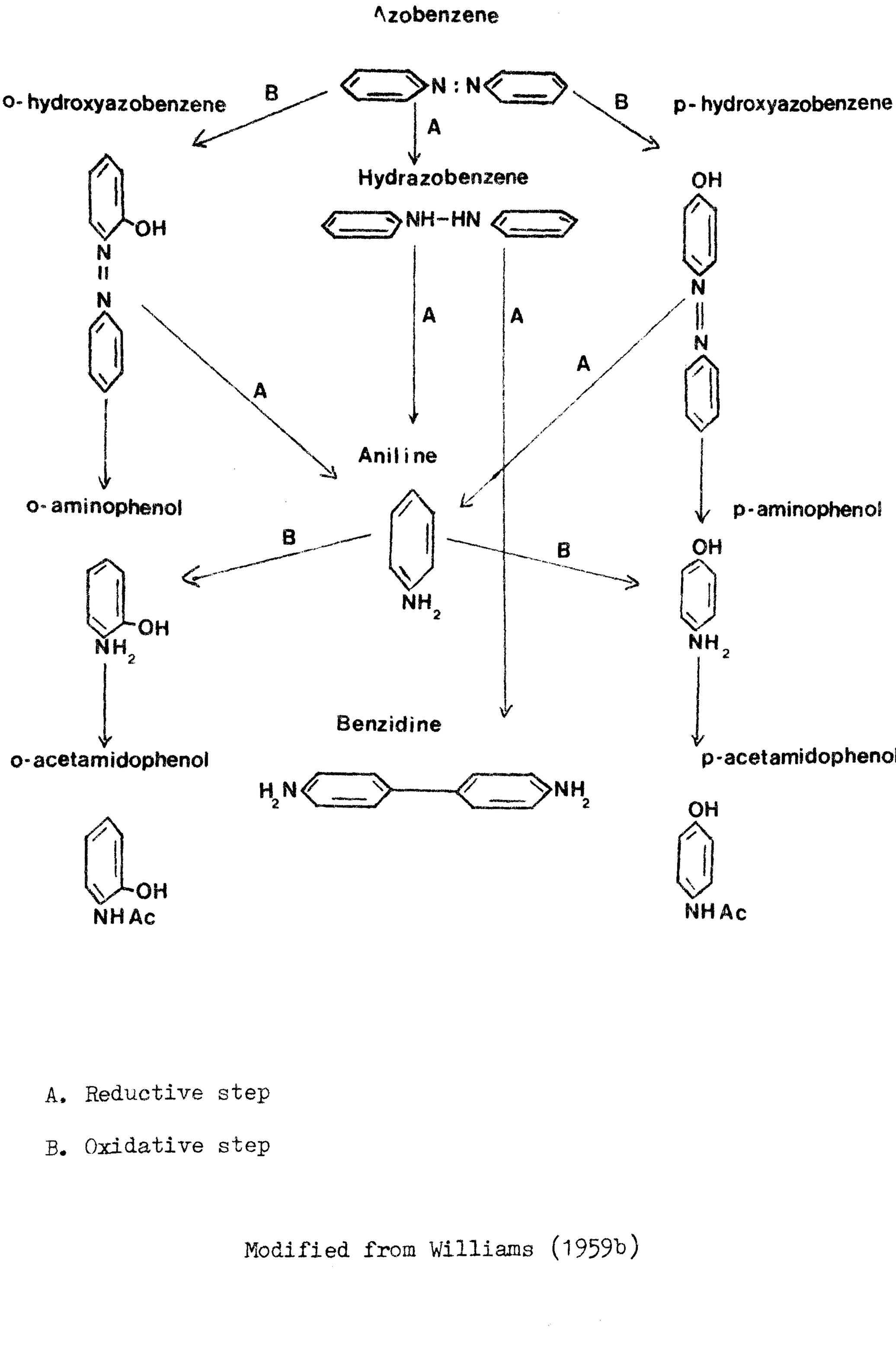
The metabolism of azocompounds in the mammal is of great interest, as it was through the realisation that the azodye Prontosil broke down *in vivo* to sulphonilamide, that the sulphonilamides were discovered and used for the treatment of bacterial infections (Williams, 1959b). Azobenzene is the simplest azo derivative. It has been shown that the azo linkage can be broken (Elson and Warren, 1944) and this was confirmed by Bray, Clowes and Thorpe (1951) who showed, using paper chromatography, that azobenzene is metabolised to yield o- and p-hydroxyazobenzene, benzidine, o- and p-acetamidophenols, o- and p-aminophenols and hydrazobenzene, some of these being conjugated with glucuronic or sulphuric acid. The p-hydroxy-compounds were the major metabolites. Figure 9 summarises the major pathway of azobenzene metabolism in the mammal.

A variety of azocompounds have now been shown to be reduced *in vivo* by enzymes localised mainly in the microsomal fraction of the liver (Mueller and Miller, 1948; 1949; 1950). Studies *in vitro* revealed that the enzyme localised in the microsomal fraction was a flavoprotein (Hernandez, Gillette and Mazel, 1967).

Azoreduction has also been reported in the cestode, *Moniezia expansa*, and in the nematode *Ascaris lumbricoides* var *suum* (Douch and Blair, 1975; Douch, 1975a; 1975b; 1976). The enzyme responsible for azoreduction was detected only in the distal cytoplasm of the cestode proglottids. No oxidative products of azobenzene were detected.

FIGURE 9

Diagram of Azobenzene metabolism in the mammal



A. Reductive step

B. Oxidative step

Modified from Williams (1959b)

1.5. CHEMOTHERAPY OF TRICHOMONAS VAGINALIS

1.5.1. Methods of assessing antitrichomonal activity

The conventional method for the determination of the susceptibility of Trichomonas vaginalis to compounds in vitro involved growth in tubes of media containing serial twofold dilutions of drug, and incubation for a set period of time, usually one to three days (Forsgren and Wallin, 1974). Usually the drug's activity was tabulated as the minimum inhibitory concentration (MIC), the minimum concentration of the drug that stopped the flagellar movement and motility of the organism. This technique was simplified by Meingassner and Thurner (1979) by using microtitre plates in place of test tubes; thus facilitating the measurement of the MIC under both aerobic and anaerobic conditions. Another method of measuring the compound susceptibility of T. vaginalis using discs containing set concentrations of a compound was reported by Smith and Domenico (1980). It was reported that all three methods gave comparable results (Smith and Domenico 1980).

Determination of susceptibility of T. vaginalis to compounds in vivo usually has been assessed using a subcutaneous infection of T. vaginalis in mice or rats. Such a subcutaenous lesion contains large numbers of flagellates (Honigberg, 1961). Doubling dilutions of drug are given to the animals and lesions are studied at various times after dosing for the presence of live parasites. Drug activity was expressed as the MIC, the lowest concentration of the drug which killed all the parasites in the lesion. More recently, in vivo drug

activity has been assessed by measuring its effect on parasites growing intravaginally in hamsters or mice. This method has the advantage of measuring the drugs effect on parsites in situ.

1.5.2. History

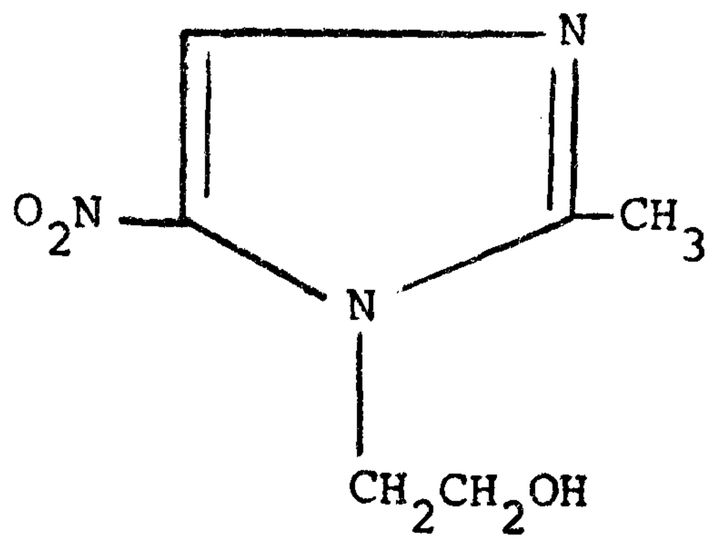
The treatment of trichomonal vaginitis has become more sophisticated over the last hundred years. Early treatment included efforts to restore the acidity of the vagina as well as kill the trichomonads. Treatments of this type involved the initial drying of the vaginal mucosa with warm air and then the application of douches of dilute vinegar. Powders containing boric acid, or acid creams and jellies, were also found useful, often in conjunction with the application of lactose to stimulate the growth of acid-producing bacteria. Insufflations of acetarsone powder (acetylamino hydroxylphenylarsonic acid) or local application of silver picrate were found to be moderately successful (Buxton and Shelanski, 1937; Strain, 1945). However, the local application of chemicals was not a satisfactory treatment, as foci of infection often remained in neighbouring structures and glands. The simultaneous application of various agents to vagina, bladder and rectum only partly overcame this obstacle. An ideal trichomonacidal agent should exert its effect systemically, to ensure its distribution to all the affected parts of the body.

In the 1950's and early 1960's several classes of compounds were known which were systemically active in experimentally induced

extravaginal trichomonad infections in mice, and intravaginal infections in hamsters and rats. These included 2-substituted 5-nitrothiazoles (Bushby and Copp, 1955), 2-substituted nitropyridines (Michaels and Strube, 1961), 2-substituted 5-nitropyrimidines (Michaels and Strube, 1961) and 1, 2 substituted 5-nitroimidazole (Cosar and Julou, 1959). It is noteworthy that these classes of compounds were all nitroheterocyclics. In clinical trials, nitropyridines and nitropyrimidines were found to be too toxic for general use, and nitrothiazoles were found to have little trichomonacidal effect (Michaels, 1968). The nitroimidazoles, however, were promising.

In 1955, French scientists had observed the growth inhibitory effect of an extract of streptomyces 6670 on Trichomonas vaginalis (See Jolles, 1977). This extract was purified and 2-nitroimidazole isolated as the active compound. A range of nitroimidazole compounds were then synthesized and their trichomonacidal activity was investigated. 2-nitroimidazoles and 4-nitroimidazoles were found to be less active and more toxic than 5-nitroimidazoles (Michaels, 1968). Of the range of 5-nitroimidazoles tested, such as dimetridazole (1, 2-dimethyl-5-nitroimidazole) and chlomizol (1-methyl-4-chloro-5-nitroimidazole), metronidazole (1-hydroxyethyl-2-methyl-5-nitroimidazole), Figure 10), was the most effective trichomonacide and was selected for the treatment of T. vaginalis in man. The trichomonacidal activity of metronidazole was reported by Cosar and Julou (1959) and the drug was launched onto the market in

Figure 10. The structure of metronidazole



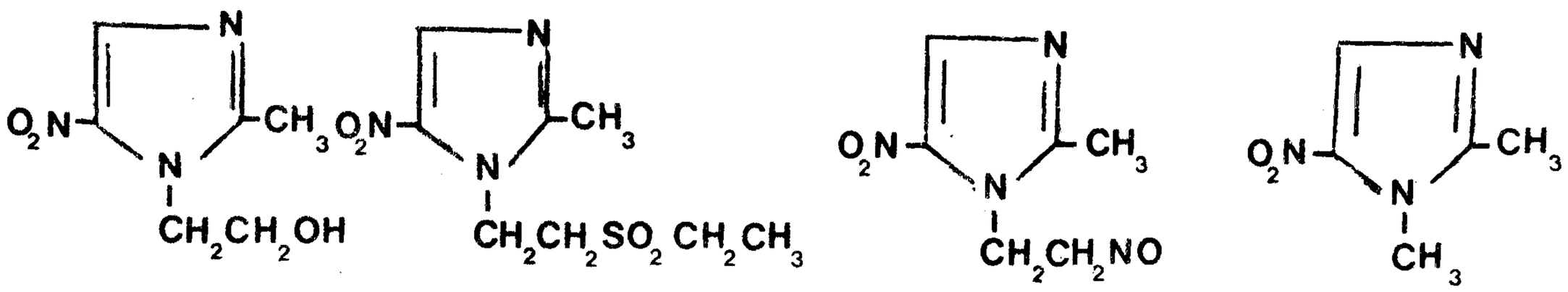
1960 for the treatment of T. vaginalis infections. Subsequently similar 5-nitroimidazoles have been developed (See section 1.5.3.1.). Other compounds such as nitrofurans, nitropyrroles and bis (thiosemicarbazones) have also been tested for antitrichomonal activity (Michaels, 1968), but the 5-nitroimidazoles such as metronidazole are, to date, the most effective antitrichomonal compounds discovered and are used extensively throughout the world.

1.5.3. Metronidazole

1.5.3.1. Spectrum of activity

The most widely used and effective antitrichomonal compound discovered to date is metronidazole, although several other 5-nitroimidazole derivatives are also very effective in the treatment of infections caused by prokoyotic and eukaryotic anaerobes (See Figure 11).

Figure 11. Metronidazole and other 5-nitroimidazole derivatives



Metronidazole Tinidazole Nimorazole Dimetridazole

The antitrichomonal activity of metronidazole was first reported by Cosar and Jolou (1959), since then metronidazole has been used very successfully in the treatment of trichomonal vaginitis (Rees, 1960; Doyle and Bruce, 1964; Porapakkham, 1967; Howes, Lynch and Kivlin, 1969). Metronidazole was found to be effective in over 95% of cases (Pereyra and Lansing, 1964; Parapakkham, 1967) and to have few side effects (Watt and Jennison, 1960). An oral dose of 500 mg metronidazole per day for 7-10 days was found to be effective (Doyle and Bruce, 1964) and the insertion of metronidazole into the vagina was found to be unnecessary. Several different regimens are now used routinely, e.g. 200 mg metronidazole, three times daily for 7 days; 800 mg in the morning and 1.2 g at night for 2 days; a single 2 g dose. All have been reported to give satisfactory results (British National Formulary, 1982).

Laboratory studies have shown that strains of Trichomonas vaginalis were killed in vitro in three days by concentrations of 0.6 to 1.0 µg metronidazole per ml. culture medium (Jennison, Stenton and Watt, 1961). Blood and urine estimations show that metronidazole is rapidly absorbed from the intestine, and excreted by the kidney. Even with the 7 day metronidazole regimen, however, blood and urine metronidazole levels are maintained at a level high

enough to cause parasite death throughout the complete treatment period (Jennison, Stenton and Watt, 1961).

Metronidazole and other 5-nitroimidazoles are also active against a range of protozoa other than T. vaginalis, such as Entamoeba histolytica (Powell, McLeod, Wilmot and Elsdon-Drew, 1966; Tanowitz, Wittner, Kress and Rosenbaum, 1973; Everett, 1974; Wittner, Rosenbaum and Kress, 1975), Giardia lamblia (Felix and Ouryoux, 1962; Rambatta, 1971; Andersson, Forsell and Sterner, 1972; Wolfe, 1975; Jokipii and Jokipii, 1980), Histomonas (Lucas, 1961) and Balantidium (Garcia-Leverde and de Bonilla, 1975).

In recent years 5-nitroimidazoles, especially metronidazole, have been found to have activity against Bacteroides spp. (Nastro and Finegold, 1972) and have been used in the treatment (Ingham, Rich, Selkon, Hale, Roxby, Betty, Johnson and Uldall, 1975; Feldman, 1976) as well as in the prophylaxis of anaerobic infections (Gillespie and McNaught, 1978). In colon surgery, metronidazole prophylaxis has been used, reducing the incidence of postoperative infections (Fiddion, 1978). The killing activity of metronidazole has been found to occur against a wide range of anaerobic bacteria (Rissing, Moore, Newman, Crockett, Buxton and Edmonson, 1980).

Other 5-nitroimidazole compounds, such as tinidazole and nimorazole, have a broadly similar spectrum of activity, but none have supplanted metronidazole from its role as the drug of choice for a wide range of bacterial and protozoal diseases.

1.5.3.2. Basis of selective toxicity

A variety of infections of man and of his domestic animals can be treated successfully with 5-nitroimidazole derivatives. A common feature of the infectious agents is that they are anaerobes. This suggested that similarities in the metabolism of these organisms are responsible for their susceptibility to these compounds.

It has been shown that the addition of metronidazole or other nitroimidazoles to cultures of Trichomonas vaginalis (Edwards and Mathison, 1970; Edwards, Dye and Carne, 1973; Müller, Lindmark and Mack, 1978) and of Clostridia spp (O'Brien and Morris, 1972; Coombs, 1976) caused a cessation of hydrogen production, indicating that the action of these compounds could be related to metabolic processes dependant on ferredoxin-linked reactions, and therefore localised in the hydrogenosomes of trichomonads. Both O'Brien and Morris (1972) and Coombs (1976) showed that the inhibition of hydrogen production was for a finite period of time, which was directly proportional to the concentration of drug present. This demonstrates that metronidazole does not act by directly inhibiting hydrogenase, but rather by utilizing the electrons normally consumed in hydrogen production. It has been shown that interferance with hydrogen production is not responsible as such for the toxicity of nitro-imidazoles in trichomonads. Other nitroderivatives, such as 2, 4-dinitrophenol (Müller, Nseka, Mack and Lindmark, 1979) and nitrofurans (Edwards, Dye and Carne, 1973) can also act as electron acceptors and do not have the same toxic effects.

Carbon dioxide production was not inhibited by metronidazole (Coombs, 1976) under a nitrogen atmosphere. Indeed, under a carbon monoxide atmosphere, CO_2 production was enhanced, for a period directly proportional to the concentration of the drug added. This suggested that, directly or indirectly, metronidazole was acting as an electron acceptor for the pyruvate:ferredoxin oxidoreductase system responsible for the production of CO_2. However, metronidazole did not stimulate CO_2 production if oxygen was present, suggesting that oxygen stopped the drug acting as an electron acceptor. Edwards and Mathison (1970) reported that metronidazole has no effect on the production of acetyl CoA in Trichomonas vaginalis and this was confirmed using Clostridium pasteurianum by Coombs (1976), although the latter noted that acetyl CoA production was enhanced under a carbon monoxide atmosphere.

Edwards, Dye and Carne (1972) suggested that if nitroimidazoles act as electron acceptors, there would be an accumulation of hydrogen ions within the parasite, unless the nitro-group of the compound was reduced. No accumulation of hydrogen ions has been detected. It has been shown that metronidazole can be reduced by sodium dithionite (Edwards, Dye and Carne, 1972; Coombs, 1976), by cell-free extracts of Clostridia spp. (Coombs, 1973; Müller, Lindmark and McLaughlin, 1976; Coombs, 1976) and by trichomonads (Lindmark and Muller, 1974; 1976; Muller, Lindmark and McLaughlin, 1976) with a concomitant loss of its 320 nm absorbance maximum. This absorbance is due to the nitro-group, which indicates that

reduction involves this group.

The metabolism of metronidazole within the cell maintains a concentration gradient of unreduced parent drug for its continued diffusion into the cell. This would explain the rapid uptake of the large quantities of radiolabelled metronidazole that it absorbs (Ings, McFadzean and Ormerod, 1974; Müller, Lindmark and McLaughlin, 1976).

Metronidazole was not reduced by ferredoxin-free Clostridium pasteurianum unless ferredoxin or methyl viologen was added (Coombs, 1976), and then the rate of reduction was proportional to the concentration of electron acceptor added. This author suggested that the reduction of metronidazole was dependant upon pyruvate: ferredoxin oxidoreductase, ferredoxin and anaerobic conditions. Müller, Lindmark and McLaughlin (1976) showed that the reoxidation of reduced ferredoxin by metronidazole occurred under aerobic conditions. Metronidazole has a redox (half-wave) potential of -0.56v and can therefore act as a better electron acceptor than ferredoxin (Edwards and Mathison, 1970), both the nitro-group and the imidazole ring of the drug conferring the properties of an efficient electron sink. Competition for electrons generated by the production of acetyl-CoA would occur and this would inhibit the hydrogenase system. Müller and Lindmark, (1976) reported that metronidazole was probably reduced by reduced ferredoxin in a purely chemical reaction.

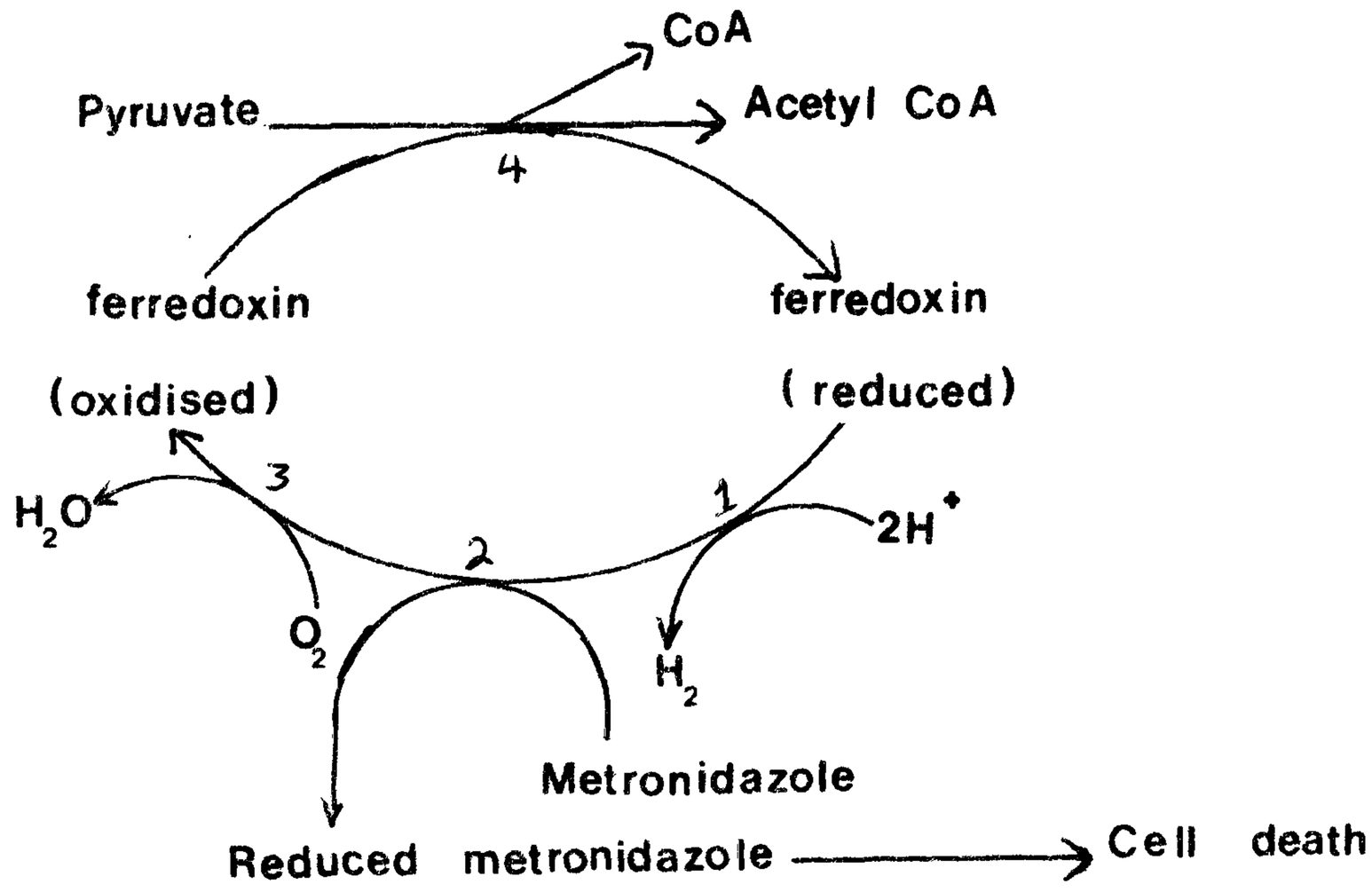
The proposed mechanism of action of metronidazole is given in Figure 12. Reduced ferredoxin can be reoxidised by three mechanisms. Hydrogenase (1) is the pathway in normal cells. In the presence of metronidazole, hydrogenase is inhibited and metronidazole is reduced (2). If oxygen is present, (1) and (2) cease, probably due to the greater electronegativity of oxygen for electrons. Reaction (4) is catalysed by pyruvate:ferredoxin oxidoreductase. The reduction of metronidazole is the key to its antimicrobial activity, and it is postulated that it is a reduced metabolite that is the agent that causes cell death.

The specificity of action of metronidazole to anaerobic organisms is thought to be due to their possession of the metabolic capability to reduce the nitro-group of the drug. Aerobic cells are apparently unable to do so. It has been shown that drug sensitive organisms grown in the presence of oxygen are less sensitive to metronidazole (Müller, 1982). This is related to the inhibition by oxygen of metronidazole reduction, and probably involves the reoxidisation of reduced ferredoxin by oxygen, removing the means of reducing the metronidazole. A second possible explanation for this finding is that O_2 may reoxidise reduced metronidazole within the cell.

Studies of the reduction of metronidazole by sodium dithionite and the inhibition of hydrogen production by the drug, by cell-free homogenate, indicated that metronidazole reduction was a four electron process (Coombs, 1976; Müller, 1976). A four electron reduction

Figure 12

The mechanism of action of metronidazole



1. Hydrogenase
2. Non-enzymatic
3. Aerobic reaction
4. Pyruvate: ferredoxin oxidoreductase

indicates that a hydroxylamine derivative was produced (See Figure 13), although this has never been confirmed by isolation. More recently it has been suggested that the reduction of metronidazole occurs in one electron steps (Müller, 1983), assumed to lead firstly to the formation of a free radical anion which can be reoxidised by oxygen to the original compound with the production of superoxide, a toxic derivative of oxygen (Perez-Reyes, Kalyanaraman and Mason, 1979). Further reduction gives a nitroso derivative, a nitroso-free radical and a hydroxylamine derivative (See Figure 13). It is assumed that one or more of these intermediates represents the cytotoxic species, although none of these transient compounds have been isolated and only the existence of the one electron reduction free-radical anion has been shown by electron paramagnetic resonance spectroscopy (Perez-Reyes, Kalyanaraman and Mason, 1979).

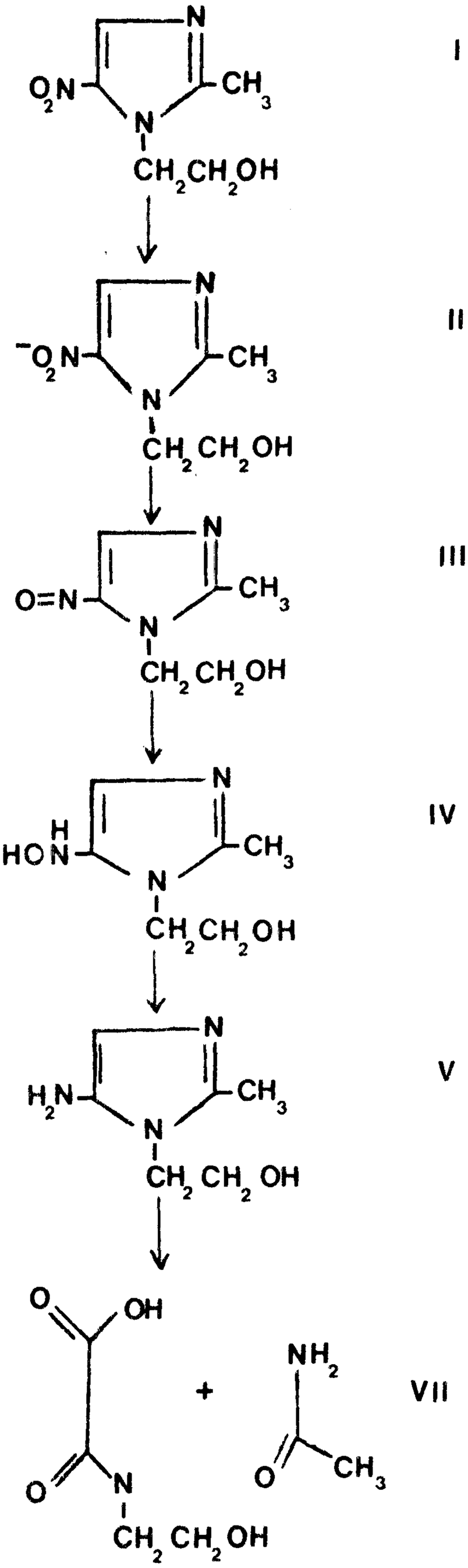
The complete reduction of metronidazole to acetamide was reported by Beaulieu, McLafferty, Koch and Goldman (1981), who used acetamide production from cultures of Trichomonas vaginalis and Entamoeba histolytica containing metronidazole as a chemical indicator of the production of the intermediate thought to exert a killing effect on the cell.

1.5.3.3. Site of action

How the parasite is killed has not yet been elucidated, but it is thought that certain short lived derivatives or free-radicals are responsible for the toxic action (Müller, Lindmark and McLaughlin,

Figure 13

The reduction of metronidazole



I Metronidazole

II Radical anion

III Nitroso derivative

IV Hydroxylamine

V Amino derivative

VI N-(2-hydroxyethyl) oxomic acid

VII Acetamide

Modified from Chrystal, Koch, McLafferty and Goldman (1980)

1977) and that specific intracellular sites are damaged. Micrographs of Trichomonas vaginalis cells exposed to high concentrations of 5-nitroimidazoles showed a reduction in number of, and structural damage to, the hydrogenosomes (Buchner and Edwards, 1975; Carosi, Filice, Suter and Dei Cas, 1977). The former authors also noted some damage to the nuclear membrane, although this occurred less quickly, however the latter authors were unable to detect any damage to the nucleus.

In 1974, Ings et al., suggested that DNA might be the major site of action of nitroimidazoles when they reported that radio-activity from ^{14}C-metronidazole bound to T. vaginalis was released on the addition of DNAase, but not RNAase, and that chemically reduced metronidazole inhibited nucleic acid synthesis. Unreduced metronidazole had no effect on nucleic acid synthesis. Plant and Edwards (1976) reported that reduced metronidazole and tinidazole inhibited DNA synthesis and degraded the existing DNA. RNA synthesis was unaffected. The binding of metronidazole to nucleic acids was also demonstrated by La Russo, Tomasz, Muller and Lipman (1977) who suggested that metronidazole reduced by dithionite in vitro yields a short lived, activated compound or compounds which covalently binds to the guanine and cytosine of mammalian, bacterial and phage DNA. This work was complemented by Edwards (1977) who reported that dithionite reduced metronidazole caused strand breaks in phage and mammalian DNA. However, results indicating DNA strand breakage with dithionite-reduced metronidazole are not reliable, as dithionite itself induces strand breaks in DNA (Cone, Hasan, Lowen and Morgan, 1976).

The interaction of electrolytically reduced metronidazole and DNA was studied by Knight, Skolimowski and Edwards (1978), who reported that this resulted in a loss of helix content and strand breakage. It was reported that reduced 5-nitroimidazole-induced damage to DNA was related to its base composition (Rowley, Knight, Skolimowski and Edwards, 1980) and was associated with the release of thymidine phosphates from DNA (Knox, Knight and Edwards, 1981a). These authors reported that misonidazole causes cleavage of the phosphodiester bonds around thymine residues in DNA, and that approximately 5-6% of the total thymidine is released irrespective of the total thymidine content of the DNA. This work was extended by Knox, Knight and Edwards (1981b) who showed a correlation between drug-induced damage and electron affinity in a range of nitroimidazoles, and that this correlation arises as a result of the different rates of reduction of the drugs, which are themselves a direct function of their relative electron affinity. La Russo, Tomasz, Kaplan and Müller (1978), however, were unable to detect strand breaks or loss of helix content by reduced metronidazole, with mammalian, phage and bacterial DNA. Weight was added to this by Mitelman, Strombeck and Ursing (1980) who were unable to detect chromosomal aberrations in patients on long-term metronidazole treatement.

There is good evidence that <u>in vitro</u> reduced metronidazole can damage DNA in some way. However, it is not possible from the evidence available to make firm conclusions on the site of action <u>in vivo</u> of the active metabolite of metronidazole. There could be differences between prokaryotes, in which evidence for DNA damage is reasonable,

and eukaryotes such as T. vaginalis, in which the situation is complicated by the physical separation between the DNA in the nucleus and drug activation in the hydrogenosomes.

1.5.3.4. Resistance to metronidazole

In the 1960's there were reports of Trichomonas vaginalis infections refractory to treatment (Robinson, 1962; Squires and McFadzean, 1962; Diddle, 1967), although the parasites isolated from these refractory patients were not found to be resistant to metronidazole in vitro (Squires and McFadzean, 1962). It was suggested that failure to respond to treatment was due to metronidazole inactivating organisms in the vagina (Nicol, Evans, McFadzean and Squires, 1966; McFadzean, Pugh, Squires and Whelan, 1969; Edwards, Thompson, Tomusange and Hanson, 1979), failure to complete the treatment course (Keighley, 1971) or to reinfection (Roe, 1977).

In the laboratory, lines of Tritrichomonas foetus were developed by in vivo exposure to metronidazole, that exhibited resistance to several nitroimidazoles as tested both in vivo and in vitro (Actor, Ziv and Pagano, 1969; Meingassner and Mieth, 1976). Meingassner, Mieth, Czok, Lindmark and Müller (1978) reported the existence of a strain of T. foetus which had been grown in sublethal concentrations of metronidazole and which showed in vitro resistance to metronidazole under aerobic, but not anaerobic conditions. Lines of Trichomonas vaginalis resistant to metronidazole in vivo have also been induced by in vivo exposure to metronidazole, however in these cases no

significant *in vitro* resistance was observed (de Carneri, Achilli, Montiand Trane, 1969; Benazet and Guillaume, 1971). Jennison, Stenton and Watt (1961) were unable to induce resistance to metronidazole in *T. vaginalis in vitro*, as were other workers (Squires and McFadzean, 1962). However, Coombs (1976) developed a line of *T. vaginalis* by growth in the presence of sublethal concentrations of metronidazole, which showed a slight decrease in sensitivity to the drug.

In 1978, Thurner and Meingassner isolated a strain of *T. vaginalis* with a decreased metronidazole sensitivity, from a patient who had not responded to several courses of treatment. This strain showed resistance to metronidazole, tinidazole and nimorazole both *in vivo* and *in vitro*. *In vitro* the resistance to metronidazole and the other nitroimidazoles only occurred under aerobic conditions. Subsequently, three metronidazole resistant strains of *T. vaginalis* were isolated from patients in the United States of America in 1978 and 1979. These isolates showed resistance to metronidazole both *in vivo* and *in vitro*. *In vitro*, however, the resistance again could be detected under aerobic conditions only (Müller, Meingassner, Miller and Ledger, 1980; Müller, 1982). Heyworth, Simpson, McNeillage, Robertson and Young (1980) also reported the isolation of a strain of *T. vaginalis*, resistant *in vitro* to metronidazole, from a patient who had not responded to metronidazole treatment.

In all lines of metronidazole resistant *T. vaginalis* isolated from patients to date, *in vitro* metronidazole resistance has only been detected under aerobic conditions. Since it is necessary for

oxygen to be present for the in vitro expression of resistance, and that the presence of ascorbate, a strong reducing agent, in the culture medium (Meingassner, Mieth, Czok, Lindmark and Müller, 1978) suppresses this resistance, it suggests that oxygen is involved in some way. It appeared possible that oxygen may be entering the hydrogenosomes of these parasites under aerobic conditions and inhibiting the reductive activation of metronidazole.

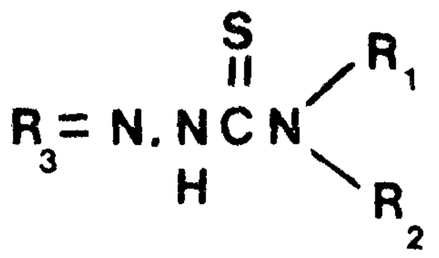
Metronidazole resistance has also been reported for bacteria. Metronidazole-resistant mutants of Bacteroides fragilis were isolated after treatment of the bacteria with N-methyl-N^1-nitro-N-nitroguanidine and ethyl methane sulfonate (Britz and Wilkinson, 1979). All the metronidazole resistant strains had lowered levels of pyruvate dehydrogenase compared with parent cultures. A consequence of this would be lowered rates of ferredoxin reduction, and therefore of metronidazole reduction in these strains, causing lowered metronidazole sensitivity (Britz, 1981). This metronidazole resistance was found under both aerobic and anaerobic conditions. This suggested that the mode of resistance to metronidazole exhibited by these organisms was different from that exhibited by clinical isolates of Trichomonas vaginalis and Tritrichomonas foetus.

1.5.4. Thiosemicarbazones

Thiosemicarbazones (See Figure 14) containing highly varied substituents have been found to possess chemotherapeutic properties against a wide range of microorganisms and diseases (Levinson, 1980).

Figure 14

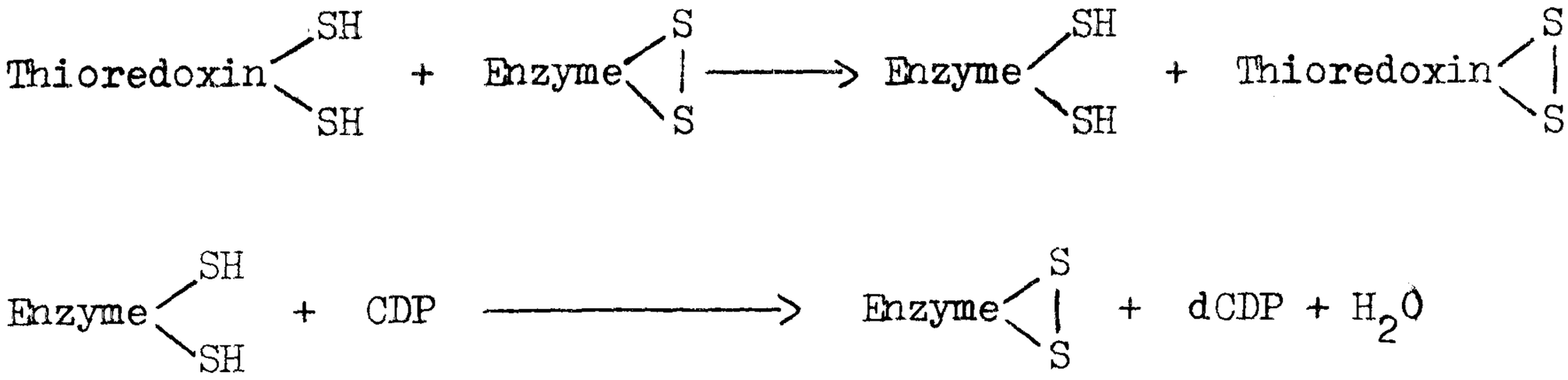
Basic Structure of a thiosemicarbazone



R_1, R_2, R_3 : Variable substituents

Figure 15

Diagram of the mechanism of action of E. coli ribonucleotide reductase



CDP Cytidine diphosphate

dCDP deoxycytidine diphosphate

Modified from Preidecker et al. (1980)

In addition to other activities, these compounds, often in chelation with transition metals, appear to inhibit the function of nucleic acids and related enzymes.

1.5.4.1. Antitumor activity and mode of action

Several thiosemicarbazones have been found to have antitumor activity (Brockman, Thomson, Bell and Skipper, 1956; French and Freedlander, 1958; Barry, Conalty and O'Sullivan, 1966). However, these compounds are apparently ineffective unless transition metal ions are present (Booth and Sartorelli, 1966; Petering, Buskirk and Crim, 1967; Van Giessen, Crim, Petering and Petering, 1973). It has been shown (Van Giessen, Crim and Petering, 1968) that thiosemicarbazones can form stable chelates with transition metals (Crim and Petering, 1967; Kessel and McElhinney, 1975) and the effect of the copper chelate of a thiosemicarbazone on various metabolic processes has been studied (Kessel and McElhinney, 1975), showing a correlation between chelation and an ability to inhibit DNA synthesis. Booth and Sartorelli (1966) showed that a combination of cupric chloride and a thiosemicarbazone inhibited the synthesis of DNA, RNA and proteins in sarcoma 180 ascites cells, although little or no inhibition was caused by the cupric chloride or thiosemicarbazone alone. Subsequently, many authors have reported that DNA synthesis was inhibited by thiosemicarboazones and their chelates (Booth and Sartorelli, 1967; Kessel and McElhinney, 1975; Minkel and Petering, 1978). Inhibition of RNA and protein synthesis also occurred, but

to a much smaller extent.

In 1970, Moore, Zedeck, Agrawal and Sartorelli showed that the interferance of DNA synthesis by a thiosemicarbazone was due to the inhibition of ribonucleoside diphosphate reductase, an enzyme responsible for the conversion of ribonucleotides to deoxyribonucleotides. They also noted that dithiothreitol (DTT) and dithioerythritol (DTE) were partially competitive with the inhibitor, and that ferrous ions enhanced the inhibitory effect of the compound. A relationship between the inhibition of ribonucleoside diphosphate reductase by a thiosemicarbazone and the concentration of DTT and DTE was reported by Sartorelli, Agrawal, Tsiftsoglou and Moore (1977) who noted that the inhibition was more pronounced at a low thiol concentration than at a higher one. They suggested that the thiosemicarbazone binds at or near to the site on the enzyme normally occupied by the thiols. The inhibitory effect of thiosemicarbazones and their transition metal chelates on ribonucleotide reductase in tumor cells has been confirmed by a number of authors (Chang and Cheng, 1978; Cory and Fleischer, 1980; Preidecker, agrawal, Sartorelli and Moore, 1980; Sato and Cory, 1981).

The reaction mechanism of the ribonucleotide reductase from Escherichia coli has been investigated (Thelander, 1974; Thelander, Larsson, Hobbs and Eckstein, 1976) and is probably as follows (See Figure 15).

The antagonism of thiosemicarbazone inhibition by the addition of thiol groups noted by Moore et al. (1970) and Sartorelli et al.

(1978) could be explained by the competition of these thiols with those associated with the enzyme for binding to the thiosemicarbazone, and therefore inhibiting the amount of enzyme associated with the thiosemicarbazone.

More recently, another mechanism of action has been postulated for thiosemicarbazones; the inhibition of amino acyl t-RNA synthetase activity (Rhode, Cordell, Webster and Levinson, 1977). It was found that thiosemicarbazone:copper complexes, but not the thiosemicarbazone or copper alone, bind to tRNA (Mikelens, Woodson and Levinson, 1976). Rhode et al. (1977) proposed that tRNA synthetase inhibition was due to such an interaction between tRNA and the copper:thiosemicarbazone complex. Since the binding of tRNA by amino acyl tRNA synthetases is sensitive to changes in the overall shape of the tRNA, they suggested that the binding of the thiosemicarbazone:copper complex to the tRNA induced a conformational change, adversely affecting its substrate properties, and therefore inhibiting tRNA synthetase.

Since the thiosemicarbazones contain compounds with such varied substitutents, it is very likely that different compounds could have activity at different sites.

1.5.4.2. Antiviral and antibacterial activity

The antiviral activity of thiosemicarbazones was first reported by Hamare, Bernstein and Donovik (1950) who found that benzaldehyde thiosemicarbazone derivatives were active against

vaccinial infection in mice when given orally. Isatin β-thiosemicarbazone was shown to be a potent inhibitor of pox virus replication in cultured animal cells (Sheffield, Bauer and Stephenson, 1960), affecting the late stages of the virus growth cycle (Woodson and Joklik, 1965). This was confirmed by Cooper, Moss and Katz (1979), who reported a 95% inhibition of viral protein synthesis. In 1969, Sidewell, Arnett, Dixon and Schabel demonstrated that purine-6-carboxaldehyde thiosemicarbazone was effective in suppressing both the cytopathic effect and the titres of human megalovirus. Activity against Herpes Simplex virus was reported by Shipman, Smith, Drach and Klayman (1981) who showed that the thiosemicarbazones inhibited viral replication to a greater extent than cellular DNA and protein synthesis.

It is only recently that thiosemicarbazones have been reported to have antibacterial activity. 2-Acetylpyridine thiosemicarbazones have been shown to be very active against Neisseria gonorrhoeae and N. meningitides (Dobek, Klayman, Dickenson, Scovill and Tramont, 1980). Inhibition of gram-negative bacilli, namely Pseudomonas, Shigella, Proteus and Escherichia coli also occurred, but only at such high concentrations as to rule out their use as chemotherapeutic agents. However, the sensitivity of Neisseria spp. to thiosemicarbazones was so great (MIC:0.002-0.008 μg/ml) with the drugs above, against both penicillin-susceptible and -resistant strains, that these compounds are of continuing interest as possible anti-bacterial agents. Little

is known of the basis of the antibacterial activity of thiosemicarbazones, although DNA synthesis has been implicated (See section 1.5.4.1.). It is not yet clear whether any of these compounds will have clinical use as antibacterial agents.

1.5.4.3. Antiprotozoal activity

The potential of thiosemicarbazones as antiprotozoal agents has only been reported over the last few years. Klayman, Bartosevich, Griffin, Mason and Scovill (1979) showed that a wide range of 2-acetylpyridine thiosemicarbazones have antimalarial activity. This work was continued on a small range of compounds in an attempt to elucidate which of the substitutents of the compound gave the greatest antimalarial activity (Klayman, Scovill, Bartosevich and Mason, 1979).

Thiosemicarbazones, in particular the 2-acetylpyridine thiosemicarbazones, have also been shown to have activity against Trypanosoma cruzi (Wilson, Revankar and Tolman, 1976), both in vivo and in vitro, and against Trypanosoma rhodesiense in vitro (Casero, Klayman Childs, Scovill and Desjardins, 1980). In the latter case, the authors suggested that a potential site for activity may be the iron-containing parasite-specific enzyme, glycerol-3-phosphate oxidase, which is essential for energy producing metabolism in the blood-form of the trypanosome. They support this by noting that thiosemicarbazones are avid chelators of transition metals, and that a structurally similar thiosemicarbazone is thought to form a ligand with iron within its target organism (French, Blanz,

DoAmaral and French, 1970). This thiosemicarbazone has also been shown to inhibit the growth and suppress RNA synthesis in, Escherichia coli (Brown, Stancato and Wolfe, 1981). DNA and protein synthesis were also inhibited, but this could be a consequence of the suppression of RNA synthesis. This compound apparently does not require an exogenous transition element. E. coli RNA polymerase and DNA polymerase, however, contain zinc (Scrutton, Wu and Goldthwaite, 1971; Slater, Mildvan and Loeb, 1971), which may form part of the drugs target. The activity of thiosemicarbazones against other parasitic protozoa, including Trichomonas vaginalis, has not been reported. It seemed feasible, however, that the biochemical pathways unique to anaerobic protozoa may be possible targets for these compounds, especially as it was known that transition metals are important constituents of these parasites.

1.6. COMPOUNDS AFFECTING DNA REPAIR

There have been no reports on the ability of T. vaginalis to repair damaged DNA or the enzymes involved in the process. However, there have been suggestions that DNA is the primary target of the main antitrichomonal drugs, 5-nitroimidazoles such as metronidazole (Knight, Skolimowski and Edwards, 1978).

In 1978, Knight, Skolimowski and Edwards reported that reduced metronidazole caused DNA strand breaks, although La Russo, Tomasz, Kaplan and Muller (1978) were unable to detect any strand breaks. Olive (1979) reported an increase of breaks in the DNA of mouse L929 cells in the presence of metronidazole. To date, the ability of

metronidazole to cause DNA strand breaks *in vivo* has not been assessed.

Dimethyl sulphate (DM^c) is a well known alkylating agent, which alkylates oxygen atoms in DNA (Newbold, Warren, Medcalf and Amos, 1980) and thus interferes with DNA base-pair hydrogen bonding. This may be the major DNA modification responsible for the induction of GC $\longrightarrow$AT transition mutations in bacteriophages by alkylating agents (Lawley and Martin, 1975). DMS has also been used in previous studies involving poly (ADP-ribose) polymerase (Durkacz, Omidijii, Gray and Shall, 1980) as a DNA-damaging agent.

Ethidium bromide is a compound which intercalates between DNA bases causing transcription errors and helix loss. It has been shown to inhibit DNA supercoiling (Benyajati and Worcel, 1976) and to inhibit the incorporation of titrated dTMP into DNA (Mattern and Painter, 1979) in permeabilised Chinese hamster ovary cells.

A group of thiosemicarbazones (2-acetylpyridine thiosemicarbazones) have been found to inhibit DNA synthesis (Booth and Sartorelli, 1966). Other thiosemicarbazones have been shown to inhibit ribonucleotide diphosphate reductase (Moore, Zadeck, Agrawal and Sartorelli, 1970). It has been postulated that some thiosemicarbazones may inhibit tRNA synthetase (Rhode, Cordell, Webster and Levinson, 1977). Poly (ADP-ribose) polymerase has not been postulated as a target for thiosemicarbazone activity, but the envolvement of these compounds in the inhibition of DNA synthesis suggested that thiosemicarbazones might be of interest in this experiment.

1.7. AIMS OF THE PROJECT

The main purpose of this project was to increase our knowledge of the metabolism of Trichomonas vaginalis and how this can be disrupted by drug action. It was hoped that this would indicate ways in which to selectively kill the parasites with new drugs. To these ends, several approaches were followed. Firstly, to compare metronidazole-resistant and metronidazole-sensitive lines of T. vaginalis in an attempt to understand the mechanism or mechanisms of drug resistance and other factors that affect the parasite's susceptibility to metronidazole. Secondly to develop an in vitro drug-screening system to test the sensitivity of T. vaginalis to potential antitrichomonal compounds and drug combinations, and to elucidate the mode of action of compounds with potent antitrichomonal activity. Thirdly, to investigate the ability of T. vaginalis to metabolise xenobiotics, which are metabolised in the mammal by both reductive and oxidative steps, and so to discover more about the anaerobic nature of the parasite and the restrictions this imposes upon it.

2. MATERIALS AND METHODS

2.1. PARASITES AND GROWTH CONDITIONS

2.1.1. Parasite lines

Seven lines of Trichomonas vaginalis were used: Clone G3, a clone isolated in this laboratory from a line originally obtained in 1973 from The Wellcome Research Laboratories, Beckenham, Kent (Coombs, 1976); Line IR78, a metronidazole-resistant line isolated from the vagina of a patient after three unsuccessful courses of treatment with metronidazole (Meingassner and Thurner), and obtained from Dr. J.G. Meingassner (Sandoz Forschungsinstitut, Vienna, Austria) in 1978; Lines LRS (0.6) and LRS (2.0), developed in this laboratory from clone G3 by growth in vitro under increasing drug pressure, until they were grown in medium containing metronidazole at 0.6 and 2.0 µg/ml respectively; Line 965691, obtained from May and Baker Ltd., Dagenham, Essex, England; Lines Albany, Boston and Fall River, isolated from patients after unsuccessful metronidazole treatment (Müller, Meingassner, Miller and Ledger, 1980) and obtained from Dr. J.G. Meingassner. The line of Leishmania mexicana mexicana used had been isolated in 1962 and was obtained from Dr. R.A. Neal (The Wellcome Research Laboratories, Beckenham, Kent) in 1972. Promastigotes were obtained by transformation of isolated amastigotes.

2.1.2. Growth of Parasites

Clone G3 and lines LRS (0.6), LRS (2.0), IR78, Albany, Boston and Fall River of Trichomonas vaginalis were usually grown axenically at 37°C in sterile, screw-capped, bijou bottles containing approximately 5 ml GTM medium (Table 1).

Table 1. Constituents of GTM culture medium

L-ascorbic acid	3.7 g/l	Panmede	1.0 g/l
L-cysteine-HCl	2.5 g/l	Bactopeptone	5.0 g/l
D-glucose	10.0 g/l	Lablemco powder	3.0 g/l
		Distilled deionised water	1 litre

The medium was adjusted to pH5.8, autoclaved for 10 minutes at 15 lbs/in^2 and stored for up to two weeks at 4°C. Newborn calf serum, heat inactivated by 30 minutes incubation at 56°C, was added to 10% (v/v), just prior to use. The parasites were cultured stationary with air as the gas phase (Coombs, 1976). Lines G3 and LRS were subcultured every 24 and 48 hrs respectively, and lines IR78, Albany, Boston and Fall River every 72 hrs, by the addition of 0.2 mls of late log-phase culture (about 2 x 10^5 parasites) into approximately 4.8 mls of fresh culture medium.

Line 965691 was maintained in a similar manner using modified Bushby's medium (Bushby and Copp, 1955), (Table 2).

Table 2. Constituents of modified Bushby's medium

Tryptone broth	30 g/l	Calcium pantothonate 0.05% soln.	1 ml/l
Dextrose	20 g/l		
Neutralised liver digest	18 g/l	Distilled deionised water	1 litre

The medium was adjusted to pH6.0, autoclaved for 10 minutes at 15 lbs/in^2 and stored for up to two weeks at 4°C. Heat inactivated horse serum was added to 20% (v/v) just prior to use.

Leishmania promastigotes were obtained by transformation of isolated amastigotes and grown at 26°C in HOMEM medium (Berens, Brun and Krassner, 1976) with 10% Foetal Calf Serum. Only promastigotes that had been subpassaged 3–8 times were used in this investigation. Parasites were subpassaged every 96 hrs.

Parasite numbers were determined using an improved Neubauer Haemocytometer. Checks for contaminating organisms were made by microscopic observation using a Leitz phase-contrast microscope at the time of each subculture. Contaminated cultures were subcultured with 500 units of Crystamycin/ml culture medium. If the contamination was present at the next subculturing then the contaminated cultures were discarded and a fresh culture taken from the stabilate.

2.1.3. Variations in growth conditions

Growth studies of clone G3 and line IR78 in media containing a range of concentrations of acetate, L-lactate, L-cysteine, L-ascorbate, L-methionine, glutathione and dithiothreitol, at a range of pH's, and under different oxygen, nitrogen and carbon dioxide tensions, were carried out at 37°C using 20 ml universal bottles containing 5 ml of GTM medium and fitted with a tight fitting suba-seal (Gallenkamp and Co., Ltd., London, England). Appropriate gaseous conditions were attained by gassing cultures (1 litre/min) with mixtures of nitrogen, oxygen and carbon dioxide for 3 minutes initially, and for 2 minutes every subsequent 12 hrs. Gas mixtures were obtained using a gas mixing apparatus in which the gas flow rates are controlled by needle valves (Negretti and

Zambra, Southampton, England), and the apparatus was calibrated by monitoring gas flow rates using measuring cylinders (Hart and Coombs, 1981), (See Figure 1.).

The gas flow from each of the gas cylinders was calibrated in the following manner. The flow rate was firstly standardised using the calibrated regulators (R) to give gas pressures of 29 lbs/in^2 (nitrogen and oxygen) or 5 lbs/in^2 (carbon dioxide). With the rate of gas issuing from each gas cylinder standardised, the gas passed through a calibrated needle valve (CV) and the flow rate through the valve at each setting was measured by use of a measuring cylinder, the bottom of which was fitted with a teat containing detergent. With gas passing through the measuring cylinder, the teat was squeezed, the detergent level was raised above that of the gas inlet and a bubble was formed. The time taken (in minutes) for the bubble to reach the top of the one litre cylinder gave the rate of gas flow in litres/minute. The gas flow from each gas cylinder was calibrated individually using the needle valve settings. The flow rate of each gas could then be set at the required rate and the gases mixed in the mixing chamber. This apparatus was designed and constructed by Dr. A.C. Taylor of this department. Gas mixtures are given throughout as the percentage (v/v) in the gas phase.

Routine drug screening of all drugs, under aerobic and anaerobic conditions (without the use of gas mixing apparatus), was carried out using microlitre plates. The use of these multiwell plates is discussed in section 2.2.1.

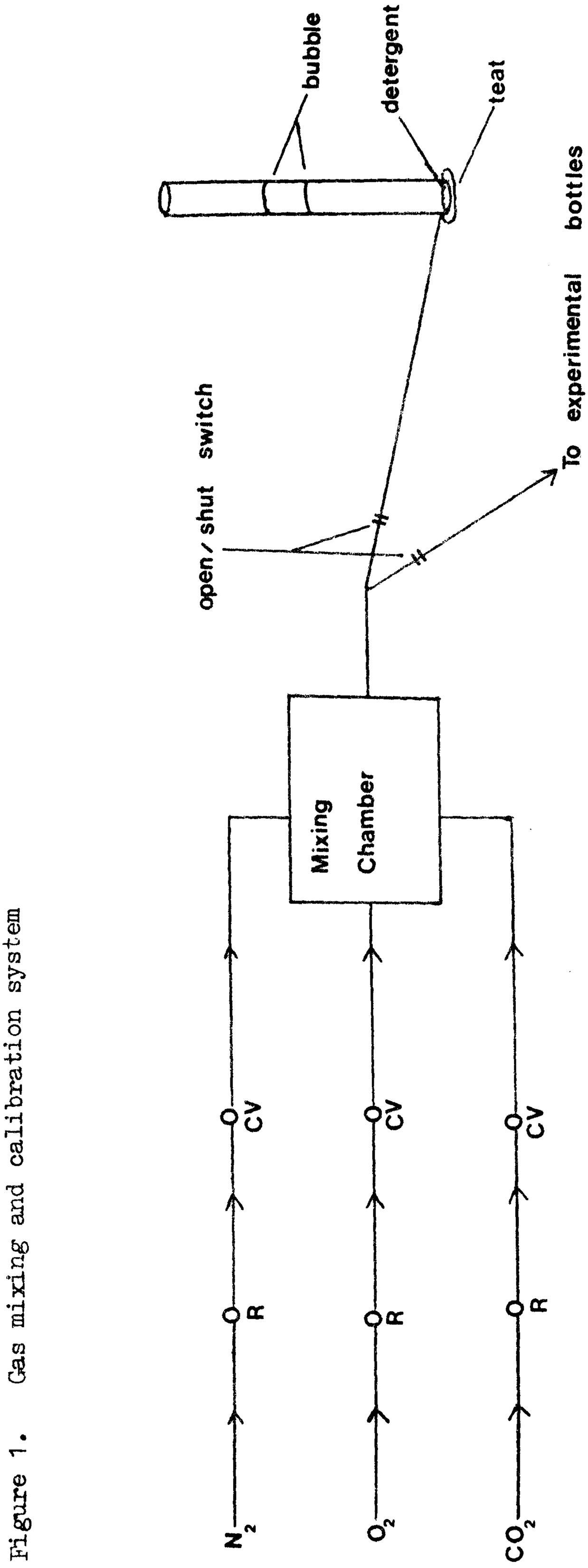


Figure 1. Gas mixing and calibration system

2.1.4. Cryopreservation of Trichomonas vaginalis

5 ml of culture medium containing 5% (v/v) DMSO was added to 5 ml of axenic, late log-phase culture, containing approximately 5×10^5 parasites/ml, by 3 additions at 15 minute intervals. This mixture was aseptically transferred into 1 ml stabilate tubes and frozen using liquid nitrogen and methanol, at a rate of 3-4°C/minute from room temperature to 0°C and at 1°C/minute from 0°C to -40°C. At -40°C the stabilate tubes were transferred into liquid nitrogen for storage. Parasites stored in this way maintained viability for several years. The parasite lines were reinitiated from stored parasites at least every 6 months, to minimise the effect of changes that occurred on continuous subculture.

2.1.5. Materials

Metronidazole (1-hydroxy ethyl-2-methyl-5-nitroimidazole) was provided by May and Baker Ltd., Dagenham, Essex, England. Other compounds were obtained from the following sources: l-ascorbate, l-acetate, L-lactate, D-glucose, D-dextrose, BDH Laboratory Chemical Division, Poole, Dorset, England; "Lab. lemco" powder, tryptone broth, neutralised liver digest, Oxoid Ltd., London, England; panmede, Paines and Byrne Ltd., Greenford, Middlesex, England; newborn calf serum, horse serum, Gibco-Biocult, Paisley, Scotland. All other chemicals were of analar grade and were obtained from Sigma Chemical Co., Ltd., Poole, Dorset, England. Deionised and distilled water was used in all solutions.

2.2. METHODS OF ASSESSING IN VITRO ANTITRICHOMONAL ACTIVITY

2.2.1. In vitro drug screening methods

Trichomonas vaginalis were incubated in round-bottomed, multiwell plates, containing 96 wells (Flow Laboratories Ltd., Ayrshire, Scotland) in 300 µl GTM medium with added drug (0-580 µM) at 37°C under both aerobic and anaerobic conditions. The latter conditions were produced by a BBL Gas Pak 150 hydrogen and carbon dioxide generating system (Becton, Dickinson U.K. Ltd., Wembley, Middlesex, England). The aerobic experimental plates were incubated in the incubator inside a large (25 x 15 x 12 cm) plastic sealed container, along with a beaker containing 25 ml water, so that the wells would not dry out. The effect of cysteine on the killing activity of the thiosemicarbazones (TSC1 and TSC2) on Leishmania mexicana mexicana promastigotes was carried out in multiwell plates under aerobic conditions, in Homem medium, with and without the addition of 12.7 mM cysteine HCl. Any pH change caused by the addition of the cysteine was countered by the addition of 0.1 M NaOH. These cultures were incubated at 26°C and parasite number and condition assessed . Incubation usually was for 24 hr, and the number and condition of parasites at the end of incubation was assessed using a Leitz inverted phase contrast microscope. Parasite number was assessed using a three level scheme:'++' indicating similar numbers of live cells as in the control well; '+' indicating live cells, but fewer than in the control, and '-' indicating no live cells present. Lack of flagellar and body motility was taken to mean that the parasite was dead. Drug activity was expressed as the minimum inhibitory concentration (MIC),

the lowest concentration in which no motile parasites were observed at the end of the experiment. All drugs were dissolved in water where possible, or in analar methanol diluted in water. In the experiments, only methanol concentrations of 2% or less were used, concentrations shown to have no inhibitory effect on the growth of the parasite. All drugs were filter sterilised, using a millipore filter (pore diameter 20 um) prior to use.

The drugs assessed using this method were: metronidazole, nitrofurans (nitrofurantoin and compound 4), 4-nitrodiazoles (compounds 1, 2 and 3), isothiazoles (compounds 5,6 and 7), thiosemicarbazones (TSC1, 2 and 3), quinone compounds (compounds 8 and 9), non-nitro heterocyclics (compounds 10 and 11), ethidium bromide, dimethyl sulphate, benzamide, 2-, 3- and 4-aminobenzamide, leupeptin, antipain. The structure of metronidazole, nitrofurantoin, compounds 1 to 11 and TSC1 to 3 are given in section 3.1.

In experiments to measure the antitrichomonal activity of thiosemicarbozones on Trichomonas vaginalis, cysteine and ascorbate were omitted from the medium. Experiments involving dimethyl sulphate were carried out in a fume cupboard.

In some experiments T. vaginalis was incubated in 10 ml test-tubes, in 5 ml of Bushby's medium with added drug (0-100 ug/ml in doubling dilutions) at 37°C, under aerobic conditions. The compounds tested by this method were: aniline, nitrobenzene, 4-nitrophenol and azobenzene. The number and condition of parasites was assessed by the microscopic examination of a wet preparation of parasite culture using the scheme outlined above.

The production of thiosemicarbazone killing curves was carried out using an axenic 100 ml culture containing approximately 2.7 x 10^6 cells per ml which was dispensed equally into five sterile 20 ml universal bottles, Thiosemicarbazones (TSC1 and TSC2) were added, at concentrations of 1 µM and 5 µM each, to four of the cultures, the fifth 20 ml culture, without added thiosemicarbazone, was used as a control. The cultures were incubated at 37°C. Small samples were removed from each culture using a sterile pipette after 0,30,60,120,180,240,300 and 360 minutes. The samples were used to determine the parasite density in each culture, using an improved Neubauer haemocytometer.

2.2.2. Procedure in experiments involving acetate and lactate

Experiments involving the effect of acetate, lactate or pH change on the killing effect of metronidazole were performed by adding metronidazole (0-120 µM in doubling concentrations) to GTM medium containing a range of acetate or lactate concentrations (0-10 mM in doubling concentrations) or to medium adjusted to pH 5.2 or pH 4.6, the pH obtained upon the addition of 10 mM acetate and 10 mM lactate respectively to GTM medium (usually pH 6.0). Parasites were incubated at 37°C under aerobic and anaerobic conditions as in section 2.2.1. Their number and condition was assessed using a Leitz inverted phase contrast microscope, and drug activity was assessed as in section 2.2.1.

2.2.3. Materials

The compounds assessed using the drug screening methods were

provided either by May and Baker Ltd., Dagenham, Essex, England, or by BDH Laboratory Chemical Division, Poole, Dorset, England.

2.3. PRODUCTION OF CELL-FREE HOMOGENATES

2.3.1. Trichomonas vaginalis

Axenic 200 ml cultures containing approximately 5×10^5 parasites per ml were harvested by centrifugation at 1430 g for 20 minutes at 4°C. Parasites were washed three times in a solution containing 0.11 M NaCl, 2.8 mM D-glucose and 0.19 M sodium phosphate, pH 7.4, resuspended to a cell density of approximately 1×10^8 cells/ml in a solution containing 0.25 M sucrose, 0.025 M kCl, 5 mM $MgCl_2$, 5 mM EDTA, 30 mM mercaptoethanol, 0.05 M tris-HCl, pH 7.5 and gassed with argon (1 litre/minute) over the surface of the solution, so that the surface was agitated, for 3.5 minutes. The tube containing the parasite suspension was sealed with a tight fitting suba-seal and gassed again for a further 2 minutes. Cell breakage was achieved by three cycles of freezing (-80°C) and thawing (37°C). In most cases, homogenates were assayed immediately, but some were immediately frozen to -70°C and assayed within 96 hrs.

2.3.2. Mouse liver

The liver was excised from a freshly killed female NIH mouse (Hacking and Churchill, Huntingdon, Cambridge), cut up into small pieces, rinsed twice in 10 volumes of 0.1 M tris-HCl, pH 7.3 buffer, resuspended in 3 volumes of the same buffer and homogenised with 20 strokes of a tight fitting pottor homogeniser at a speed of 10 revolutions per second. The resultant cell-free homogenate was

centrifuged at 1430 g for 10 minutes at 4°C using an MSE Chillspin centrifuge. The supernatant was removed and centrifuged at 24000 g for 60 minutes at 4°C using an MSE Prepspin 50 centrifuge, to remove the remaining cell debris. The supernatant was removed and either assayed immediately or frozen at -70°C for up to 4 days prior to use.

2.3.3. Materials

Suba-seal caps were obtained from A. Gallenkampand Co., Ltd., Braeview Place, Nerston, East Kilbride, Glasgow, Scotland. EDTA and mercaptoethanol were obtained from Sigma Chemical Co., Ltd., Poole, Dorset, England. All other chemicals were of analar grade and were obtained from BDH Laboratory Chemical Division, Poole, Dorset, England.

2.4. STUDIES ON THE XENOBIOTIC METABOLISM OF *TRICHOMONAS VAGINALIS*

Two methods of investigating the potential of *T. vaginalis* to metabolise azobenzene and nitrobenzene were used. (a) Using living parasites, (b) Using cell-free homogenates.

2.4.1. Incubation conditions and extraction procedure

a) Whole cells

Axenic cultures of *T. vaginalis* were grown in 100 mls of modified Bushby's medium at 37°C for 24 hrs, alone or in the presence of nitrobenzene or azobenzene at 25 µg/ml. At the end of the incubation the complete 100 ml culture, both cells and medium,

was subjected to 3 cycles of freezing (-80°C) and thawing (37°C) to break the cells, and the resultant homogenate was extracted three times into equal volumes of chloroform to which 0.1 M NaOH had been added, to raise the pH to pH 8.0. The extracts were evaporated to dryness in a vacuum oven at 50°C and resuspended either in 1-2 mls of chloroform, if they were to be analysed using thin layer chromatography, or in 1 ml of methanol, if they were to be subjected to high performance liquid chromatography (HPLC).

b) Cell-free homogenates

Azobenzene or nitrobenzene (25 µg) was incubated at 37°C with 500 µl of T. vaginalis cell-free homogenate, for 30 minutes under aerobic or anaerobic conditions. Two control phials, one containing homogenate but no parent compound, and the other parent compound but no homogenate, were incubated anaerobically at 37°C for 30 minutes. Anaerobic conditions were attained using a Forma Scientific anaerobe system, model 1024, containing a 37°C incubator. All incubations were conducted both with and without the addition of pyruvate (?50 mmoles), coenzyme A (5 mmoles) and methyl viologen (100 mmoles). At the end of the incubation the mixtures were extracted using the same system that was used for whole cell incubates.

2.4.2. Thin layer chromatography

The compounds in the samples were separated by ascending thin layer chromatography (Williams and Kirby, 1948). 10-40 µl samples

of the extracts, and 5 µg of the standards of the parent compounds and their mammalian metabolites, were spotted onto Camlab polygram Sil G/U.V.$_{254}$ thin layer chromatography plates. These were then placed upright in solvent tanks containing solvent to a depth of approximately 1 cm. The tanks were sealed and the solvent was seen to move up the plate over a period of hours. The plates were removed when the solvent front was approximately 1 cm from the top of the plate, the position of the solvent front was marked and the plates allowed to dry in air. The run was complete after approximately 5.5 hr using solvents I and II, and after 1.5 hr using solvent III.

After drying, the plates were examined under a 254 nm U.V. light source. The plates fluoresced under light of this wavelength and samples and standards showed up as dark spots on the fluorescing background. The samples and standards were examined by comparing the distance they and the solvent had migrated. This was expressed as the Rf value (i.e. distance of a sample spot from the origin/ distance of solvent front from the origin). An identical Rf value of a sample and a standard was taken as a positive identification of the sample. The standards used in this way were nitrobenzene, aniline, o-, m- and p-aminophenol, o-, m- and p-nitrophenol, azobenzene, hydrazobenzene. Three solvents were used:-

I. Butan-1-ol: acetic acid: H_2O (4:1:5 v/v) (Robinson, Smith and Williams, 1951).

II. Propan-1-ol: ammonia (7:3 v/v).

III. Methanol: toluene: acetic acid (8:45:4 v/v).

2.4.3. High performance liquid chromatography

Chromatography of standards and extracted samples was performed on a 25 cm reverse phase column packed with ODS hyperseal, 5 µm diameter beads, using a Waters Associates inc. chromatography pump, solvent delivery system, model 6000A, equipped with a Pye-Unicam LC-U.V. detector set at 290 nm. The mobile phase consisted of methanol:water (1:1 v/v).

The apparatus was calibrated for each standard compound by measuring the heights of absorbance peak obtained with a range of different concentrations of each compound, and comparing them with that of an added internal standard. The results were used to give standard curves of compound concentration vs. peak height ratio for each standard. The time taken from the injection of a compound to a peak registering is known as the retention time. The compounds in the extracted samples were identified by comparing the retention time and shape of the peak produced with peaks obtained on the injection of standards. 10 µls of extract was injected into the system each time, and replicate analyses of each extract was carried out.

2.4.4. Assessment of extraction efficiency

100 µg/ml standards of nitrobenzene, 3-aminophenol and aniline were extracted three times into chloroform at pH 8.0 as in section 2.4.1. The final 1 ml sample obtained was then used for HPLC in order to elucidate the efficiency of the extraction procedure. This was done by measuring the absorbance peak height of two equal concentrations of each compound, one of which was fresh, the other had been processed through the extraction procedure. Identical

peak heights for the two samples would indicate that the extraction procedure was 100% efficient.

2.4.5. Materials

Pyruvate, coenzyme A and methyl viologen were obtained from Sigma Chemical Co., Ltd., Poole, Dorset, England. All other compounds were provided by May and Baker Ltd., Dagenham, Essex, and supplied by BDH Laboratory Chemical Division, Poole, Dorset, England.

2.5. ENZYME ASSAYS

2.5.1. Metronidazole reduction

The reduction of the nitro-group of metronidazole was followed by measuring the decrease in absorbance at 320 nm using a Pye Unicam SP8000 spectrophotometer. The absorbance peak at 320 nm is due to the 5-nitro group of the compound, and this disappears on reduction of the group. The reduction was followed at 37°C under anaerobic conditions. The standard reaction mixutre contained 100 µmol metronidazole, 12.5 mmol pyruvate, 8 mmol methyl viologen, 250 µmol coenzyme A, 125 µmol mercaptoethanol, 50 µg cell-free homogenate protein in 600 µl, 0.1 M phosphate buffer, pH 7.0. Anaerobic conditions were achieved by gassing the reaction mixture prior to the addition of cell-free homogenate, in a 2 mm path length cell, fitted with a suba-seal, with argon for 2 minutes. The cell-free homogenate, was prepared under anaerobic conditions (section 2.3.1.), withdrawn from its anaerobic storage tube by microsyringe, and injected into the sealed cell to start the reaction.

2.5.2. Nitrobenzene and azobenzene reduction

The absorbance spectrum of nitrobenzene has a peak at 315 nm due to the nitro-group. Upon reduction of this group the absorbance disappears. Therefore, the reduction of the nitro-group of nitrobenzene was monitored by following the decrease in absorbance at 315 nm. The reduction was followed at 37°C under anaerobic conditions. The reaction mixture contained 0.12 mM nitrobenzene, 1.25 mmol coenzyme A, 62.5 mmol pyruvate, 40 mmol methyl viologen, 250 µg cell-free homogogenate protein in 3.4 ml, 0.1 M potassium phosphate buffer, pH 7.2. The reaction solutions were dissolved and then agitated for 10 minutes in an anaerobic environment produced in a Forma Scientific anaerobic hood system, model 1024, and then allowed to stand on ice, in this anaerobic environment for a further 10 minutes, to ensure that they contained no oxygen. The constituents of the reaction were then added to the cell which was then sealed. The cell was then taken out of the anaerobic chamber and the reaction followed in a Pye Unicam SP800 spectrophotometer.

In a similar way, the reduction of the azo-group of azobenzene was followed by measuring the decrease in absorbance, due to the azo-group, at 440 nm. 0.08 mM azobenzene was added to the reaction mix in place of nitrobenzene.

In both nitrobenzene and azobenzene reductions, the reaction was started by the addition of coenzyme A.

2.5.3. NADH oxidase (E.C. 1.6.99.3.) and NADPH oxidase (E.C. 1.6.99.1.)

NADH and NADPH both have absorbance maxima at 340 nm, whereas NAD^+ and $NADP^+$ do not. The oxidation of NADH and NADPH at 37°C were followed by monitoring the decrease in absorbance at 340 nm. The reaction mixture contained 0.12 µmol NADH or NADPH, 50 µg cell-free homogenate protein, and 0.1 M phosphate buffer, pH 7.2, to 1 ml total volume. For the NADPH oxidase assay 100 µmol FMN was added. In both assays the reaction mixtures were open to the atmosphere and cell-free homogenate was added to start the reaction (Tanabe, 1979).

2.5.4. Lactate dehydrogenase (E.C. 1.1.1.27.)

The activity of lactate dehydrogenase was followed at 37°C by measuring the decrease in absorbance at 340 nm due to the oxidation of NADH. The reaction mixture contained 0.6 µmol NADH, 7.5 µmol pyruvate, 25 µg cell-free homogenate protein in 600 µl, 0.1 M tris-HCl, pH 7.6. Anaerobic conditions were achieved by gassing the reaction mixture, in a cell fitted with a suba-seal, with argon for 2 minutes. A similar method was previously reported in use with T. vaginalis by Arese and Cappuccinelli (1974).

2.5.5. Hexokinase (E.C. 2.7.1.1.)

The activity of trichomonal hexokinase was followed at 37°C by linking it with glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49.) and measuring the increase in absorbance at 340 nm due to the formation of NADPH. The reaction mixutre contained 100 nmol $NADP^+$, 400 nmol EDTA, 12 µmol $MgCl_2$, 300 nmol ATP 9 µmol D-glucose,

330 m units of glucose-6-phosphate dehydrogenase (Grade II) from yeast, 50 µg T. vaginalis cell-free homogenate protein in 600 µl, 0.1 M tris-HCl, pH 7.3. Anaerobic conditions were achieved by gassing the reaction mixture, in a cell fitted with a suba-seal, with argon for 2 minutes.

The activities of purified yeast hexokinase and the isofunctional enzyme in mouse liver were measured aerobically using a similar reaction mixture as with T. vaginalis, but using a volume of 1.5 ml. Either 27 m units of hexokinase from yeast or 50 µg protein from mouse liver cell-free homogenate supernatant were added. This method was previously reported by Wellerson and Kupferberg (1962).

2.5.6. Phosphofructokinase (E.C. 2.7.1.11.)

The activity of phosphofructokinase at 37°C was followed using a modification of the method of Makkada, Schaefer, Simon and Neu (1974), by linking the enzyme to aldolase (E.C. 4.1.2.13.), triose-phosphate isomerase (E.C. 5.3.1.1.) and glycerol-3-phosphate dehydrogenase (E.C. 1.1.1.8.) and following the decrease in absorbance at 340 nm due to the oxidation of NADH. The reaction mixture contained 400 nmol fructose-6-phosphate, 360 nmole ATP, 300 nmol NADH, 1.3 µmol $MgCl_2$, 9 µmol KCl, 1.8 µmol DTT, 200 µg aldolase from rabbit muscle, 4 units glyceraldehyde-3-phosphate dehydrogenase/triose phosphate isomerase from yeast, 50 µg T. vaginalis cell-free homogenate protein in 600 µl 0.1 M tris-HCl, pH 7.3. Anaerobic conditions were achieved by gassing the reaction

mixture in a cell fitted with a suba-seal, with argon for 2 minutes. Phosphofructokinase assays using purified enzyme from rabbit muscle were performed aerobically using 1.5 ml total assay volume, the reaction mix components being proportionately increased, with 0.42 units of phosphofructokinase being added. In each case the ATP was added last, to start the reaction.

2.5.7. Pyruvate kinase (E.C. 2.7.1.40.)

The activity of pyruvate kinase at 37°C was followed using a modification of the method used by Valentine and Tanaka (1966), by linking the enzyme to lactate dehydrogenase (E.C. 1.1.1.27.) and following the decrease in absorbance at 340 nm due to the oxidation of NADH. The reaction mixture contained 240 nmole ATP, 1.35 µmol PEP, 60 nmol NADH, 4 µmol $MgCl_2$, 45 µmol KCl, 19 units of lactate dehydrogenase from pig heart, 50 µg T. vaginalis cell-free homogenate protein in 600 µl, 0.1 M tris-HCl, pH 7.3. Anaerobic conditions were achieved as in the other enzyme assays. Pyruvate kinase assays using purified enzyme from rabbit muscle or mouse liver homogenate supernatants were performed aerobically using 1.5 ml total volume, the reaction mix components being proportionately increased. 20 µls of pyruvate kinase or 50 µg of protein from mouse liver cell-free homogenate supernatant were added.

2.5.8. Effect of inhibitors on enzyme activity

The effect of a range of compounds on enzyme activity was assessed by comparing the rate of activity of each enzyme in the presence and absence of each compound. The inhibitory effect of

each compound was expressed as the percentage inhibition of the enzyme activity at a given inhibitor concentration.

2.5.9. Proteinase

Proteinase activity was assessed using azocasein as the substrate, by the method described by Coombs (1982). The reaction mixture contained 10 mg azocasein and approximately 500 µg *T. vaginalis* cell-free homogenate protein to a total volume of 750 µl with a buffered solution consisting of 0.25 M sucrose, 25 mMKCl, 5 mM $MgCl_2$, 5 mM EDTA, 50 mM tris-HCl, pH 6.0 or pH 7.5. Proteinase activity was assessed both in the presence and absence of 1.3 mM dithiothreitol (DTT). Several compounds were tested for proteinase inhibition, these were leupeptin, antipain and the thiosemicarbazones TSC1 and TSC2.

The effect of an inhibitor was assessed by preincubating it with cell-free homogenate for 1 hr at 37°C at 3 times the final concentration before the reaction was started by the additions of 500 µl substrate. Inhibitors were dissolved in buffer where possible, or in methanol. In the latter case the inhibitory activity was assessed by comparison with the appropriate methanol control.

Activity was also assessed at a range of pH's, from pH 5.0 to pH 8.0 using the above buffered solution adjusted to the appropriate pH by the addition of 1 M HCl or 1 M NaOH.

The reaction was stopped by the addition of 750 µl of 5% (v/v) trichloroacetic acid which had been cooled to 4°C. The precipitate was pelleted by centrifugation at 10,000 g at room temperature (18°C)

for 3 minutes using an Eppendorf centrifuge, and the absorbance at 366 nm of the supernatant fluid was measured using an MSE Spectro-plus spectrophotometer. One unit (U) of activity was defined as that which caused hydrolysis of 1.0 Mg azocasein/ minute under assay conditions. The $E^{1\%}_{366}$ of azocasein was taken as 32.

2.5.10. Materials

Metronidazole, azobenzene and nitrobenzene were provided by May and Baker Ltd., Dagenham, Essex, England. Aldolase, from rabbit muscle (4.1.2.13) (9 U/mg at 25°C, fructose as substrate); glucose-6-phosphate dehydrogenase (grade II), from yeast (1.1.1.49.) (140 U/mg at 25°C, glucose-6-phosphate as substrate); glycerol-phosphate dehydrogenase/triose phosphate isomerase, from yeast (1.1.1.8./5.3.1.1.); hexokinase, from yeast (2.7.1.1.) (140 U/mg at 37°C, ATP and glucose as substrates); pyruvate kinase, from rabbit muscle (2.7.1.40.) (500 U/mg at 37°C, PEP as substrate); phosphofructokinase, from rabbit muscle (2.7.1.11.) (130 U/mg at 37°C, fructose-6-phosphate and ATP as substrates); Lactate dehydrogenase, from pig heart (1.1.1.27.)(1100 U/mg at 37°C, pyruvate as substrate); and sodium pyruvate were obtained from Boehringer Co., (London) Ltd., Lewes, England. All other compounds were of analar grade and were obtained from either BDH Laboratory Chemical Division, Poole, Dorset, England, or Sigma Chemical Co., Ltd., Poole, Dorset, England.

2.6. DETERMINATION OF COMPOUND CONCENTRATIONS

2.6.1. Lactate

The concentrations of lactate in media in which T. vaginalis had been growing were determined by a modification of the method of Gutman and Wahlefeld (1974). The reaction mixture contained 4 µmol NAD^+, 50 units of lactate dehydrogenase, from pig heart, 20 µls of sample, glycine (0.4 M) and hydrazine (0.5 M) buffer, pH 9.0, to 1.5 mls. 20 µmol of perchloric acid was added to control solutions to stop any reaction occurring. The lactate concentration was assessed by monitoring the increase in absorbance at 340 nm due to NADH formation. This was produced from lactate in the reaction: Lactate + NAD^+ $\longrightarrow$ Pyruvate + NADH + H^+. Thus the total amount of NADH formed was directly proportional to the concentration of lactate initially present. The reaction at 37°C was followed using an SP8000 U.V. recording spectrophotometer and the total change that occurred measured. A range of standard lactate concentrations in culture medium, were assayed using this method, and a calibration curve of medium lactate concentration vs. change in absorbance at 340 nm was constructed. Samples of media which had been used to grow cultures of T. vaginalis were then assayed, and the lactate concentrations estimated.

2.6.2. Cell preparation for glutathione or thiol determinations

50 ml samples of late log phase T. vaginalis culture were harvested as in section 2.3.1. and washed 3 times in 0.1 M phosphate buffer, pH 8.0 containing 0.005 M EDTA, and resuspended in a total of 2.0 mls of this buffer and 0.42 mls 25% (v/v) phosphoric acid.

Cell breakage was achieved by 3 cycles of freezing (-80°C) and thawing (37°C). The resultant homogenate was centrifuged at 20,000 g for 30 minutes at 4°C in an MSE prepspin ultracentrifuge. The supernatant was removed and stored at -70°C until the glutathione or thiol determinations were carried out.

To assess the effect of thiosemicarbazones on glutathione and thiol levels, four parallel cultures (approximately 5 x 10^5 organisms /ml) were incubated for 3.5 hr at 37°C in the presence of thiosemicarbazone (TSC1 or TSC2) at concentrations of either 1 µM or 5 µM. A fifth culture with no added drug acted as a control. At points throughout the incubation period 10 ml samples were removed from each culture and processed as described above, until thiol and glutathione determinations were carried out as described in sections 2.6.3. and 2.6.4.

2.6.3. Glutathione

Glutathione (GSH) was determined by the method of Hissin and Hilf (1976). 100 µl of T. vaginalis homogenate supernatant, prepared as described in section 2.6.2., was made up to 2.9 ml with 0.1 M sodium phosphate buffer, pH 8.0, containing 5 mM EDTA. A series of standards (0-1.5 µg reduced glutathione in 100 µl buffer) were treated similarly. 100 µl of 0.1% (w/v) orthophthaldehyde (OPT) in absolute methanol was added to form a fluorescent compound. Some samples were spiked with an internal standard of 0.5 µg GSH, and GSH was estimated as above. After 20 minutes incubation at room temperature, fluoresence was measured using a Perkin Elmer fluoresence spectrophotometer, model MPF4, excitation wavelength

350 nm, emission wavelength 420 nm.

Concentrations of sample of glutathione were calculated by comparison with GSH standards. All standards and samples were assayed in duplicate. Both GSH standards and OPT were prepared just prior to use.

2.6.4. Thiol

The non-protein thiol content of T. vaginalis homogenate supernatant was determined by using Ellman's reagent (Ellman, 1959). 30 µl samples, prepared as in section 2.6.2., were made up to 3.0 ml with 0.05 M potassium phosphate buffer, pH 7.4, and the absorbance at 412 nm measured, using buffer as a blank. 20 µl 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) was added and the mixture left at room temperature for 10 minutes. The absorbance at 412 nm was measured again, using buffer as a blank, and the change in absorbance (Δ E) at 412 nm calculated. The total concentration of thiol was estimated by comparison with GSH standards in the range 0-20 nmole/ml in buffer. Some samples were spiked with an internal GSH standard. All samples were assayed in duplicate.

2.6.5. Protein

Protein concentration was estimated by the method of Lowry, Rosebrough, Farr and Randall (1951). To 0.2 ml sample was added 1.0 ml reagent 1 (2% (w/v) $Na_2 CO_3$ in 0.1 M NaOH, 1% (w/v) copper sulphate solution, 2% (w/v) NaK tartrate; 98; 1; 1 v/v) and the mixture incubated for 10 minutes at room temperature (18°C). 0.1 ml of reagent 2 (2N Folin and Ciocalten's phenol reagent;

distilled water; 1; 1 v/v) was added and the mixture incubated for a further 30 minutes at room temperature. The absorbance at 750 nm was measured. By comparison with a standard curve, constructed using standards containing 0-50 µg bovine serum albumen, increasing by 5 µg steps, sample protein concentration was calculated.

2.6.6. Materials

EDTA, NAD^+ and Folin's reagent were supplied by Sigma Chemical Co., Ltd., Poole, Dorset, England. Copper sulphate, sodium potassium tartrate, glycine, hydrazine, l-lactate and phosphoric acid were supplied by BDH Laboratory Chemical Division, Poole, Dorset, England. All other compounds were supplied by May and Baker Ltd., Dagenham, Essex, England.

2.7. THIOSEMICARBAZONE CHELATE PRODUCTION

2.7.1. Chelate formation

Possible thiosemicarbazone chelate formation was followed by continuously monitoring the spectrum of TSC1, from 300-700 nm, upon the addition of solutions of $MnSO_4$, $MgCl_2$, $ZnSO_4$, NaCl, KCl, and $CaCl_2$. All compounds were dissolved in 50% Methanol and monitoring was carried out at room temperature. In an attempt to elucidate the molar ratio of TSC1 to metal in the chelates, 10 µm, 100 µM and 500 µM concentrations of TSC1 were titrated against solutions of $MnSO_4$, $ZnSO_4$ and $CuSO_4$ at concentrations of 15-2000 µM. Measurements were made of the change in absorbance at

480 nm upon addition of each amount of $CuSO_4$, and at 460 nm and 345 nm with $MnSO_4$ and $ZnSO_4$ respectively. These were the wavelengths at which the difference in absorbance between TSC1 and respective chelates had been found to be maximal. When all the TSC1 had been chelated, the addition of excess metal did not cause any further change in absorbance.

The effect of 5 mM concentrations of compounds containing thiol groups (cysteine, DTT, glutathione, homocysteine) on the stability of the chelate was also investigated. This was done by monitoring the spectrum of a chelate upon addition of thiol compounds and for 30 minutes subsequently. It was anticipated that the spectrum would alter if the chelate was disrupted.

2.7.2. Materials

The thiosemicarbazone (TSC1) was supplied by May and Baker Ltd., Dagenham, Essex, England. Cysteine, DTT, glutathione and homocysteine were obtained from Sigma Chemical Co., Ltd., Poole, Dorset, England. All other compounds were obtained from BDH Laboratory Chemical Division, Poole, Dorset, England.

Drugs and Solvents

Metronidazole and the nitrofurons (nitrofurantoin and compound 4) were dissolved in distilled water. Dimethyl sulphate and ethidium bromide were dissolved in 100% ethanol. All other drugs were dissolved in 100% methanol.

Abbreviations

ATP, Adenine 5'triphosphate; DTNB, 5,5'-dithiobis-2-nitrobenzoic acid; DTT, dithiothreitol; EDTA, ethylenediaminetetra acetate; FMN, flavin mononucleotide; GSH, glutathione; HPLC, high-performance liquid chromatography; LRS, laboratory developed resistant strain; MIC, minimum inhibitory concentration; NAD^+, nicotinamide adenine dinucleotide (oxidised form); NADH, nicotinamide adenine dinucleotide (reduced form); $NADP^+$, nicotinamide adenine dinucleotide phosphate (oxidised form); NADPH, nicotinamide adenine dinucleotide phosphate (reduced form); OPT, orthophthaldehyde; PEP, phosphoenol pyruvate; U.V., ultra-violet.

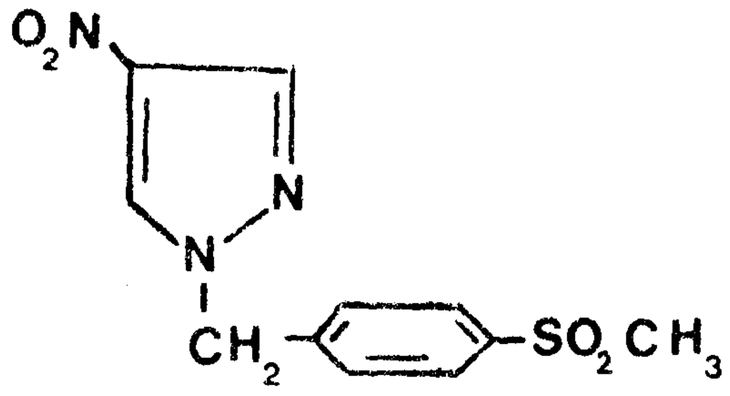
3. RESULTS

3.1. SENSITIVITY OF TRICHOMONAS VAGINALIS TO A RANGE OF COMPOUNDS: VARIATIONS WITH CULTURE CONDITIONS

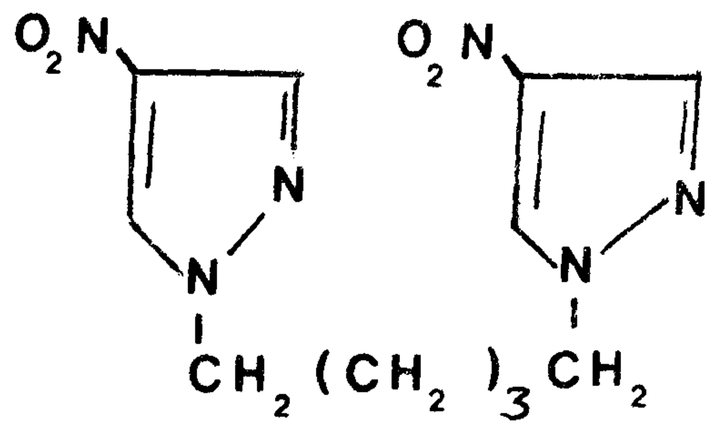
The sensitivity of three lines of T. vaginalis to a range of compounds under aerobic and anaerobic conditions, using the methods given in section 2.2.1., are given in Table 1. It was found that each of the nitroheterocyclic compounds tested (5-nitroimidazole, 4-nitrodiazoles and nitrofurans) were active against T. vaginalis, clone G3. The 4-nitrodiazoles however, were relatively inactive against line IR78 under both aerobic and anaerobic conditions, whereas the 5-nitroimidazole (metronidazole) was inactive only under aerobic conditions. In contrast, the nitrofurans were active against all three parasite lines under aerobic and anaerobic conditions. Line LRS (0.6), the parasite line grown in the laboratory under in vitro drug (metronidazole) pressure, and at the time of these experiments grown routinely in medium containing 0.6 µg metronidazole/ml, was affected by by nitro-compounds differently to line IR78. Metronidazole was active against this line under both aerobic and anaerobic conditions, although higher concentrations were required to kill all the cells than with clone G3. The 4-nitrodiazoles, although relatively effective against it and clone $G3_1$ differed with individual compounds. The thiosemicarbazones had poor activity against all three parasite lines in comparison to metronidazole when tested using this method. All other compounds tested were inactive against all three parasite lines at concentrations of 100 µM. The structures of these compounds are given in Diagram 1.

Diagram 1

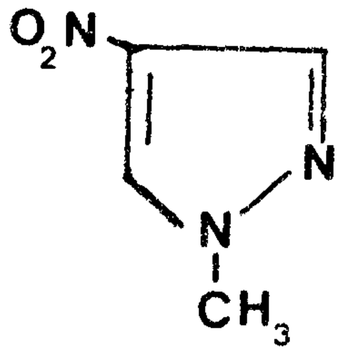
Structure of compounds tested for antitrichomonal activity



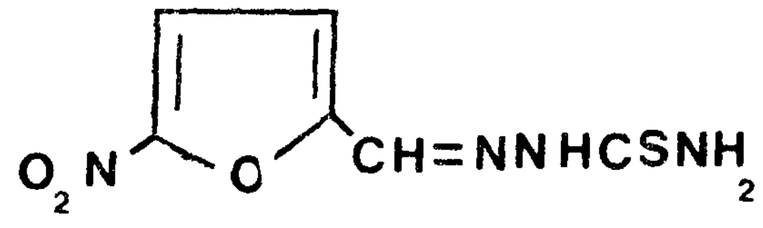
compound 1



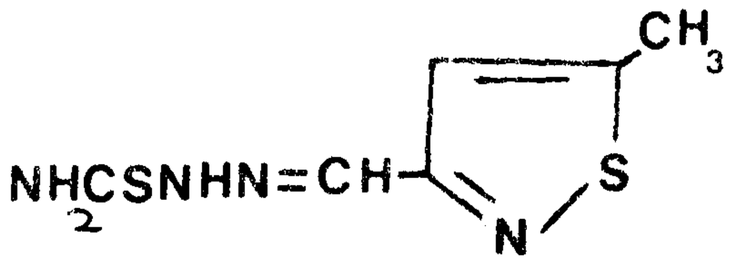
compound 2



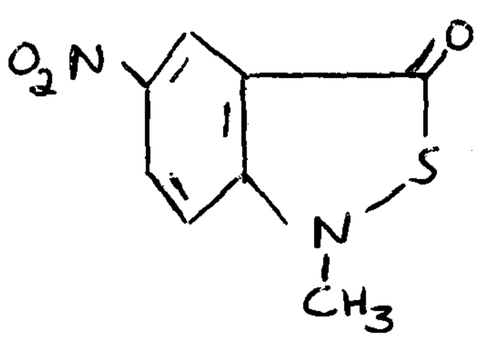
compound 3



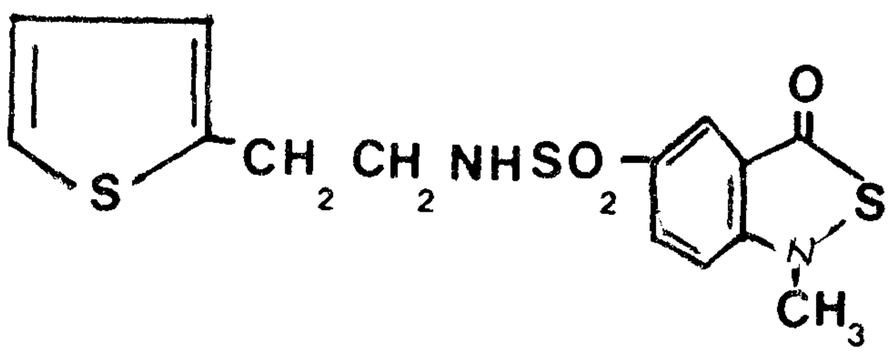
compound 4



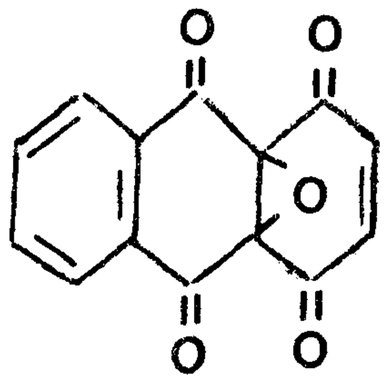
compound 5



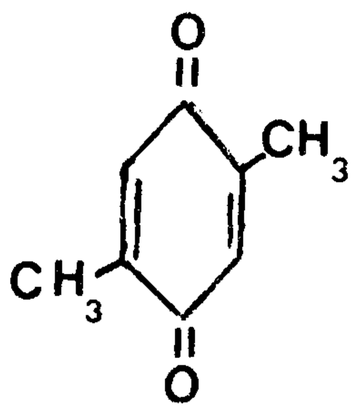
compound 6



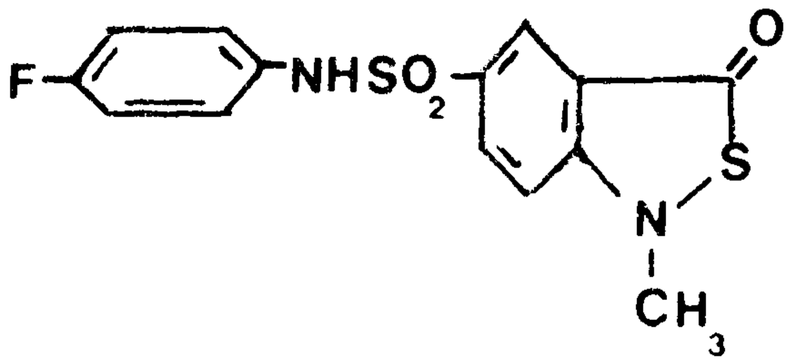
compound 7



compound 8

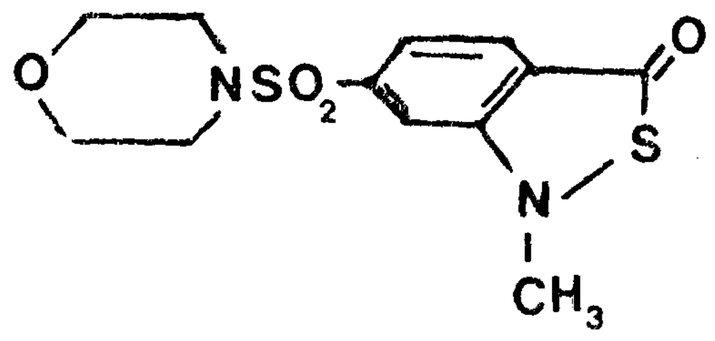


compound 9

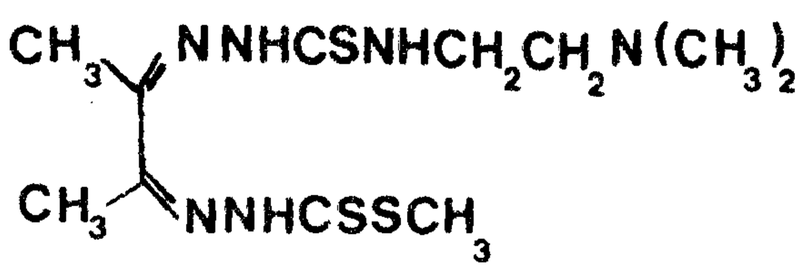


compound 10

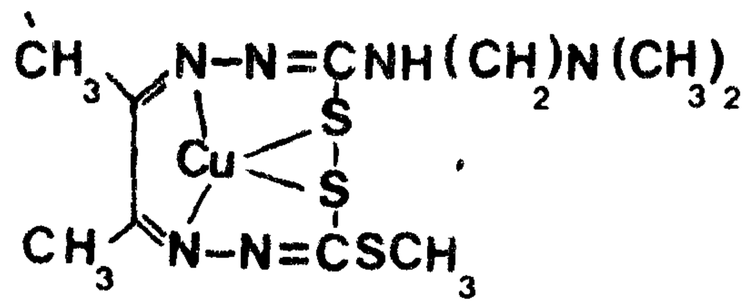
Diagram 1 contd.



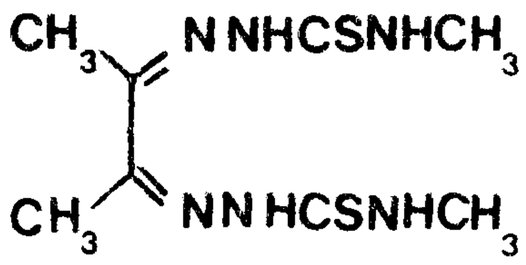
Compound 11



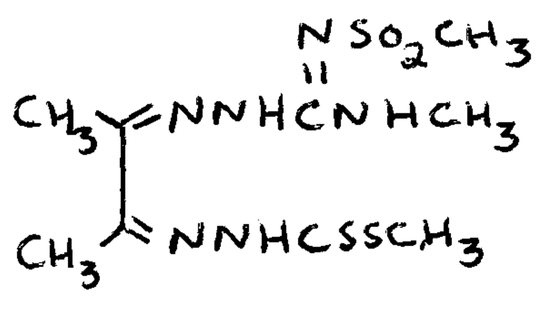
TSC 1



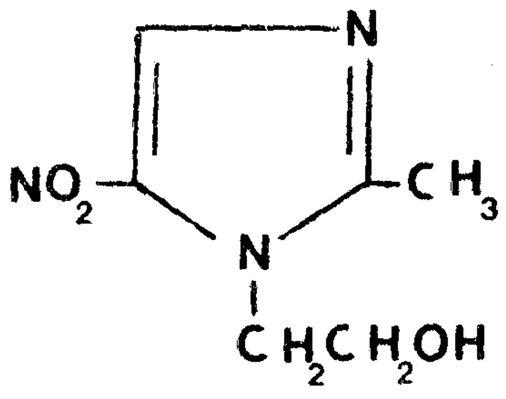
TSC 2



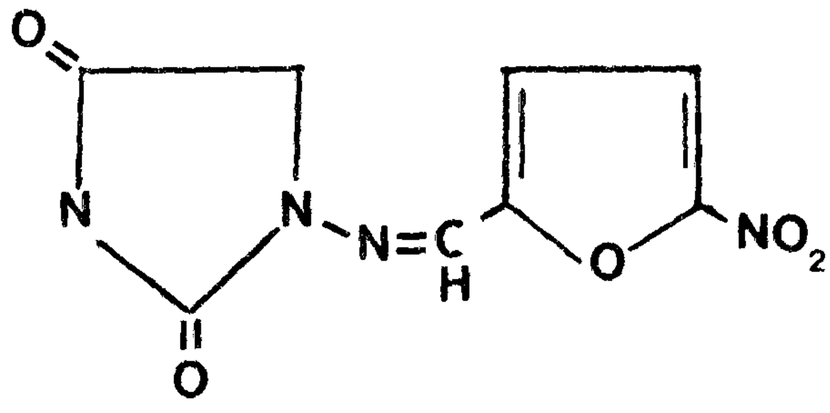
TSC 3



TSC 10



Metronidazole



Nitrofurantoin

The sensitivity of T. vaginalis, line 965691, to all the above compounds, except metronidazole and nitrofurantoin, was tested using the test-tube screening method described in section 2.2.1. The results of this method are given in Table II. They show that the minimum inhibitory concentrations (MIC) obtained using this method were, in most cases, similar to those produced by the microtitre plate method. Using the test-tube method, however, the thiosemicarbazones (TSC1 and TSC10) and the isothiazoles were found to be much more active. To test if the differences in activity were dependant upon the parasite strain, the culture medium, or the assessment method, the sensitivities of clone G3 and line 965691 to a thiosemicarbazone (TSC10) and to an isothiazole (compound 7) were measured using both the methods outlined in section 2.2.1., using both GTM and Bushby's medium. The results obtained are given in Table III. They show that for each compound the MIC was lower using Bushby's medium than with GTM medium.

The sensitivities of line 965691 to both of the above compounds (compound 7 and TSC10) were tested in GTM medium, Bushby's medium, and in Bushby's medium with added ascorbate (12.7 mM and 2.7 mM) or added cysteine (12.7 mM or 2.7 mM), using the aerobic microtitre plate method. 12.7 mM cysteine or ascorbate was used as this is the concentration normally present in GTM medium, 2.7 mM concentrations were used to study the effect of lower levels of the two compounds. The results are given in Table IV. The addition of 12.7 mM cysteine to Bushby's medium raised the MIC of TSC10 to 20 μM, a point between the MIC's found using GTM (100 μM) and Bushby's medium without additives (< 5 μM). The addition of

2.7 mM cysteine or either concentration of ascorbate had no discernable effect. The addition of cysteine or ascorbate raised the MIC of compound 7 to a lesser extent, but not to that using GTM medium. The addition of 2.7 mM ascorbate had a greater effect than that of 12.7 mM ascorbate. This result suggests that high concentrations of ascorbate may not inhibit the activity of this compound. The addition of 12.7 mM cysteine and 12.7 mM ascorbate together to Bushby's medium had a similar effect to the addition of 12.7 mM cysteine alone on the MIC of TSC10, but had a greater effect on the MIC of compound 7 than using either compound by itself.

3.2. ANTITRICHOMONAL ACTIVITY OF METRONIDAZOLE

The sensitivities of clone G3 and lines LRS (2.0), Boston, Fall River and Albany to metronidazole measured under aerobic and anaerobic conditions are given in Table V. It was found that lines IR78, Boston, Fall River and Albany were resistant to metronidazole, but only under aerobic conditions, whereas line LRS (2.0) was less sensitive to the drug than clone G3 under both aerobic and anaerobic conditions.

3.3. STUDIES ON METRONIDAZOLE RESISTANT T. VAGINALIS

3.3.1. Reduction of metronidazole by homogenates of T. vaginalis

Metronidazole was reduced by cell-free homogenates of T. vaginalis only if all the assay constituents given in section 2.5.1. were present, with the single exception of methyl viologen.

Reduction occurred in the absence of methyl viologen, but at a much lower rate, about one twentieth, than when methyl viologen was present. If oxygen was present the reduction did not occur. The addition of NADH, NADPH or FMN did not enhance the reaction or replace the requirement for any of the assay constituents. The metronidazole reducing activity decreased by approximately 20% over 5 hr, when the cell-free homogenate was kept on ice under a flow of argon. If the homogenate was not kept on ice under argon the activity decreased by approximately 50% in 1 hr. The rates at which metronidazole was reduced by cell-free homogenates of three lines of T. vaginalis (clone G3, lines LRS (2.0) and IR78) are given in Table VI. The results show that homogenates of line LRS (2.0) reduced the drug much more slowly than those of either clone G3 or line IR78.

3.3.2. NADH oxidase and NADPH oxidase activities

NADH oxidation catalysed by cell-free trichomonal homogenate took place under aerobic conditions only, and the addition of CoA or FMN did not enhance the reaction. The enzyme was stable at $4^{\circ}C$ for a three hour period, but after 6 hr activity had decreased by 20%. NADPH oxidation took place under aerobic conditions only, and was enhanced by FMN. Without FMN present the NADPH oxidase activity of T. vaginalis clone G3 homogenate was approximately 20 nmoles/min/mg cell-free homogenate protein i.e. 20 fold less than in the presence of FMN. For this reason FMN was added to all NADPH oxidase assays to enhance the reaction. The NADH oxidase and NADPH oxidase activities of cell-free homogenates of the three

lines are given in Table VI. The two activities of line IR78 were found to be significantly lower than those of clone G3, and the NADPH oxidase activity was also found to be significantly lower than that of line LRS (2.0). The enzyme activities of clone G3 and line LRS (2.0) were not significantly different.

3.3.3. Growth studies

The growth of clone G3 and line IR78 in GTM medium under anaerobic and aerobic conditions, and in media containing lower concentrations of cysteine and ascorbate are given in Figures 1-4. It was found that with clone G3 under anaerobic conditions, reduced concentrations of cysteine and ascorbate resulted in a lower growth rate and a lower maximum parasite density. The reduction in growth rate was much greater with line IR78 (Fig. 3) than that with clone G3 (Fig. 1). Under aerobic conditions the lowering of cysteine and ascorbate concentrations similarly resulted in lower growth rates and lower maximum parasite densities, but under these conditions the effect was much more marked. With line IR78 rapid cell death occurred in the absence of added cysteine and ascorbate and when they were present at 1.3 mM and 2.1 mM respectively. With clone G3, there was parasite growth even in the absence of added cysteine.

Although high concentrations of both cysteine and ascorbate were required for optimal growth under aerobic conditions, the parasites were more sensitive to changes in the cysteine concentration (Table VII). When the cysteine concentration was varied

the ascrobate concentration was constant at 21 mM and when the ascorbate concentration was varied the cysteine concentration was constant at 12.7 mM. Since the effects of cysteine were most obvious under aerobic conditions, other thiol-containing compounds were tested for their effect on growth under these conditions. The results are given in Table VIII. The replacement of cysteine by other agents resulted in reduced growth, however growth was still greater than if there were no thiol compounds added. Perhaps surprisingly, methionine was the most effective replacement for cysteine.

The growth of clone G3 and line IR78 in the medium without cysteine or ascorbate under various oxygen, nitrogen and carbon dioxide tensions is shown in Figures 5 and 6 respectively. It was found with both strains that as the oxygen tension was increased then growth was lessened. Premature death of all the parasites was caused at oxygen tensions of 15% and 20% with clone G3, although only after an initial increase in parasite numbers, and growth rate and maximum density achieved were reduced at lower oxygen tensions compared with the anaerobic cultures. With line IR78 those effects were more marked, premature cell death occurring at oxygen tensions of 10% and above, and with no initial increase in parasite numbers. In this experiment the growth rate of line IR78 under an argon atmosphere was rather less than that found in some other experiments using similar conditions e.g. Figure 3. The possible explanation for this could be that despite flushing with argon, a level of oxygen was present in the incubation vessel, and this was sufficient to slightly inhibit parasite growth.

A 5% carbon dioxide concentration was found to enhance slightly growth of both lines compared with growth under an argon atmosphere.

3.3.4. Effect of acetate and lactate on the sensitivity of *T. vaginalis* to metronidazole

The sensitivities of *T. vaginalis* clone G3 to metronidazole under aerobic and anaerobic conditions, in GTM medium with added 5 mM **L**-lactate, **L**-acetate, **L**-pyruvate and **L**-succinate, and at different pH's, are given in Table IX. Parasites grown in medium containing 5 mM lactate or acetate were less sensitive to metronidazole under aerobic, but not under anaerobic conditions, than those grown in medium without the added salts. Neither succinate nor pyruvate had any effect on parasite sensitivity to metronidazole. PH had no effect. The effect of different concentrations of lactate and acetate on metronidazole sensitivity is given in Table X. Increasing the lactate or acetate concentrations to 10 mM did not enhance the inhibition, and the addition of 0.5 mM lactate or acetate to cultures had no detectable effect, whereas 2 mM lactate or acetate raised the aerobic MIC to approximately half that found in the presence of a 5 mM concentration.

It was feasible that the effects of additions of lactate and acetate upon the sensitivity of the parasite to metronidazole may be due to the lower pH which they produced, however this was not the case (Table IX).

Metronidazole reduction by, and NADH oxidase and NADPH oxidase activities in, parasite homogenates were not affected by the addition of 5 mM lactate or 5 mM acetate to the assay mixtures.

The addition of 5 mM lactate or acetate to the growth medium did not affect parasite growth significantly under aerobic conditions, although it was reduced appreciably by 10 mM of the compounds (Figures 7 and 8), nor did it affect the specific activities of NADH oxidase or NADPH oxidase in parasite homogenates, or the rate at which the homogenates catalysed the reduction of metronidazole (Table XI).

As lactate is a major excretory product of T. vaginalis, the effect of growth in 5 mM lactate was investigated by studying the activity of parasite lactate dehydrogenase. It was found that the activity (5.1 $\pm$ 0.3 umoles/min/mg protein, mean of four experiments $\pm$ one standard deviation) of lactate dehydrogenase in cell-free homogenates of parasites (clone G3) grown in culture medium with 5 mM lactate added was significantly greater than the activity (3.8 $\pm$ 0.3 umoles/min/mg protein) in cell-free homogenates of parasites grown in GTM medium alone. In addition, the concentration of lactate produced by the parasite growing for 24 hr in GTM medium which 5 mM lactate added was significantly less than that produced from cells growing in GTM medium alone (Table XII).

3.4. SYNERGISTIC ANTITRICHOMONAL DRUG COMBINATIONS

The sensitivities of T. vaginalis clone G3 to metronidazole, a thiosemicarbazone (TSC1), ethidium bromide, dimethyl sulphate (DMS) and various benzamides, under both aerobic and anaerobic conditions, are given in Table XIII. The thiosemicarbazone (TSC1) was highly active against T. vaginalis. Dimethyl sulphate was

more active than metronidazole, but less active than TSC1. Ethidium bromide and the benzamides did not kill all the parasites, even at 1 mM and 5 mM respectively. The results were similar under both aerobic and anaerobic conditions. The trichomonacidal concentrations of metronidazole, TSC1, ethidium bromide and dimethyl sulphate in combination with sub-inhibitory concentrations of the benzamides are given in Table XIII. It was found that the addition of benzamide or the aminobenzamides to cultures containing dimethyl sulphate reduced the MIC of DMS approximately one hundred fold. This effect was independant of oxygen. The benzamides, however, did not alter the effectiveness of metronidazole or TSC1. Ethidium bromide was no more effective in combination with benzamide and aminobenzamides than by itself.

3.5. THE XENOBIOTIC METABOLISM OF AZOBENZENE AND NITROBENZENE BY TRICHOMONAS VAGINALIS

3.5.1. Antitrichomonal activity of azobenzene and nitrobenzene

The sensitivities of T. vaginalis to azobenzene, nitrobenzene and two of their mammalian metabolites, aniline and p-nitrophenol, are given in Table XIV. All four compounds killed the parasite, but high concentrations were required compared with metronidazole.

3.5.1.1. Reduction of azobenzene and nitrobenzene by T. vaginalis

As one of the mammalian metabolites (aniline) of both compounds is a reductive metabolite, the production of which involves the reduction of the nitro-group of nitrobenzene or the azo-group of azobenzene, the ability of T. vaginalis cell-free homogenate to reduce these compounds was investigated.

The reduction was followed at 440 nm with azobenzene and at 315 nm with nitrobenzene, wavelengths at which the azo-group and nitro-group absorb maximally. These absorbance maxima disappear on reduction. The addition of pyruvate and coenzyme A to T. vaginalis cell-free homogenate and the compound, was necessary for the reduction to occur. Reduction took place under anaerobic conditions only. The addition of NADH to the assay mix had no effect, but the addition of 40 mmoles methyl viologen enhanced the rate of reduction approximately five fold.

3.5.2. Detection of metabolites by Thin Layer Chromatography

Possible metabolites of nitrobenzene and azobenzene metabolism in T. vaginalis were looked for using thin layer chromatography (TLC). Since nitrobenzene and azobenzene were reduced by cell-free homogenates of the parasite, reductive metabolites were considered good candidates as metabolic products.

The Rf values of 5 µg standards of nitrobenzene and azobenzene, and their main mammalian metabolites were elucidated using the solvents and methods given in section 2.4.2. The Rf values of these compounds are given in Table XV. The Rf values show that it is possible to distinguish between azobenzene and two of its main mammalian metabolites, benzidine and 4-aminophenol, using solvents I or III. However, hydrazobenzene could not be distinguished from azobenzene in this manner, whereas aniline was not detected using any of the three solvents. Nitrobenzene and its main mammalian metabolites, the aminophenols and the nitrophenols, were found to be separated using solvent III, and to a lesser extent with solvent I. Separation of the different species of these groups of compounds was not possible, except in the case of 4-aminophenol which had a much

lower Rf value than 2- or 3-aminophenol with solvent III. Using solvents I and III, 2-nitrophenol could not be detected. Solvent II was the least useful solvent. With this solvent it was not possible to separate aminophenols and nitrophenols, although detection of 2-nitrophenol was possible. This method was very sensitive, a 0.1 µg sample of 4-aminophenol could be detected using solvent I.

Chromatography of extracts of parasites grown in the presence of azobenzene produced spots with Rf values identical to that of the parent compound. These spots could have been due to the parent compound, to hydrazobenzene, or to a mixture of both, as it was not possible to separate these two compounds using these solvents. No other metabolites could be positively identified. Similarly chromatography of extracts of T. vaginalis grown in the presence of nitrobenzene demonstrated the presence of nitrobenzene itself, but no metabolites. A function of this method was the inability to detect aniline. This is the major purely reductive metabolite of both nitrobenzene and azobenzene, but its production by T. vaginalis could not be detected by this method.

3.5.3. Detection of metabolites by High Performance Liquid Chromatography

The metabolism of nitrobenzene and azobenzene by T. vaginalis was investigated using HPLC. This method is more sensitive than TLC, giving greater separation of compounds, and quantification is much more precise.

Standards of azobenzene, nitrobenzene, and their main mammalian metabolites were injected into the column and the retention time of each compound and the shape of its absorbance at 290 nm was studied. The retention time of all the compounds are given in Table XVI. The results show that using compound standards, azobenzene and its mammalian metabolites, hydrazobenzene, benzidine, aniline, 2-aminophenol and 4-aminophenol can be separated and detected using this method, as can nitrobenzene and its mammalian metabolites, 3-nitrophenol, aniline and the aminophenols. HPLC can be used to detect much smaller quantities of a compound than TLC. The detection limit of the TLC method given in section 2.5.2. was 0.1 µg of a 4-aminophenol standard. Using HPLC the same compound was detected using a 0.01 µg standard. The other standards had detection limits in the range 0.005-0.05 µg. In this series of experiments cell-free homogenates of the parasite had been incubated with 25 µg of the parent compound. If extraction was 100% efficient, then the 1 ml extraction sample would contain 25 µg parent compound and metabolites, well within the limits of detection. Experiments with intact parasites involved 2,500 µg of parent compound and detection would be easier in these cases.

No azobenzene or nitrobenzene metabolites were detected by HPLC in whole-cell incubates (section 2.4.1. (a)). Parent compound recovery rates were approximately 90% (azobenzene) and 70% (nitrobenzene) in both control (incubates lacking parasites) and experimental incubates.

Using incubates of cell-free homogenates and parent compounds

alone, no metabolites were detected, and parent compound recovery rates were similar to those given above. In incubates with added pyruvate, coenzyme A, methyl viologen, as well as nitrobenzene and cell-free homogenate, only 30% of the parent compound was recovered. In appropriate control incubates (lacking homogenate), 70% of the parent compound was recovered. However, no metabolites were detected in the experimental sample. In incubates containing pyruvate, coenzyme A, methyl viologen, azobenzene and cell-free homogenate, hydrazobenzene was detected by cochromatography. Approximately 15% of the azobenzene was reduced to hydrazobenzene and approximately 40% of the parent compound was recovered. In control incubates (lacking homogenate) approximately 75% of the parent compound was recovered.

In an attempt to elucidate the efficiency of the extraction procedure, the extraction of nitrobenzene, 3-aminophenol and aniline standards was carried out, and the compounds detected by HPLC. Approximately 70% of the nitrobenzene and 3-aminophenol was recovered, but aniline was not detected. Unextracted aniline samples were detected. A quantitative U.V. analysis of an aniline chloroform extract and its aqueous remains showed that the aniline was extracted into the chloroform. The next step in the extraction procedure was the evaporation of the chloroform to dryness, and resuspension in methanol. The aniline was not present in the methanol however, and must have been lost in the evaporation procedure, presumably due to its highly volatile nature.

3.6. ANTITRICHOMONAL ACTIVITY OF THIOSEMICARBAZONES

3.6.1. Sensitivity of T. vaginalis to thiosemicarbazones

The sensitivity of T. vaginalis to three thiosemicarbazone compounds (TSC1, TSC2 and TSC3) and of Leishmania mexicana mexicana promastigotes to TSC1, both with and without the addition to the media of compounds containing thiol groups, is given in Table XVII. The presence of cysteine in the media of cultures of Trichomonas vaginalis appreciably raised the MIC of TSC1 and TSC2, but had no effect on the trichomonacidal activity of TSC3, and in the media of cultures of Leishmania mexicana mexicana promastigotes raised the MIC of TSC1. The addition of DTT, glutathione or homocysteine also antagonised the trichomonacidal activity of TSC1 and TSC2, whereas the addition of methionine or thioglycolate had no effect on the activity of TSC1.

3.6.2. Formation of thiosemicarbazone chelates

Thiosemicarbazones are avid chelators of metals, and their chelates have been implicated in antitumor activity (Minkel and Petering, 1978). Chelates of TSC1 were formed by the method given in section 2.7.1. Only transition metals formed chelates with TSC1. Chelates of TSC1 formed on the addition of $MnSO_4$ and $CuSO_4$ had absorbance maxima at 460 nm and 480 nm, and the chelate of TSC1 formed on the addition of $ZnSO_4$ did not have the absorbance maximum at 345 nm exhibited by TSC1. Chelates were not formed on the addition of $MgCl_2$, $CaCl_2$, NaCl or KCl. Chelate formation was followed by the addition of $MnSO_4$, $CuSO_4$ and $ZnSO_4$ to TSC1, and following the absorbance change at the above wavelengths. These

results are given in Figures 9-11 respectively. They suggest that chelates were formed with equimolar concentrations of TSC1 and transition metal. The spectra of TSC1, and of its copper, manganese and zinc chelates are given in Figure 12. These chelates were stable, as judged by spectral properties, on the addition of an excess of another transition metal and on the addition of thiol-containing compounds. The effect of thiol-containing compounds on chelate formation was not investigated.

A comparison was made of the structures and antitrichomonal activities of a series of thiosemicarbazones, (including some thiosemicarbazone chelates), and their ability to form chelates (Table XVIII). It was found that in general compounds able to form chelates were more potent trichomonacides than similar compounds that could not. It was seen, also, that the chelates had greater antitrichomonal activity than the parent thiosemicarbazone.

3.6.3. Effect on proteinase activity

The proteinase activities of T. vaginalis cell-free homogenate at pH 6.0 and pH 7.5, with and without added DTT, are given in Table XIX. Activity was enhanced by the addition of DTT in both cases and was greater at the lower pH. T. vaginalis proteinase activity at a range of pH's is given in Table XX. The activity increased with the increasing acidity of the assay conditions.

T. vaginalis proteinase activity was found to be inhibited by TSC2, leupeptin and antipain, as shown in Figure 13. 90% (I90) and 50% (I50) proteinase inhibition was achieved at inhibitor

concentrations of 76 and 24 µg/ml (TSC2), 42 and 2.4 µg/ml (leupeptin), 100 and 14 µg/ml (antipain) respectively. TSC1 had no effect on the proteinase activity at concentrations of up to 100 µg/ml.

Despite their potent proteinase inhibitor activity, neither antipain nor leupeptin were trichomonacidal (Table XXI), unlike TSC2.

The effect of a range of thiol-containing compounds on the inhibition by TSC2 of T. vaginalis proteinase are given in Table XXII. It was found that the addition of DTT, glutathione, homocysteine or cysteine antagonised the inhibitory effect of TSC2 to a great extent, whereas the addition of methionine or thioglycolate had much less effect.

3.6.4. Effect on glycolytic kinases

The hexokinase, pyruvate kinase and phosphofructokinase activities of T. vaginalis clone G3 cell-free homogenates were assayed under anaerobic conditions to avoid interference from trichomonal NADH and NADPH oxidases. Under aerobic conditions the NADH oxidase present in the homogenate oxidised the NADH present in the assay mixes of pyruvate kinase and phosphofructokinase, and NADPH oxidase oxidised the NADPH produced in the hexokinase assay. This made it impossible to measure the activities of the above kinases aerobically. In each assay, all the constitutents described in the materials and methods section were found to be necessary for the reaction to proceed. The specific activities in cell-free homogenates of T. vaginalis of hexokinase, pyruvate kinase and phosphofructokinase are given in Table XXIII. All three enzymes were present with high activity.

The sensitivity of T. vaginalis clone G3 to three arsenical compounds is given in Table XXIV. Rather high concentrations of these compounds were required to kill the parasite, the trivalent arsenical melarsen oxide (A2) having the greatest trichomonacidal activity.

The effects of the thiosemicarbazone TSC1 and its chelate TSC2, and the three arsenicals upon yeast, mouse liver and T. vaginalis hexokinase activities is given in Table XXV. Compounds A2 (melarsen oxide), A3 (Mel B) and TSC1 all inhibited hexokinase from yeast, compound A2 inhibited mouse liver hexokinase, whereas only TSC1 inhibited T. vaginalis hexokinase. The inhibition by TSC1 of hexokinase from yeast was significantly greater than for the T. vaginalis enzyme.

The effects of different concentrations of compounds A2, A3 and TSC1 on the hexokinases from yeast, mouse liver and T. vaginalis are given in Figures 14, 15 and 16 respectively. In each case the I90 was greater than 100 μM. The I50's for yeast hexokinase were 2 μM (A2), 43 μM (A3), 50 μM (TSC1), for mouse liver hexokinase, 38 μM (A2) and for T. vaginalis hexokinase, 97 μM (TSC1).

The inhibitions of pyruvate kinase purified from rabbit muscle, pyruvate kinase in mouse liver cell-free homogenate supernatent and the enzyme in T. vaginalis cell-free homogenate by thiosemicarbazones and arsenicals is given in Table XXVI. Only compounds A1 (melarsen) and A2 inhibited rabbit muscle pyruvate kinase, and then only slightly. Compounds A2 and TSC1 at 100 μM inhibited mouse liver pyruvate kinase to a great extent, whereas compound A3 (Mel B) inhibited slightly. Pyruvate kinase activity from T. vaginalis was inhibited very

significantly by TSC2, but was unaffected by the other compounds.

The concentration dependences of the inhibition of mouse liver pyruvate kinase by compounds A2 and TSC1, and of T. vaginalis pyruvate kinase by TSC2 are given in Figures 17 and 18 respectively. The I90's for compounds A2 and TSC1 were both greater than 100 µM. Compound A2 was more active than TSC1, however, with the I50s being 37 µM and 86 µM respectively. The I90 of TSC2 against T. vaginalis pyruvate kinase was 100 µM, and the I50 was 32 µM.

The inhibition of phosphfructokinase purified from rabbit muscle and phosphofructokinase from T. vaginalis clone G3 cell-free homogenate by thiosemicarbazones and arsenicals is given in Table XXVII. The only inhibition observed was with TSC2, which inhibited T. vaginalis phosphofructokinase slightly.

The effect of thiol-containing compounds and methionine on the inhibition of T. vaginalis hexokinase by TSC1 and pyruvate kinase by TSC2 is given in Table XXVIII. The addition of the thiol-containing compounds antagonised the inhibition in all cases. However, the addition of methionine had no effect on the inhibition of hexokinase by TSC1 and only a slight effect on the inhibition of pyruvate kinase by TSC2.

3.6.5. Effect on the thiol content of T. vaginalis

The results described in section 3.6.1. suggested that thiols were involved in some way in the antitrichomonal activity of thiosemicarbazones. It was postulated that the addition of thiosemicarbazones to a parasite culture could have affected the non-protein bound thiol content of T. vaginalis, through the direct binding of the drug to free thiols in the cell.

As a prerequisite to investigating this possibility, parasites were incubated with thiosemicarbazones (TSC1 and TSC2) to determine the time taken to kill the parasites. This was necessary information as I wished to measure the thiol content of T. vaginalis after incubation with thiosemicarbazones, and this had to be carried out with live cells. The progressive killing effect of the two thiosemicarbazones (TSC1 and TSC2) on T. vaginalis is given in Table XXIX. TSC2 had a greater trichomonacidal activity than TSC1 over the period of this study.

The total soluble thiol content of T. vaginalis was found to be 5.2 nmoles/10^6 cells (approximately equal to 1.6 µmoles/g wet weight), (mean of 3 experiments) with a range of 3.4-6.6/10^6 cells. The total glutathione content was found to be 1.1 nmoles/10^6 cells (mean of 3 experiments (approximately 0.34 µmoles/g wet wt.), with a range of 0.8-1.5 nmoles/10^6 cells. Glutathione was approximately 21% of the total soluble thiol of the cell.

The effects of TSC1 and TSC2 on the thiol content of T. vaginalis are shown in Table XXX. In all cases it was found that there was an initial decrease in the total thiol content, but with some subsequent recovery. In the case of the lower thiosemicarbazone concentration, the initial thiol loss from the cells was approximately equimolar to the total drug present (50 nmoles). This relationship was not true for the higher thiosemicarbazone concentration, however, where the initial thiol loss was similar to the loss with the lower thiosemicarbazone concentration, i.e. only approximately one fifth of the high drug concentration. There was no obvious correlation between the loss of total free thiol and cell death.

TABLE I. Sensitivity of Trichomonas vaginalis to a range of compounds using GTM medium

Compound Type	Compound	MIC (µM) G3		LRS(0.6)		IR78		100 µg/ml AS µM
		Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic	
5-nitroimidazole	Metronidazole	10 (10)	5 (9)	15 (4)	7 (3)	>580 (5)	5 (5)	580
4-nitrodiazole	1	20 (3)	40 (3)	>60 (3)	>20 (3)	>60 (3)	60 (3)	355
	2	10 (5)	10 (3)	20 (4)	15 (3)	>100 (2)	>100 (2)	339
	3	40 (8)	40 (6)	25 (7)	20 (4)	>100 (4)	>100 (4)	787
nitrofuran	4	15 (6)	20 (4)	15 (4)	10 (2)	20 (2)	15 (2)	467
	nitrofurantoin	40 (4)	40 (2)	40 (2)	40 (2)	40 (2)	60 (2)	420
thiosemicarbazone	TSC3	> 20 (3)	>20 (3)	20 (3)	20 (3)	20 (1)	15 (3)	350
	TSC1	>100 (11)	100 (3)	60 (3)	60 (3)	60 (3)	60 (3)	386
	TSC10	60 (5)	60 (5)	60 (4)	60 (3)	20 (3)	60 (5)	296

TABLE I. (contd.)

Compound Type	Compound	MIC (μM) G3 Aerobic	G3 Anaerobic	LRS(0.6) Aerobic	LRS(0.6) Anaerobic	IR78 Aerobic	IR78 Anaerobic	100 μg/ml AS μM
iso-thiazole	5	>100 (15)	>100 (9)	>100 (7)	>100 (4)	>100 (7)	>100 (6)	530
	6	>100 (3)	>100 (5)	>100 (4)	20 (3)	>100 (7)	>80 (2)	475
	7	>100 (4)	>100 (7)	>100 (4)	60 (3)	>100 (4)	>100 (3)	280
Quinone	8	>100 (3)	>100 (2)	100 (4)	>100 (3)	>100 (3)	>100 (2)	395
	9	>100 (3)	>100 (2)	100 (3)	>100 (3)	>100 (4)	>100 (2)	655
Benzisothiazoles	10	>100 (4)	>100 (4)	>100 (3)	>100 (2)	>100 (3)	>100 (3)	300
	11	>100 (3)	>100 (2)	>100 (3)	>100 (2)	>100 (3)	>100 (3)	320

The figures are the median results from the number of experiments given in parentheses.

TABLE II. Sensitivity of T. vaginalis to a range of compounds using Bushby's medium

Compound Type	Compound	Aerobic MIC (uM) Line 965691
4-nitrodiazole	1	4 (2-8)
	2	300
	3	63
nitrofuran	4	19
thiosemicarbazone	TSC3	14
	TSC1	0.2 (0.1-0.4)
	TSC10	0.2 (0.1-0.4)
iso-thiazole	5	21
	6	19
	7	11
Quinone	8	63
	9	26
Benzisothiazole	10	19
	11	20

The figures given are the mean results of two experiments with the ranges given in parentheses where there was variation between experiments.

TABLE III. Sensitivity of T. vaginalis clone G3 and line 965691 to compounds 7 and TSC10 using different test conditions

Test method/Compound		MIC (uM) Culture medium	
Compound 7	Cell line	GTM	Bushby's
Test tubes	G3	>100	45
	965691	>100	45
Aerobic microtitre plate	G3	100	< 3
	965691	>100	10
Anaerobic microtitre plate	G3	>100	13
	965691	>100	26
TSC10			
Test tubes	G3	23	< 3
	965691	45	< 3
Aerobic microtitre plate	G3	40	N.P.
	965691	40	< 5
Anaerobic microtitre plate	G3	100	< 5
	965691	100	< 5

N.P. Experiment not performed.

The figures given are the results from a single experiment.

TABLE IV. Effect of the culture media on the MIC of trichomonacidal compounds

	Anaerobic MIC (uM) line 965691	
Culture Medium	Compound 7	TSC10
GTM	100	100
Bushby's + 12.7 mM ascorbate	35	< 5
Bushby's + 2.7 mM ascorbate	55	< 5
Bushby's + 12.7 mM cysteine	55	20
Bushby's + 2.7 mM cysteine	55	< 5
Bushby's + 12.7 mM (cysteine and 12.7 mM ascorbate)	60	20
Bushby's	30	< 5

The figures given are the results from a single experiment.

TABLE V. Metronidazole sensitivity of Trichomonas vaginalis

Parasite line	MIC (uM)	
	Aerobic	Anaerobic
G3	10 (6) 7.5-12.5	5 (4) 5.0-7.5
IR78	> 580 (5)**	5 (5) 2.5-5.0
LRS (2.0)	40 (6) 35-42.5	20 (4) 17.5-20.0
Boston	350 (3)	5 (3)
Fall River	580 (2)	5 (2)
Albany	470 (2)	5 (2)

The figures are the median results from the number of experiments given in parentheses, with ranges where there was variation between experiments.

TABLE VI. Metronidazole reduction, NADH oxidase and NADPH oxidase activities of *Trichomonas vaginalis* homogenates

	Clone G3	Line IR78	Line LRS (2.0)
Metronidazole reduction[a]	65± 9 (6)	91±18 (5)	19± 3 (6)
NADH oxidase[b]	570± 87 (10)	413±55 (10)	507±153 (9)
NADPH oxidase[c]	389±101 (12)	127±42 (7)	559±123 (6)

The figures are the means ± one standard deviation from the number of experiments given in parentheses. Units:nmoles/min/mg protein.

a. Rates with clone G3 and line IR78 are significantly different from that with the line LRS (2.0) at p = 0.01.

b. Activity of clone G3 is significantly different from line IR78 at p = 0.05.

c. Activities of clone G3 and line LRS (2.0) are significantly different from that of line IR78 at p = 0.05.

TABLE VII. Effect of different concentrations of cysteine and ascorbate on the growth of *T. vaginalis* clone G3 and line IR78 under aerobic conditions

Ascorbate	% Growth		Cysteine	% Growth	
concentration (mM)	G3	IR78	concentration (mM)	G3	IR78
21.0	100	100	12.7	100	100
16.8	80 (79-81)	95 (93-97)	10.2	85 (78-92)	86 (80-92)
12.6	75 (73-77)	89 (84-93)	7.6	73 (67-79)	52 (45-59)
8.4	62 (61-63)	85 (81-89)	5.1	54 (48-60)	42 (34-50)
4.2	61 (57-65)	81 (77-85)	2.5	36 (31-41)	28 (24-32)
2.1	54 (49-59)	78 (71-85)	1.3	27 (22-32)	18 (15-21)
0	12 (11-13)	67 (61-73)	0	15 (12-18)	6 (3- 9)

The figures given are the mean results and ranges of two experiments. Incubation was for 33 hr (clone G3) and 72 hr (line IR78) respectively.

TABLE VIII. Effect of methionine, DTT and glutathione on growth of Trichomonas vaginalis clone G3 under aerobic conditions in the absence of cysteine

Culture medium	No. of parasites/ml	% of control
GTM	3.8×10^6	100 ± 2
GTM-cysteine	8.6×10^5	23 ± 3
GTM-cysteine + 12.7 mM methionine	1.9×10^6	50 ± 2
GTM-cysteine + 6.4 mM glutathione	1.8×10^6	47 ± 3
GTM-cysteine + 6.4 mM dithiothreitol	1.1×10^6	30 ± 3

The results given are the parasite densities after 24 hr (initial cell density 1×10^5/ml) and the cell density expressed as a % of the growth in GTM medium. The figures are the mean (± range) from 2 experiments.

TABLE IX. Effect of pH and added salts upon the trichomonacidal activity of metronidazole

Culture conditions	MIC (uM)	
	Aerobic	Anaerobic
GTM (pH 6.0)	10 (15)	5 (11)
GTM (pH 5.2)	10 (2)	5 (2)
GTM (pH 4.6)	10 (2)	5 (2)
GTM + 5 mM lactate	80 (6)	5 (4)
GTM + 5 mM acetate	80 (6)	5 (4)
GTM + 5 mM succinate	10 (3)	5 (2)
GTM + 5 mM pyruvate	10 (4)	5 (2)

The figures are the median results from the number of experiments given in parentheses. T. vaginalis clone G3 was used.

TABLE X. Effect of different concentrations of lactate and acetate upon the metronidazole sensitivity of *T. vaginalis* clone G3

Lactate/Acetate concentration (mM)	Aerobic MIC (uM)	
	Lactate	Acetate
0	10 (16) (5-10)	10 (16) (5-10)
0.25	10 (2)	10 (2)
0.5	10 (2)	10 (2)
1.0	20 (2)	20 (2)
2.0	45 (2)	45 (2)
5.0	80 (6) (60-80)	80 (6) (60-80)
10	80 (3)	80 (3)

The figures are the median results from the number of experiments given in parentheses with ranges where there was variation between experiments.

TABLE XI. Effect of lactate and acetate on the activity of NADH oxidase, NADPH oxidase and the rate of metronidazole reduction by *T. vaginalis* clone G3 homogenates

	Cells grown in GTM		
	Alone	+ 5 mM Lactate	+ 5 mM Acetate
Metronidazole reduction	73 ± 11 (3)	78 ± 21 (3)	65 ± 14 (3)
NADH oxidase	603 ± 55 (4)	625 ± 106 (3)	592 ± 78 (3)
NADPH oxidase	376 ± 26 (3)	426 ± 100 (3)	404 ± 83 (3)

The figures are the mean results ± one standard deviation of the number of experiments given in parentheses.

TABLE XII. Effect of added lactate to medium upon the excretion of lactate by Trichomonas vaginalis clone G3

Culture medium	Lactate produced (mM)
GTM	13.2 ± 0.5 (3)
GTM + 5 mM Lactate	9.64 ± 0.38 (3)

The figures are the mean results ± one standard deviation of the number of experiments given in parentheses.

Initial parasite density 1×10^5 cells/ml.

Final parasite density: Control (GTM) $1.69 \times 10^6 \pm 1.1 \times 10^5$ cells/ml

Experimental (+Lactate) $1.56 \times 10^6 \pm 1.2 \times 10^5$ cells/ml

24 hr incubation growth in bijou bottle cultures of 5 ml medium.

TABLE XIII. Antitrichomonal activity of metronidazole, a thiosemicarbazone, ethidium bromide, demethyl sulphate (DMS) and benzamides, alone and in combination

Culture condition	Compound	MIC (μM) Alone	+ 100 μM benzamide	+ 1 mM 2 amino-benzamide	+ 1 mM 3 amino-benzamide	+ 1 mM 4 amino-benzamide
AEROBIC	metronidazole	15 (4)	15 (2)	15 (2)	15 (2)	15 (3)
	TSC1	0.5 (4)	0.5 (3)	0.5 (3)	0.5 (2)	0.5 (2)
	ethidium bromide	1000 (4)	1000 (3)	1000 (3)	1000 (2)	1000 (2)
	DMS	3 (4)	0.03 (2)	0.03 (2)	0.03 (2)	0.03 (3)
AEROBIC	benzamide	1000 (2)				
	2 amino-benzamide	5000 (1)				
	3 amino-benzamide	5000 (1)				
	4 amino-benzamide	5000 (2)				

TABLE XIII. (contd.)

Culture condition	Compound	MIC (μM) Alone	+ 100 μM benzamide	+ 1 mM 2 amino-benazamide	+ 1 mM 3 amino-benzamide	+ 1 mM 4 amino-benzamide
ANAEROBIC	metronidazole	10 (3)	10 (2)	10 (2)	10 (2)	10 (2)
	TSC1	0.5 (13)	0.5 (2)	0.5 (2)	0.5 (2)	0.5 (2)
	ethidium bromide	1000 (3)	1000 (2)	1000 (2)	1000 (2)	1000 (2)
	DMS	3 (3)	0.03 (2)	0.03 (2)	0.03 (2)	0.03 (2)
ANAEROBIC	benzamide	1000 (2)				
	2 amino-benzamide	5000 (1)				
	3 amino-benzamide	5000 (1)				
	4 amino-benzamide	5000 (2)				

The figures are the median results from the number of experiments given in parentheses.

TABLE XIV. Sensitivity of T. vaginalis line 965691 to azobenzene, nitrobenzene and two of their mammalian metabolites.

Compound	MIC (µg/ml)	MIC (µM)
nitrobenzene	50	400
azobenzene	50	275
aniline	50	540
p-nitrophenol	50	360

The figures are the mean results of two experiments.

TABLE XV. The Rf values of azobenzene, nitrobenzene and their main mammalian metabolites by thin layer chromatography using three solvent systems.

Compound	Rf value		
	Solvent I	Solvent II	Solvent III
nitrobenzene	0.92 (1)	N.D.	0.93 (1)
aniline	N.D.	N.D.	N.D.
2-aminophenol	0.78 ± 0.05 (3)	0.90 ± 0.01 (2)	0.20 ± 0.02 (3)
3-aminophenol	0.75 ± 0.03 (3)	0.87 ± 0.02 (2)	0.18 ± 0.01 (3)
4-aminophenol	0.68 ± 0.04 (3)	0.88 ± 0.01 (2)	0.06 ± 0.02 (3)
2-nitrophenol	N.D.	0.86 ± 0.01 (2)	N.D.
3-nitrophenol	0.81 ± 0.01 (2)	0.84 ± 0.03 (3)	0.57 ± 0.03 (2)
4-nitrophenol	0.80 ± 0.01 (2)	0.89 ± 0.03 (3)	0.56 ± 0.04 (3)
azobenzene	0.92 ± 0.01 (2)	0.84 ± 0.01 (2)	0.93 ± 0.01 (2)
hydrazobenzene	0.93 ± 0.02 (2)	0.85 ± 0.01 (2)	0.90 ± 0.02 (2)
benzidine	0.68 ± 0.01 (2)	0.82 ± 0.02 (2)	0.18 ± 0.02 (2)

The figures given are the means (± one standard deviation or range) from the number of experiments given in parentheses. N.D. Not detectable.

TABLE XVI. Retention times of nitrobenzene, azobenzene and their main mammalian metabolites using HPLC.

Compound	Retention time (minutes)	Compound	Retention time (minutes)
nitrobenzene	6.7 ± 0.1 (3)	aniline	4.7 ± 0.2 (3)
3-nitrophenol	5.0 ± 0.3 (3)	azobenzene	13.3 ± 0.1 (3)
2-aminophenol	3.4 ± 0.1 (2)	hydrazobenzene	16.6 ± 0.1 (2)
3-aminophenol	4.2 ± 0.2 (2)	benzidine	4.2 ± 0.2 (2)
4-aminophenol	3.7 ± 0.1 (2)		

The figures are the mean results one standard deviation and (± ranges) from the number of experiments given in parentheses.

TABLE XVII. Effect of thiol containing compounds on the sensitivity of *Trichomonas vaginalis* clone G3 and *Leishmania mexicana mexicana* promastigotes to thiosemicarbazones.

T. vaginalis culture medium	MIC (μM)					
	TSC1		TSC2		TSC3	
	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic
GTM (contains 12.7 mM cysteine)	80 (4)	80 (11)	80 (3)	80 (3)	20 (3)	20 (5)
GTM-cysteine	1 (7)	0.5 (18)	0.2 (6)	0.2 (12)	20 (3)	20 (4)
GTM-cysteine + 6.4 mM DTT	80 (2)	80 (3)	80 (2)	80 (2)	N.D.	N.D.
GTM-cysteine + 12.7 mM glutathione	80 (2)	80 (3)	80 (2)	80 (2)	N.D.	N.D.
GTM-cysteine + 12.7 mM methionine	0.5 (2)	1 (3)	0.5 (2)	0.5 (2)	N.D.	N.D.
GTM-cysteine + 6.4 mM homocysteine	80 (3)	80 (3)	80 (3)	80 (3)	N.D.	N.D.
GTM-cysteine + 12.7 mM thioglycolate	0.5 (2)	0.5 (2)	0.5 (2)	0.5 (4)	N.D.	20 (2)
Leishmania culture medium						
Lit medium	10 (2)	N.D.	N.D.	N.D.	N.D.	N.D.
Lit medium + 12.7 mM cysteine	80 (20)	N.D.	N.D.	N.D.	N.D.	N.D.

The figures are the median results from the numbers of experiments given in parentheses. N.D. = Not determined.

TABLE XVIII. Relationship between thiosemicarbazone structure and antitrichomonal activity.

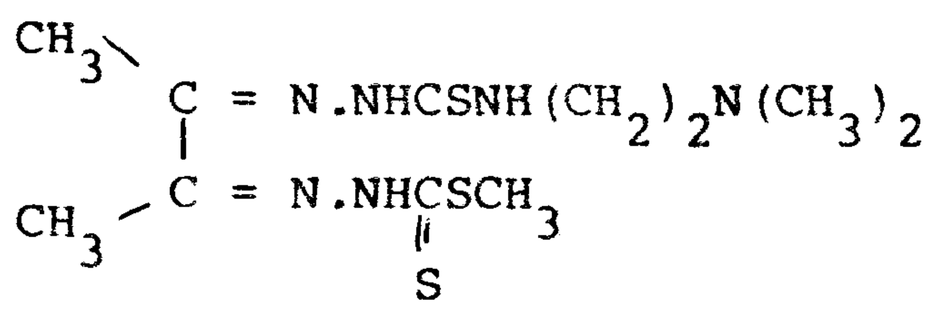
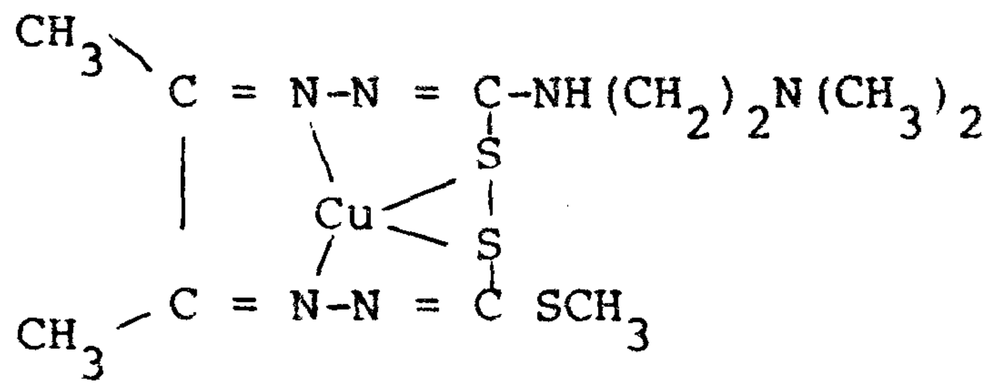
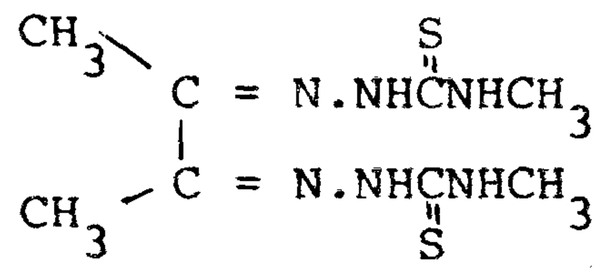
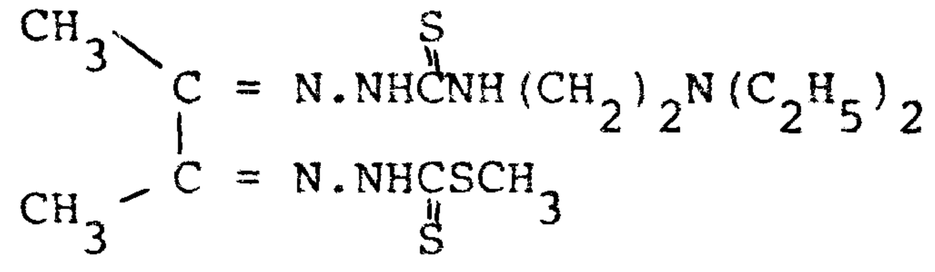
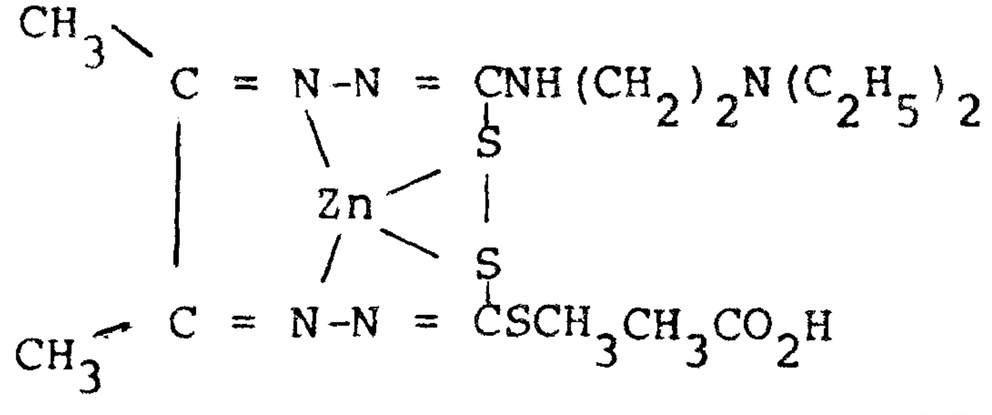
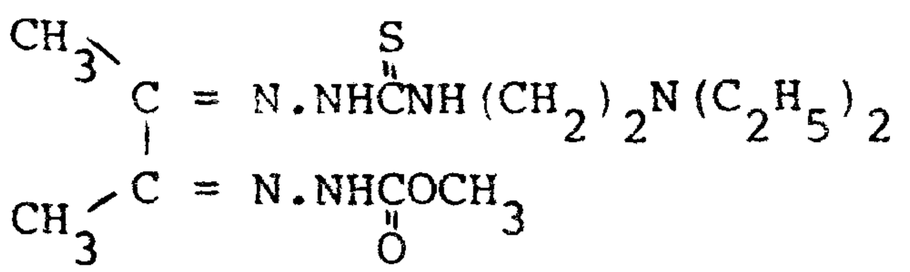
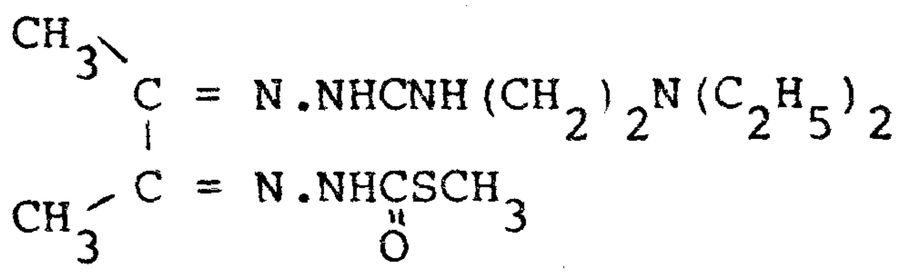
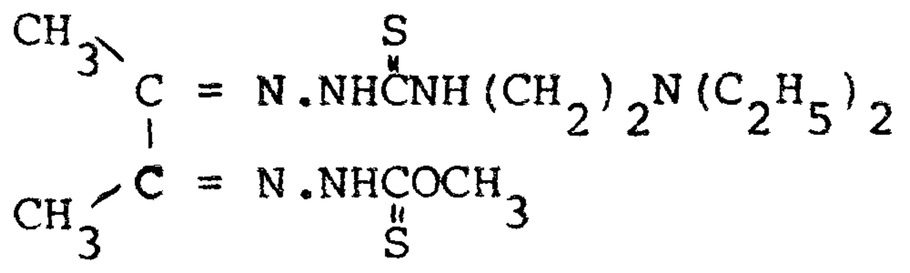
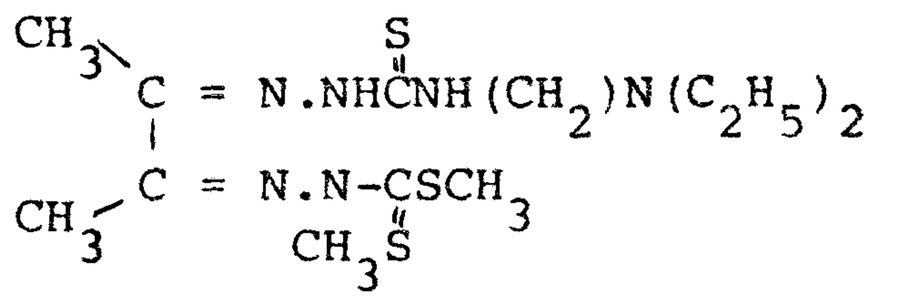
Compound	Structure	Comments	MIC (μg/ml)
TSC1		will form chelate	0.05
TSC2		copper chelate of TSC1	0.03
TSC3		will not form chelate[a]	4
TSC4		will from chelate	0.5
TSC5		zinc chelate of TSC4	0.1

TABLE XVIII. (contd.)

Compound	Structure	Comments	MIC (µg/ml)
TSC6		will not form chelate [a]	50
TSC7		will not form chelate [a]	50
TSC8		will form chelate [a]	0.8
TSC9		will not form chelate [a]	50

a. Deduction from structure of compound. No attempt was made to form chelates with those compounds.

TABLE XIX. Proteinase activity of Trichomonas vaginalis clone G3 homogenates.

Assay Conditions	Activity
pH 6.0	6.7 ± 0.9 (9)
pH 6.0 (+ 1.3 mM DTT)	10.1 ± 0.4 (3)
pH 7.5	4.0 ± 1.0 (6)
pH 7.5 (+ 1.3 mM DTT)	5.7 ± 0.2 (3)

The figures are the mean results (± standard deviation) from the number of experiments given in parentheses. Units: mU/mg protein.

TABLE XX. Proteinase activity of Trichomonas vaginalis clone G3 at a range of pH.

pH	Activity
5.0	8.5 ± 0.2 (2)
5.5	7.6 ± 0.1 (2)
6.0	7.3 ± 0.3 (2)
6.5	7.0 ± 0.1 (2)
7.0	6.4 ± 0.2 (2)
7.5	4.7 ± 0.1 (2)
8.0	1.9 ± 0 (2)

The figures are the mean results and range from the numbers of experiments given in parentheses. No DTT was present.

Units: mU/mg protein.

TABLE XXI. Sensitivity of Trichomonas vaginalis clone G3 to antipain and leupeptin.

Compound	MIC (µg/ml)
antipain	100 (2)
leupeptin	100 (2)
TSC1	0.05 (2)
TSC2	0.03 (2)

The figures are the median results of two experiments.

TABLE XXII. Effect of thiol containing compounds on the inhibition of T. vaginalis proteinase by TSC2.

	% Inhibition
Control	0 (2)
+ 100 µM TSC2	71 ± 1 (2)
+ 100 µM TSC2 + 6.4 mM DTT	5 ± 1 (2)
+ 100 µM TSC2 +12.7 mM cysteine	20 ± 2 (2)
+ 100 µM TSC2 +12.7 mM glutathione	13 ± 1 (2)
+ 100 µM TSC2 + 6.4 mM homocysteine	5 ± 1 (2)
+ 100 µM TSC2 +12.7 mM methionine	50 ± 2 (2)
+ 100 µM TSC2 +12.7 mM thioglycolate	32 (1)

The figures given are the means and ranges from the number of experiments given in parentheses.

TABLE XXIII. Hetokinase, Pyruvate kinase and phosphofructo kinase activities of Trichomonas vaginalis clone G3 homogenates.

	Activity
Hexokinase	124 ± 12 (9)
pyruvate kinase	123 ± 19 (10)
phosphofructo kinase	95 ± 6 (4)

The figures are the mean results ± one standard deviation from the number of experiments given in parentheses. Units: nmols/min/mg protein.

TABLE XXIV. Sensitivity of Trichomonas vaginalis clone G3 to arsenicals.

	MIC (μM)
Melarsen (A1)	300 (4)
Melarsen oxide (A2)	100 (4)
Mel. B. (A3)	300 (4)

The figures are the median results of the number of experiments given in parentheses.

TABLE XXV. Inhibition of hexokinase from yeast, mouse liver and T. vaginalis by thiosemicarbazones and arsenicals.

	% Inhibition of enzyme activity				
Enzyme source	100 µM (A1) Melarsen	100 µM (A2) Melarsen oxide	100 µM (A3) Mel. B.	100 µM (TSC1) Thiosemicarbazone	100 µM (TSC2) Thiosemicarbazone
yeast	0 (2)	86 ± 4 (3)	77 ± 4 (3)	72 ± 3 (3)	0 (2)
mouse liver	0 (2)	77 ± 3 (3)	0 (2)	0 (2)	0 (2)
T. vaginalis	0 (2)	0 (2)	0 (2)	55 ± 3 (3)	0 (2)

The figures are the mean results ± one standard deviation from the number of experiments given in parentheses.

TABLE XXVI. Inhibition of pyruvate kinase from rabbit muscle, mouse liver and T. vaginalis by thiosemicarbazones and arsenicals.

	% Inhibition of enzyme activity				
Enzyme source	100 µM (A1) Melarsen	100 µM (A2) Melarsen oxide	100 µM (A3) Mel. B.	100 µm (TSC1)	100 µM (TSC2)
rabbit muscle	13 ± 6 (2)	10 ± 1 (2)	0 (2)	0 (3)	0 (3)
mouse liver	0 (2)	77 ± 7 (3)	16 ± 6 (2)	58 ± 4 (3)	0 (2)
T. vaginalis	0 (2)	0 (2)	0 (2)	0 (2)	90 ± 2 (3)

The figures given are the mean results ± one standard deviation or ranges from the number of experiments given in parentheses.

TABLE XXVII. Inhibition of phosphofructo kinase from rabbit muscle and T. vaginalis clone G3 by thiosemicarbazones and arsenicals.

	% Inhibition of enzyme activity				
Enzyme source	100 uM (A1) Melarsen	100 uM (A2) Melarsen oxide	100 uM (A3) Mel. B.	100 uM (TSC1)	100 uM (TSC2)
rabbit muscle	0 (2)	0 (2)	0 (2)	0 (2)	0 (2)
T. vaginalis	0 (2)	0 (2)	0 (2)	0 (2)	22 ± 1 (2)

The figures given are the mean results and ranges of the number of experiments given in parentheses.

TABLE XXVIII. Effect of thiol compounds on the inhibition of hexokinase by TSC1 and pyruvate kinase by TSC2.

	% Activity	
	Hexokinase + 100 μM TSC1	Pyruvate kinase + 100 μM TSC2
Control (no TSC1/TSC2)	100	100
Drug + 3 mM cysteine	82 (81- 83)	95 (95- 96)
Drug + 3 mM DTT	88 (84- 92)	96 (92-100)
Drug + 3 mM glutathione	71 (67- 74)	87 (86- 88)
Drug + 3 mM thioglycolate	93 (85-100)	64 (60- 67)
Drug + 3 mM homocysteine	106 (105-107)	98 (96-100)
Drug + 3 mM methionine	44 (43- 45)	40 (35- 45)
Drug only	44 (42- 46)	23 (20- 26)

The figures given are the the mean results and ranges of two experiments.

TABLE XXIX. Trichomonacidal activity of thiosemicarbazone TSC1 and its copper chelate, TSC2.

	% Parasite survival	
	120 min	210 min
Control	100	100
1 µM TSC 1	90	87
5 µM TSC 1	86	69
1 µM TSC 2	90	70
5 µM TSC2	78	32

The figures given are the mean results $\pm$ one standard deviation of three experiments.

TABLE XXX. Effect of thiosemicarbazones on the soluble thiol content of T. vaginalis.

Time of Incubation with drug (min)	Total thiol loss (nmole)			
	TSC1 added		TSC2 added	
	50 nmoles	250 nmoles	50 nmoles	250 nmoles
0	0	0	0	0
30	44	49	36	53
60	51	47	42	31
120	22	17	0	29
210	0	N.D.	+ 29	N.D.

The figures given are the mean results of two experiments. The initial thiol content of each parasite culture was 120 nmoles, the thiol content in 2.4×10^7 cells. N.D. = Not determined.

Figures 1-4 Growth of Trichomonas vaginalis in GTM medium containing different cysteine and ascorbate concentrations. Parasite numbers are given as the number of motile parasites/ml medium. The figures are the means and range of 2 aerobic and 2 anaerobic experiments. Anaerobic conditions were achieved by passing argon over the cultures prior to incubation).

Figure 1. Clone G3 under anaerobic conditions

Figure 2. Clone G3 under aerobic conditions

Figure 3. Line IR78 under anaerobic conditions

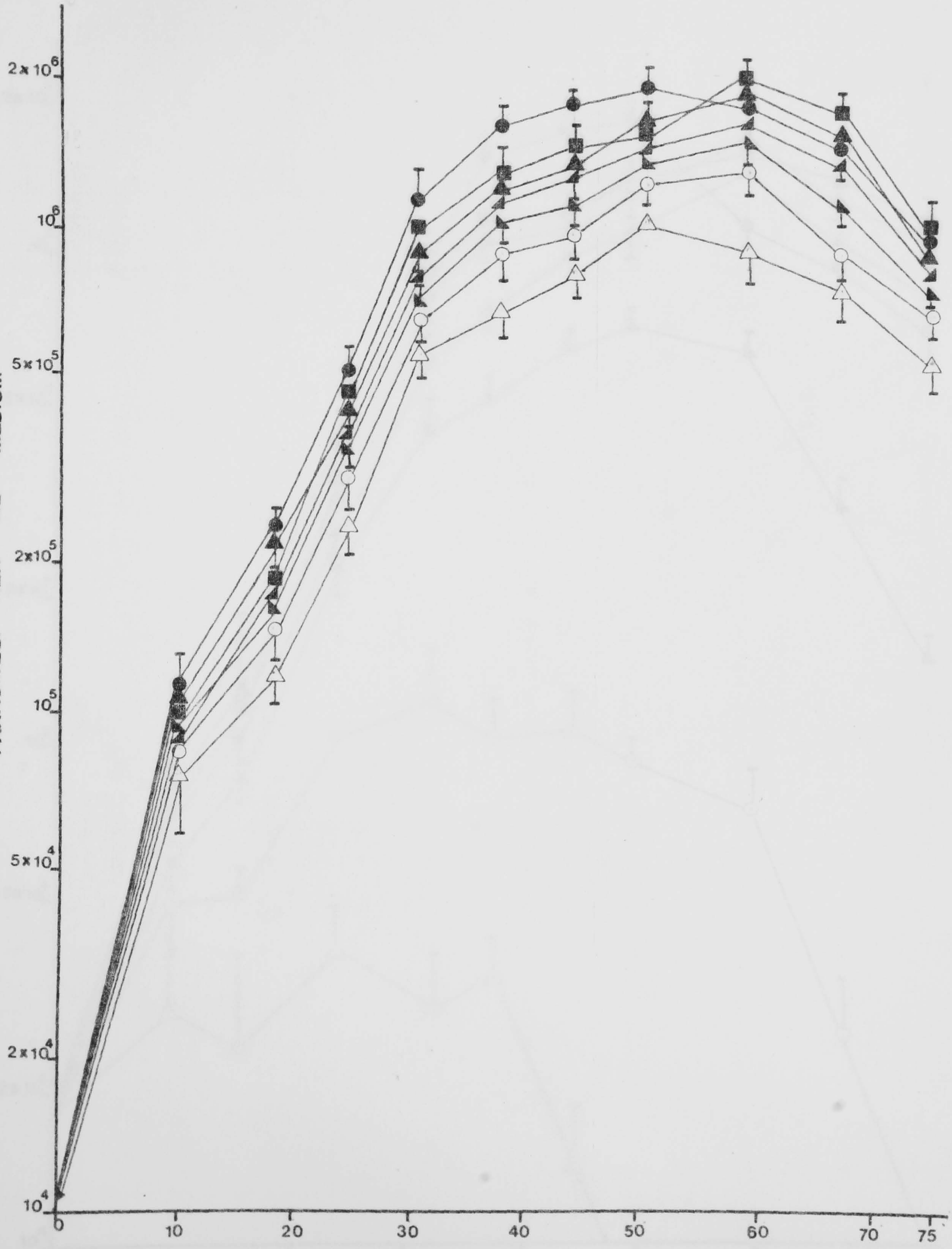
Figure 4. Line IR78 under aerobic conditions

Key for figures 1-4: cysteine and ascorbate concentrations (mM);

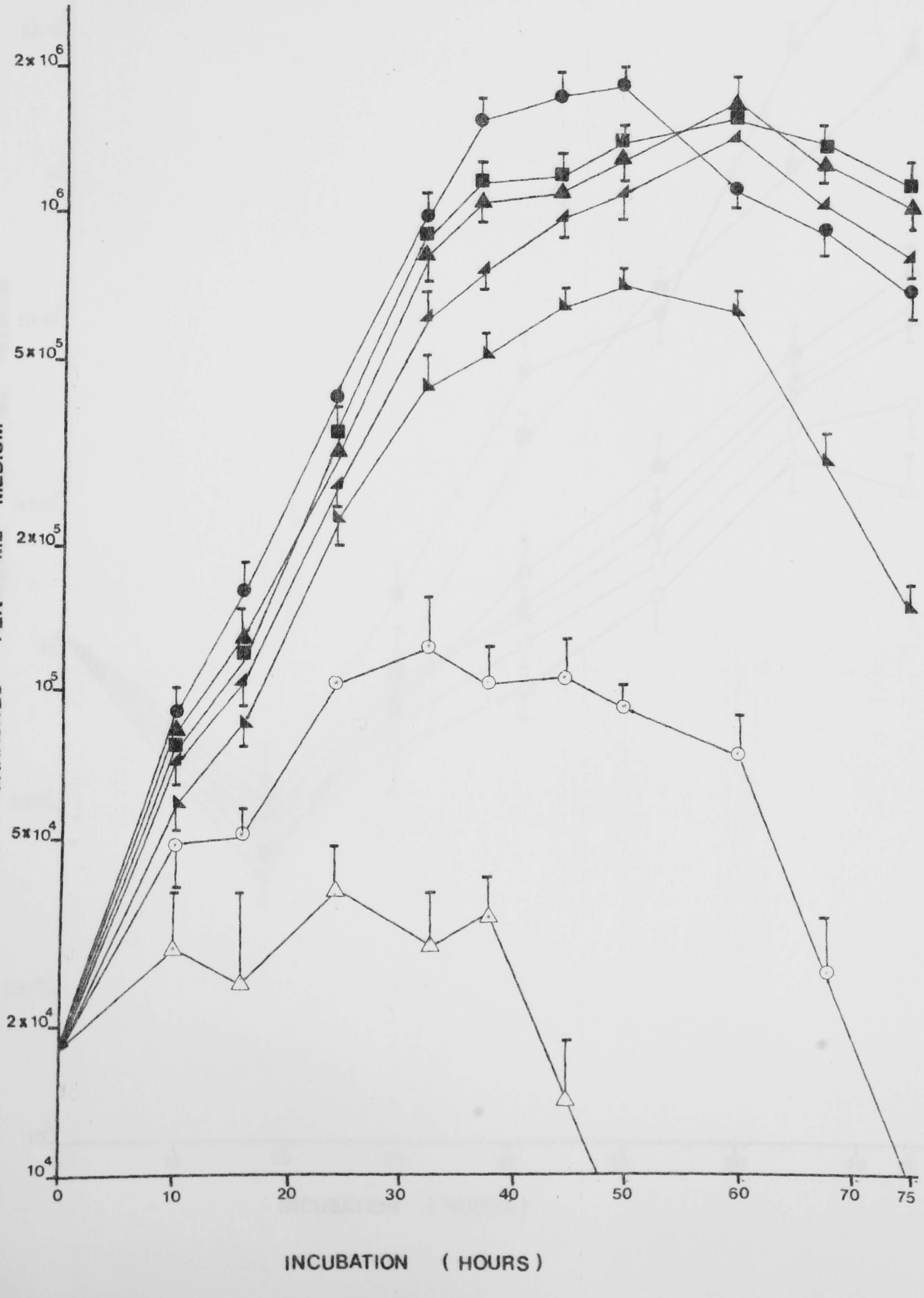
● (12.7 and 21.0, concentration in GTM medium respectively);

■ (10.2 and 16.8); ▲ (7.6 and 12.6); ◢ (5.1 and 8.4); ◣ (2.5 and 4.2); ○ (1.3 and 2.1); △ (0 and 0).

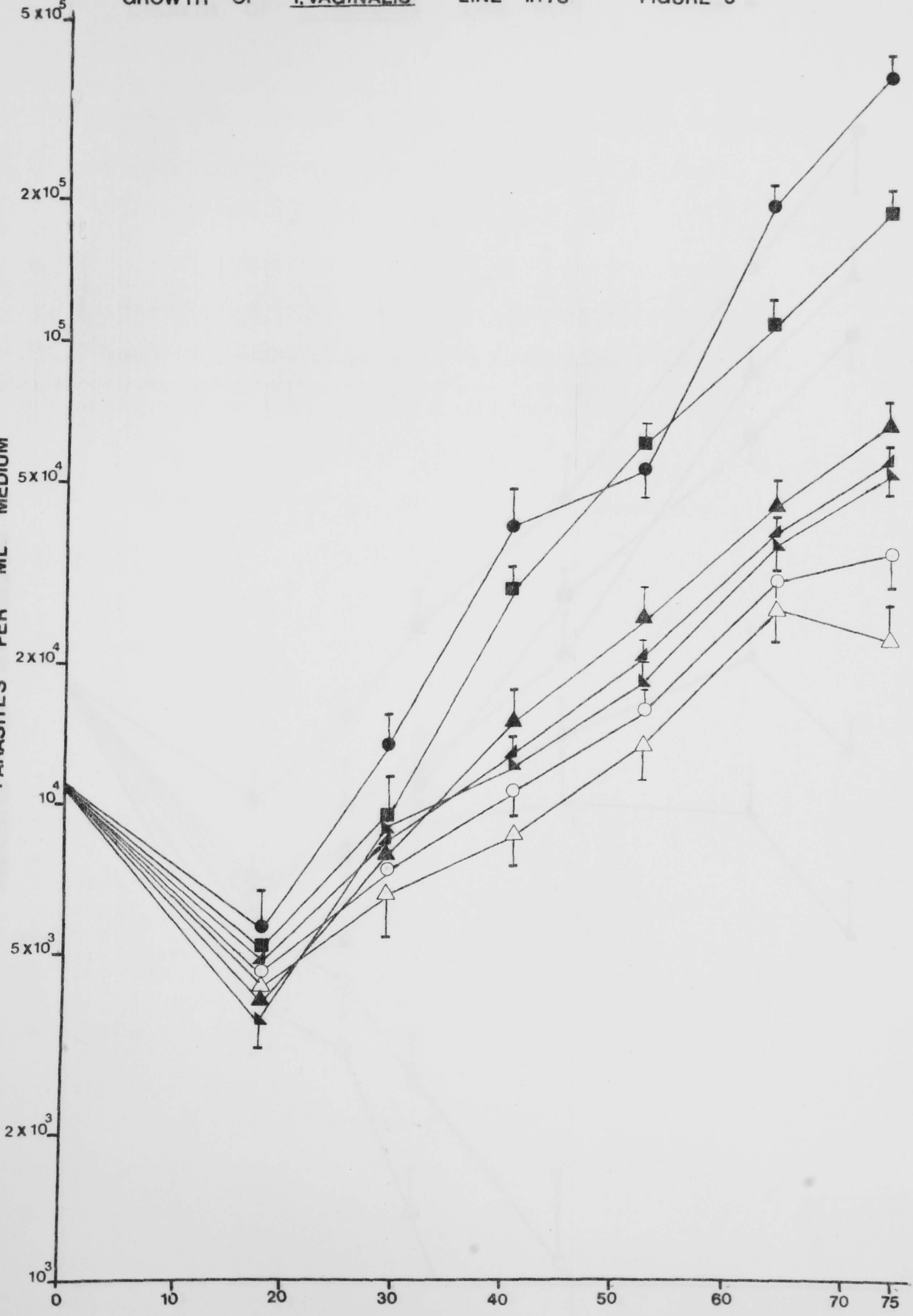
GROWTH OF T. VAGINALIS CLONE G3 FIGURE 1



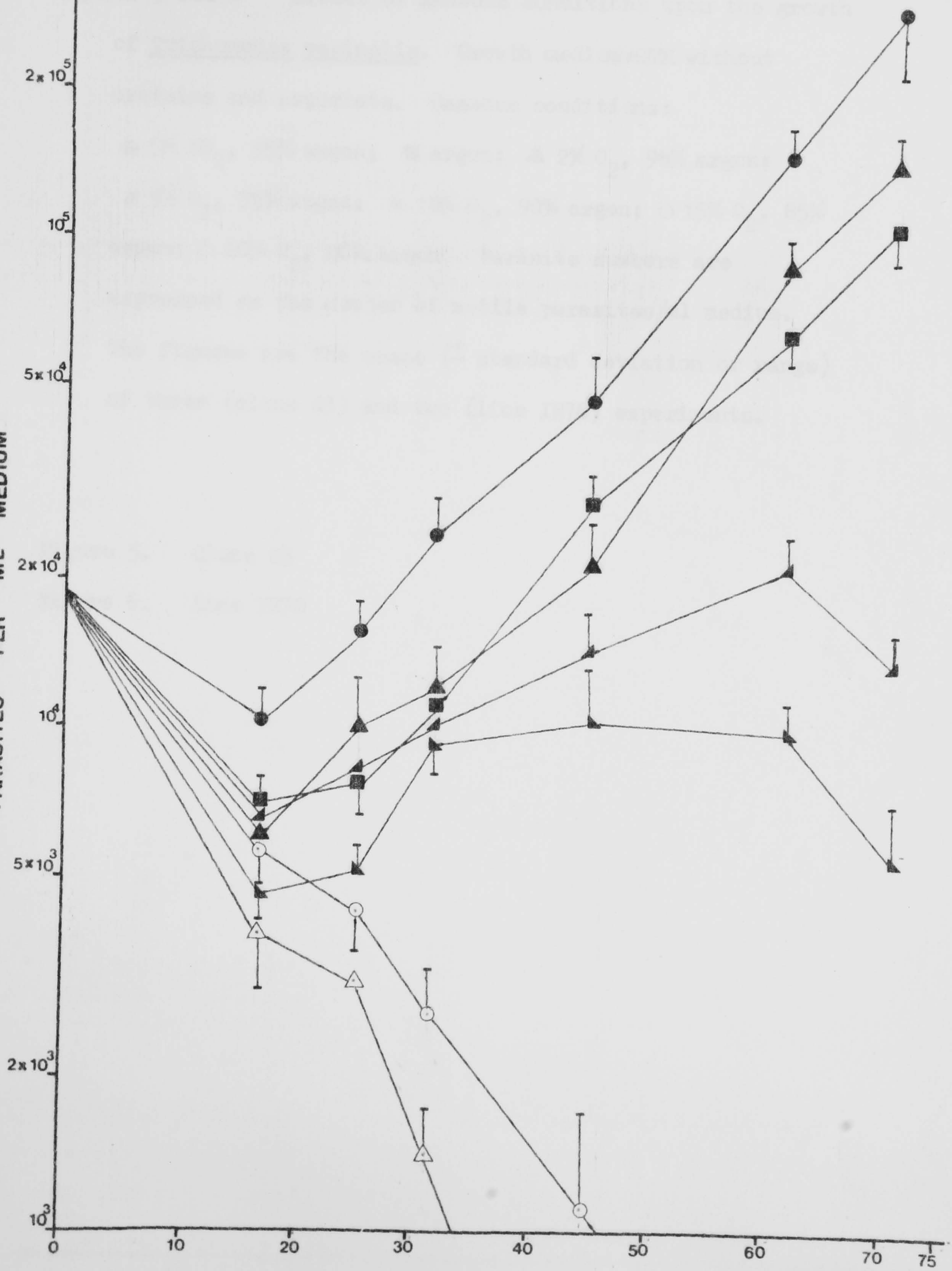
GROWTH OF T. VAGINALIS CLONE G3 FIGURE 2



GROWTH OF <u>T. VAGINALIS</u> LINE IR 78 FIGURE 3



GROWTH OF T. VAGINALIS LINE IR78 FIGURE 4



Figures 5 and 6 Effect of gaseous conditions upon the growth of Trichomonas vaginalis. Growth medium:GTM without cysteine and ascorbate. Gaseous conditions:

● 5% CO_2, 95% argon; ■ argon; ▲ 2% O_2, 98% argon; ◢ 5% O_2, 95% argon; ◣ 10% O_2, 90% argon; ○ 15% O_2, 85% argon; △ 20% O_2, 80% argon. Parasite numbers are expressed as the number of motile parasites/ml medium. The figures are the means (± standard deviation or range) of three (clone G3) and two (line IR78) experiments.

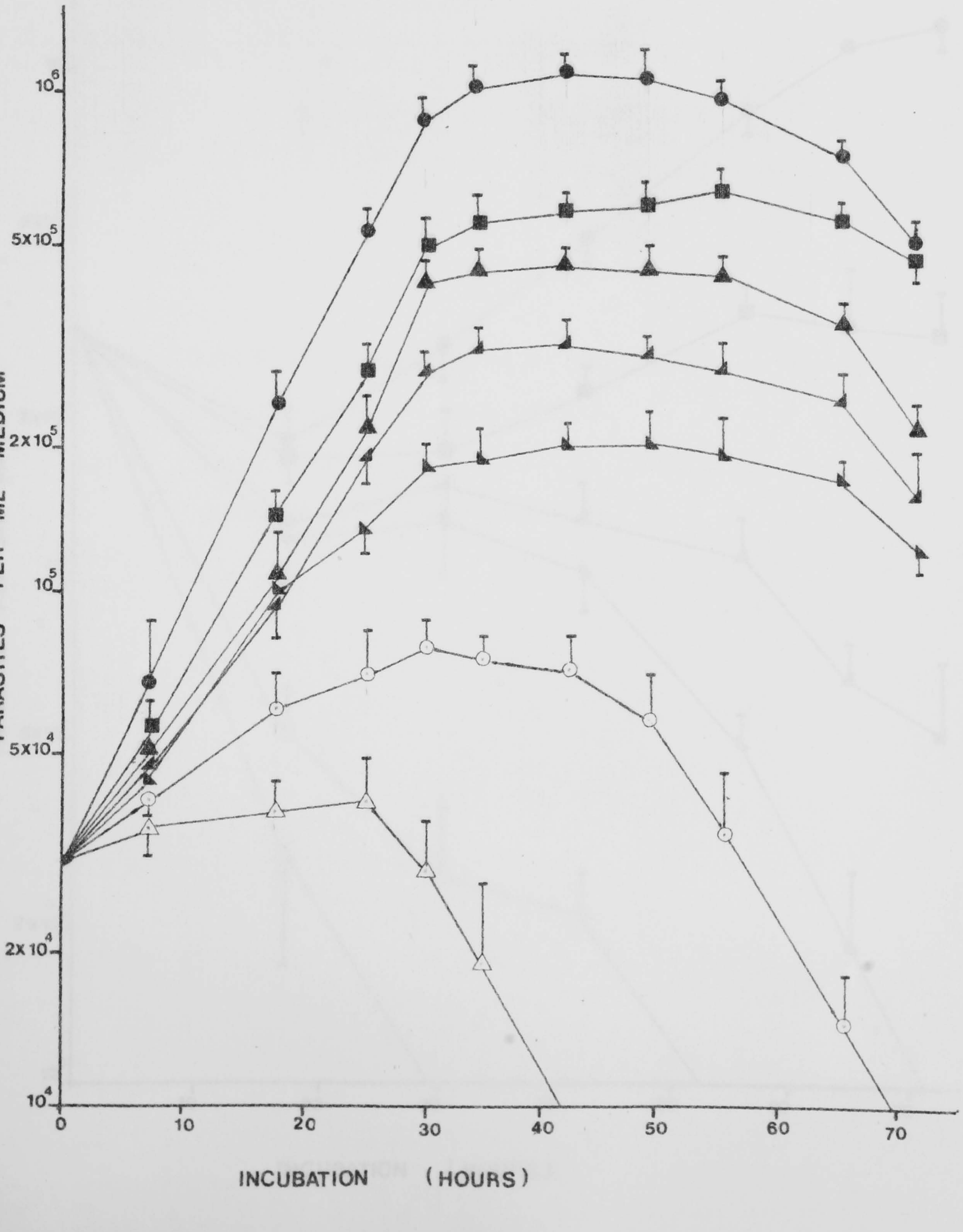
Figure 5. Clone G3

Figure 6. Line IR78

EFFECTS OF GASEOUS CONDITIONS UPON THE

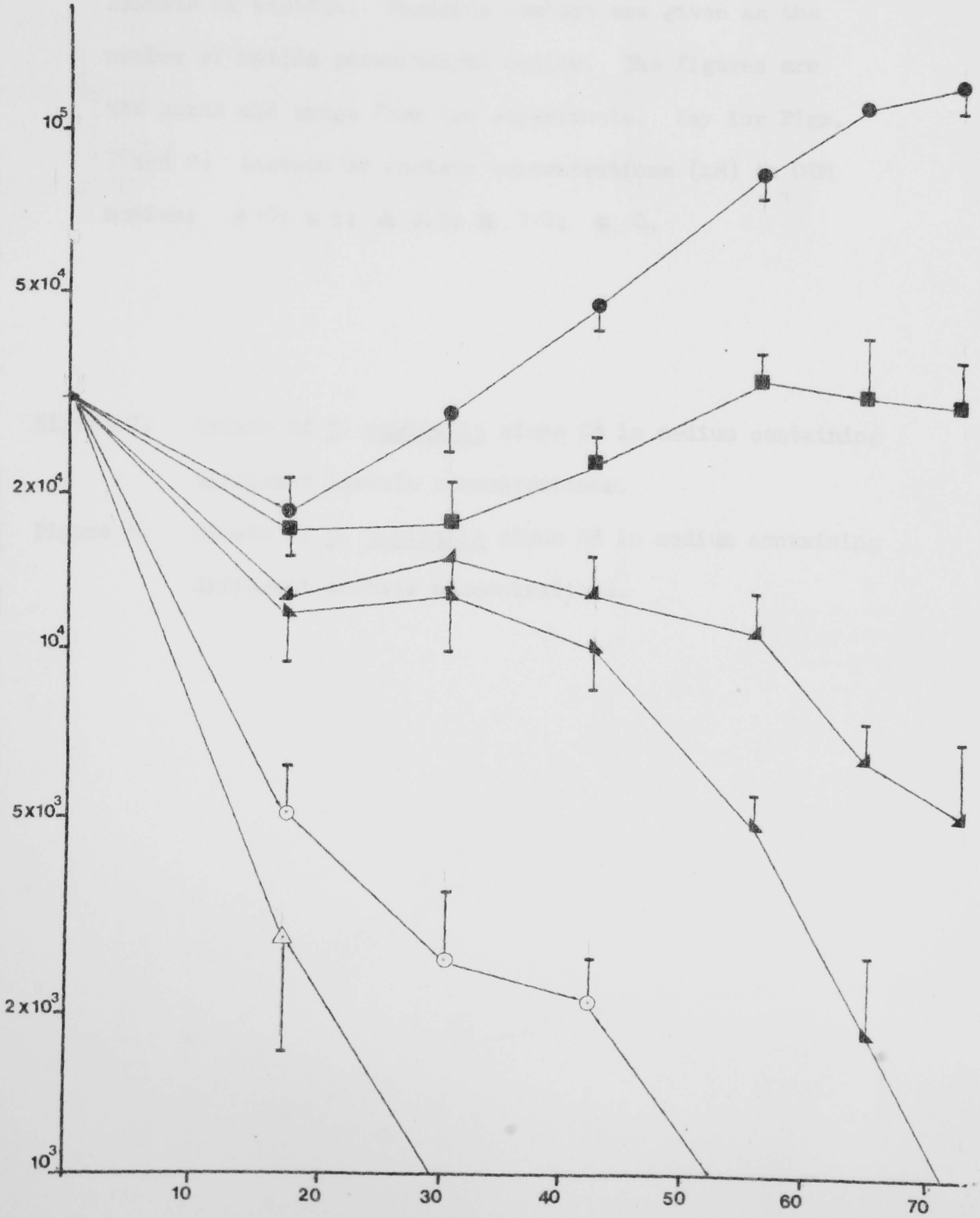
GROWTH OF T. VAGINALIS CLONE G3

FIGURE 5



EFFECTS OF GASEOUS CONDITIONS UPON THE GROWTH OF T. VAGINALIS LINE IR78

FIGURE 6



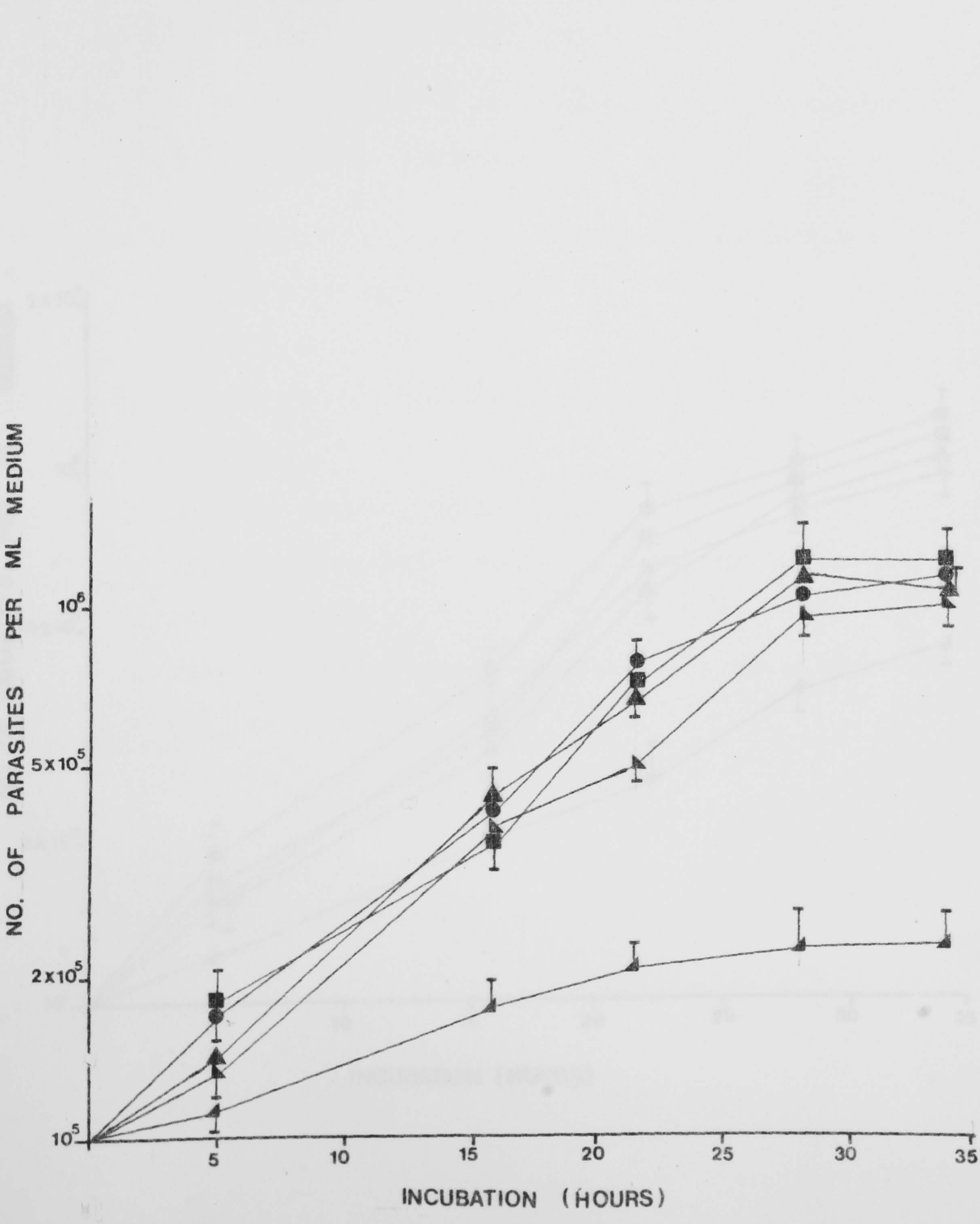
Figures 7 and 8 Growth of Trichomonas vaginalis under aerobic conditions in medium containing different concentrations of lactate or acetate. Parasite numbers are given as the number of motile parasites/ml medium. The figures are the means and range from two experiments. Key for Figs. 7 and 8: Lactate or acetate concentrations (mM) in GTM medium; ◢ 10; ◣ 5; ▲ 2.5; ■ 1·0; ● 0.

Figure 7. Growth of T. vaginalis clone G3 in medium containing different lactate concentrations.

Figure 8. Growth of T. vaginalis clone G3 in medium containing different acetate concentrations.

GROWTH OF T. VAGINALIS CLONE G 3 UNDER AEROBIC CONDITIONS IN MEDIUM WITH DIFFERENT LACTATE CONCENTRATIONS

FIGURE 7



GROWTH OF T. VAGINALIS CLONE G3 UNDER AEROBIC CONDITIONS IN MEDIUM WITH DIFFERENT ACETATE CONCENTRATIONS

FIGURE 8

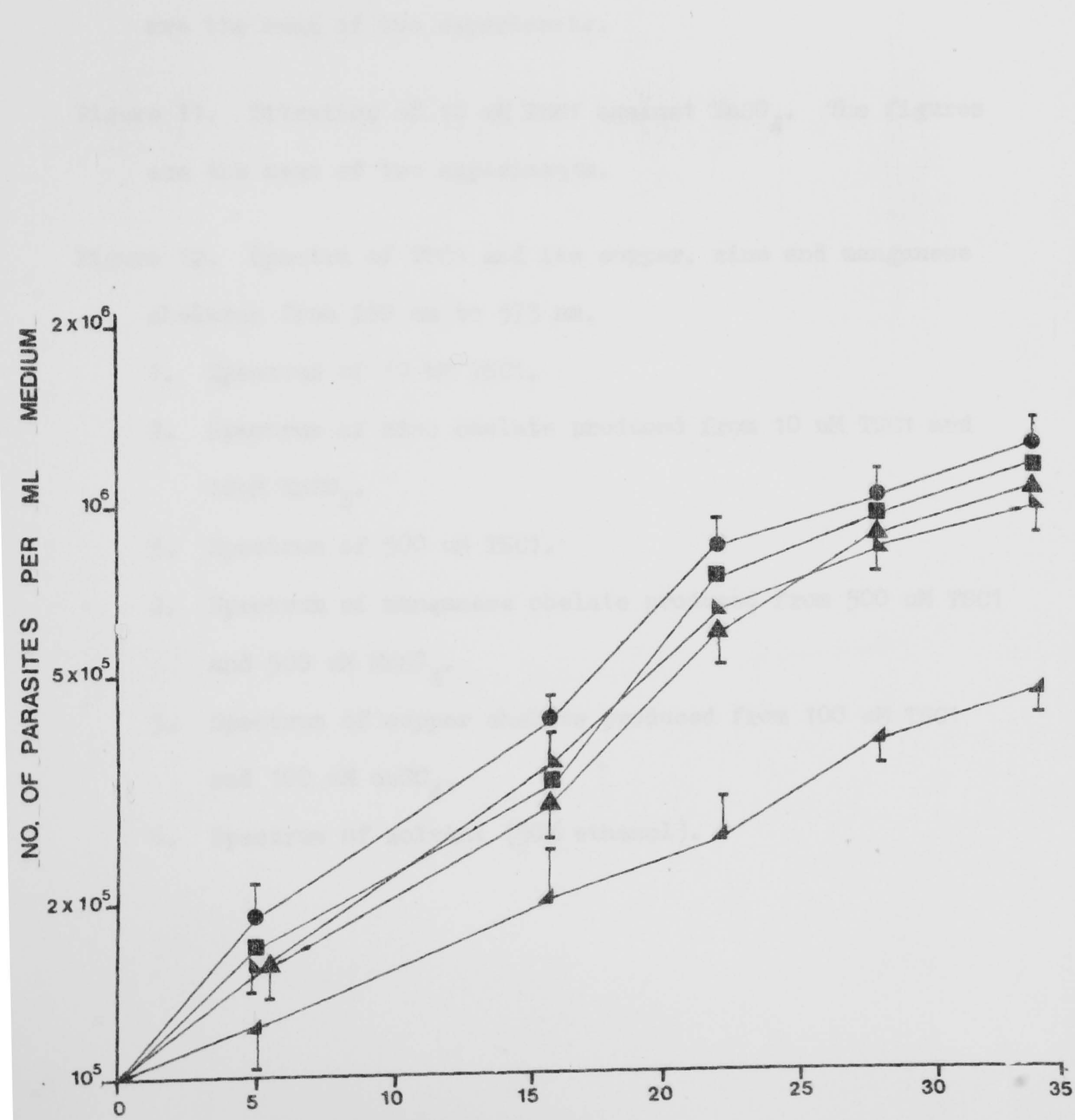


Figure 9. Titration of 500 uM TSC1 against $MnSO_4$. The figures are the mean of two experiments.

Figure 10. Titration of 100 uM TSC1 against $CuSO_4$. The figures are the mean of two experiments.

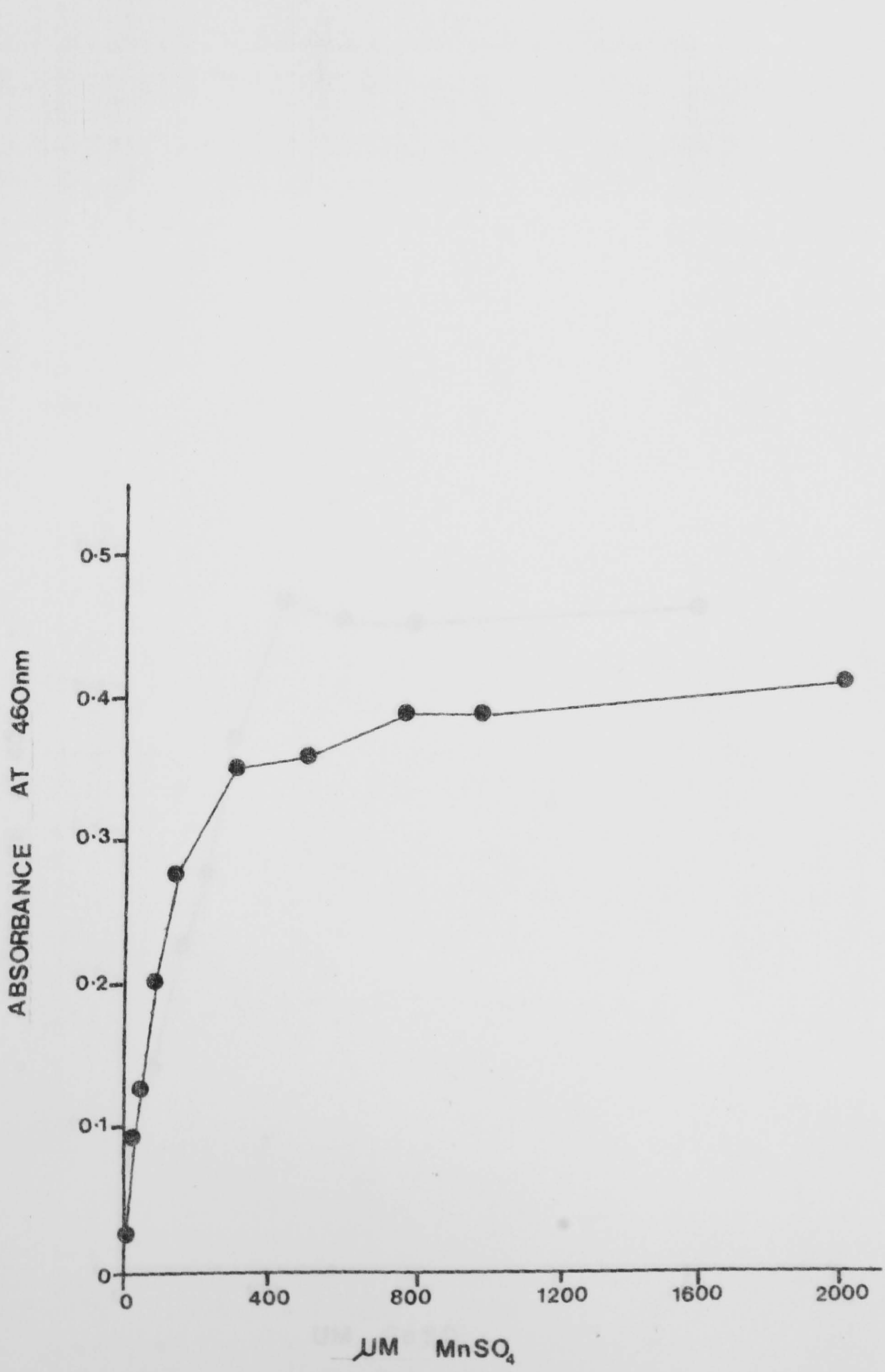
Figure 11. Titration of 10 uM TSC1 against $ZnSO_4$. The figures are the mean of two experiments.

Figure 12. Spectra of TSC1 and its copper, zinc and manganese chelates from 288 nm to 575 nm.

1. Spectrum of 10 uM TSC1.
2. Spectrum of zinc chelate produced from 10 uM TSC1 and 10uM $ZnSO_4$.
3. Spectrum of 500 uM TSC1.
4. Spectrum of manganese chelate produced from 500 uM TSC1 and 500 uM $MnSO_4$.
5. Spectrum of copper chelate produced from 100 uM TSC1 and 100 uM $cuSO_4$.
6. Spectrum of solvent (50% ethanol).

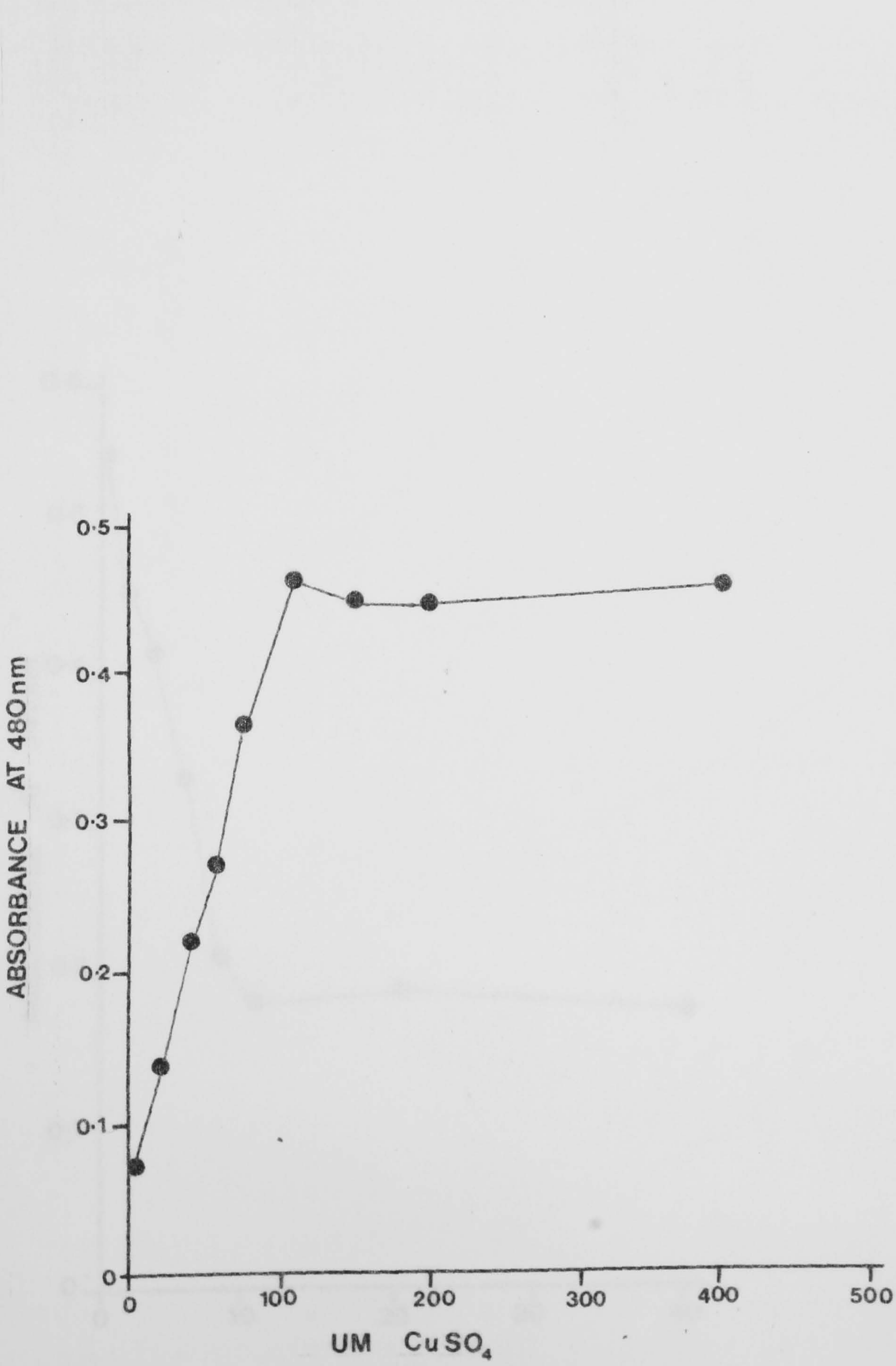
TITRATION OF 500 µM TSC I AGAINST $MnSO_4$

FIGURE 9



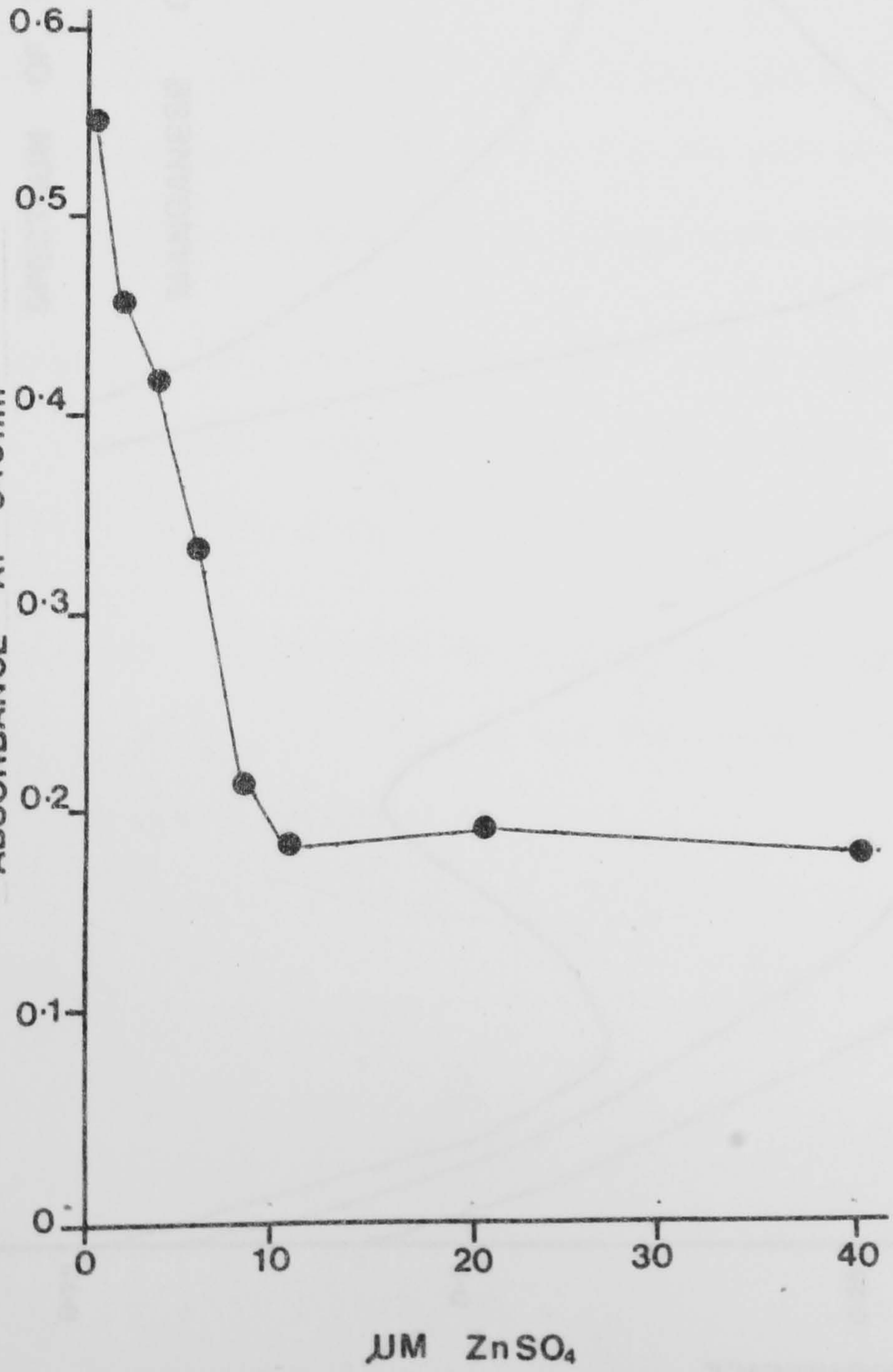
TITRATION OF 100 μM TSC1 AGAINST $CuSO_4$

FIGURE 10



TITRATION OF 10 µM TSC 1 AGAINST $ZnSO_4$

FIGURE 11



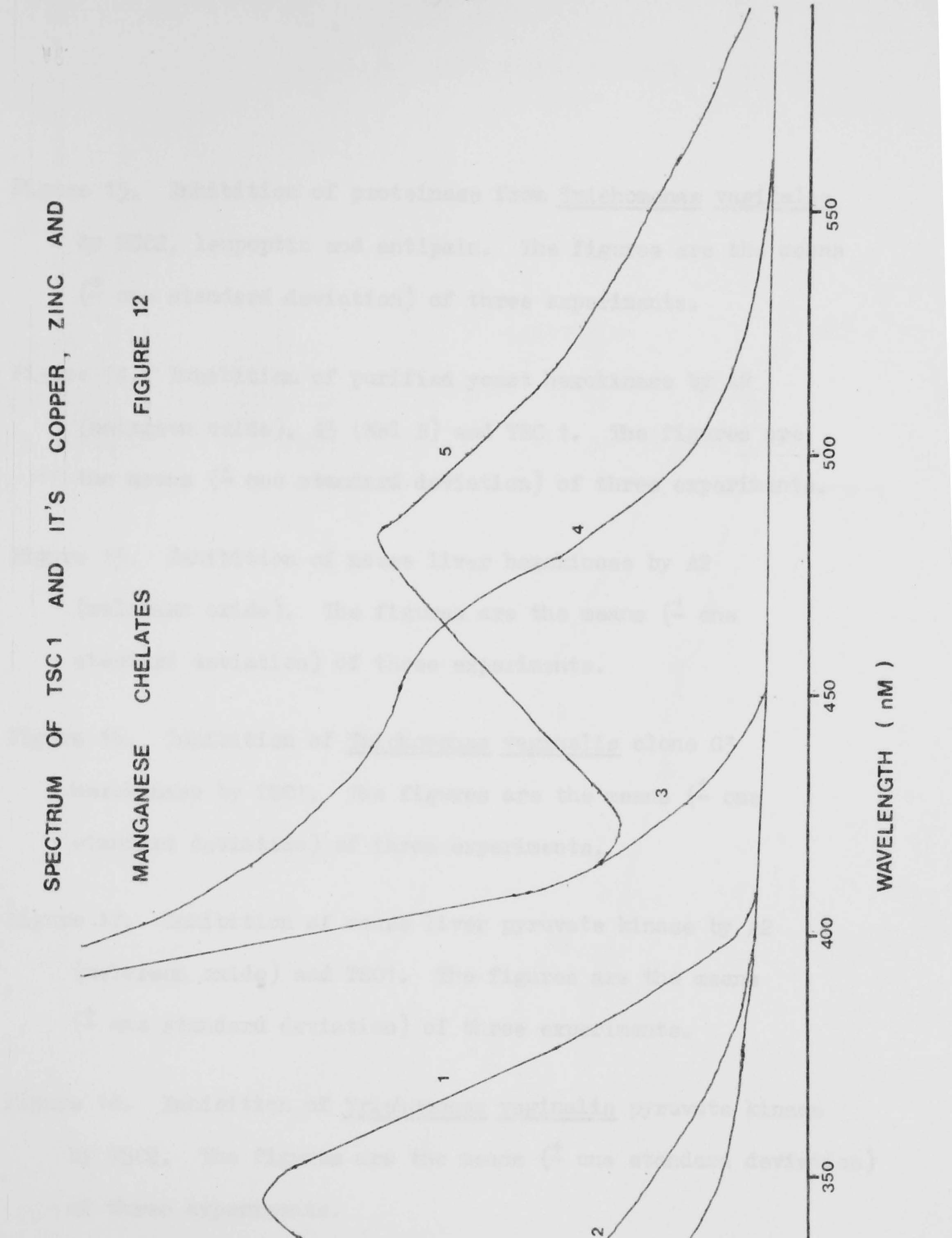

SPECTRUM OF TSC 1 AND IT'S COPPER, ZINC AND MANGANESE CHELATES
1
2
3
4
5
6
0·75
0·5
0·25
0
ABSORBANCE
300
350
400
450
500
550
WAVELENGTH (nM)

FIGURE 12

Figure 13. Inhibition of proteinase from Trichomonas vaginalis by TSC2, leupeptin and antipain. The figures are the means (± one standard deviation) of three experiments.

Figure 14. Inhibition of purified yeast hexokinase by A2 (melarsen oxide), A3 (Mel B) and TSC 1. The figures are the means (± one standard deviation) of three experiments.

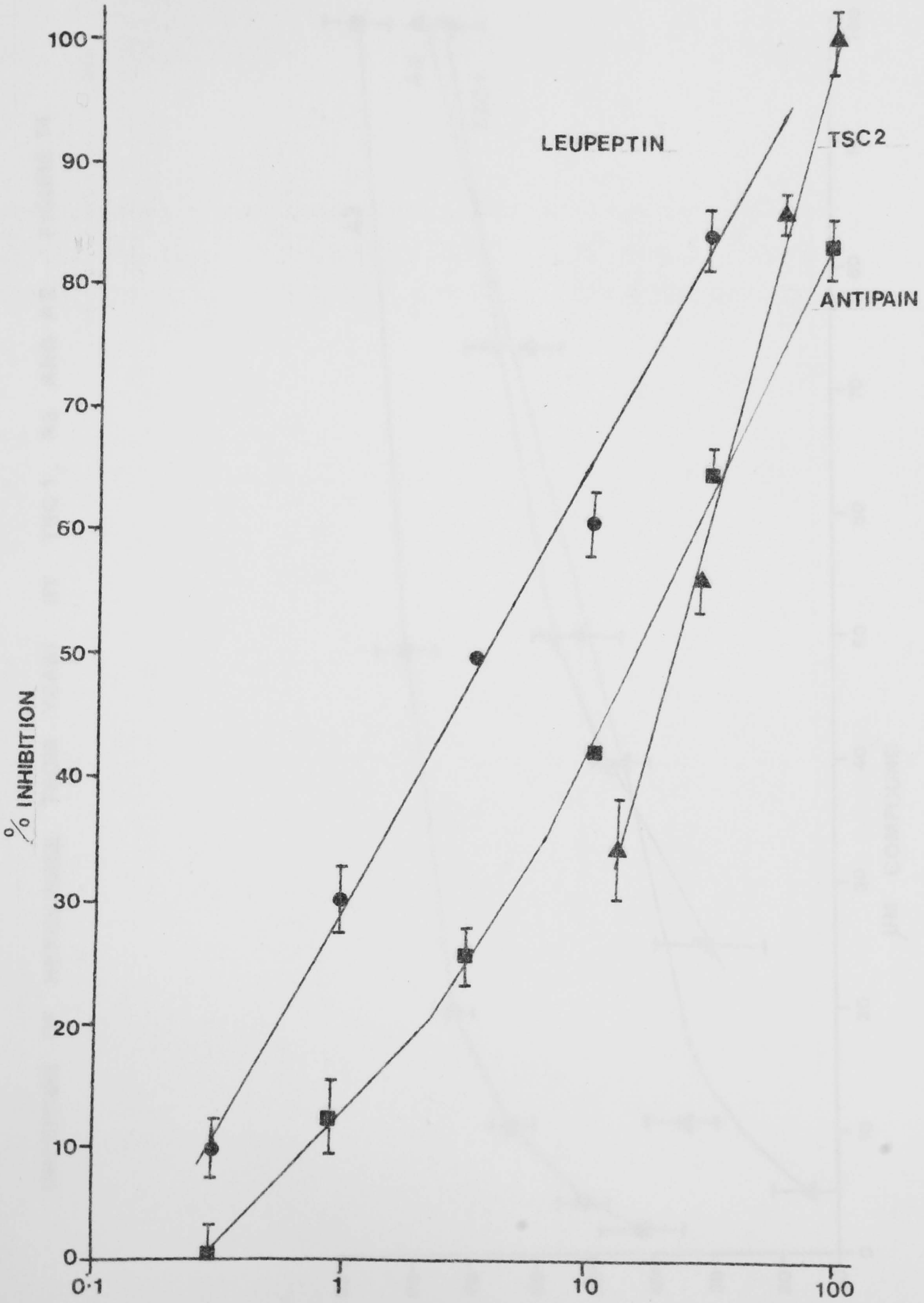
Figure 15. Inhibition of mouse liver hexokinase by A2 (melarsen oxide). The figures are the means (± one standard deviation) of three experiments.

Figure 16. Inhibition of Trichomonas vaginalis clone G3 hexokinase by TSC1. The figures are the means (± one standard deviation) of three experiments.

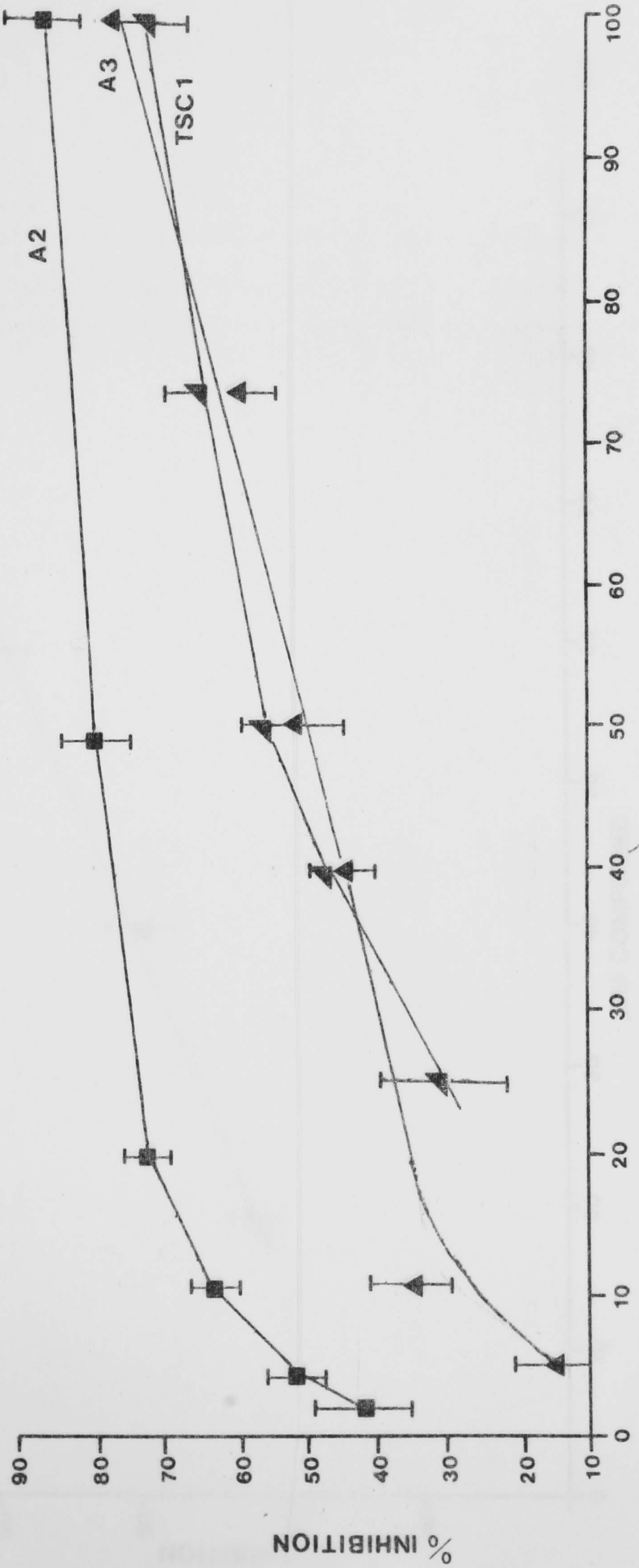
Figure 17. Inhibition of mouse liver pyruvate kinase by A2 (melarsen oxide) and TSC1. The figures are the means (± one standard deviation) of three experiments.

Figure 18. Inhibition of Trichomonas vaginalis pyruvate kinase by TSC2. The figures are the means (± one standard deviation) of three experiments.

INHIBITION OF T. VAGINALIS PROTEINASE ACTIVITY FIGURE 13



INHIBITION OF HEXOKINASE FROM YEAST BY TSC1, A3 AND A2 FIGURE 14



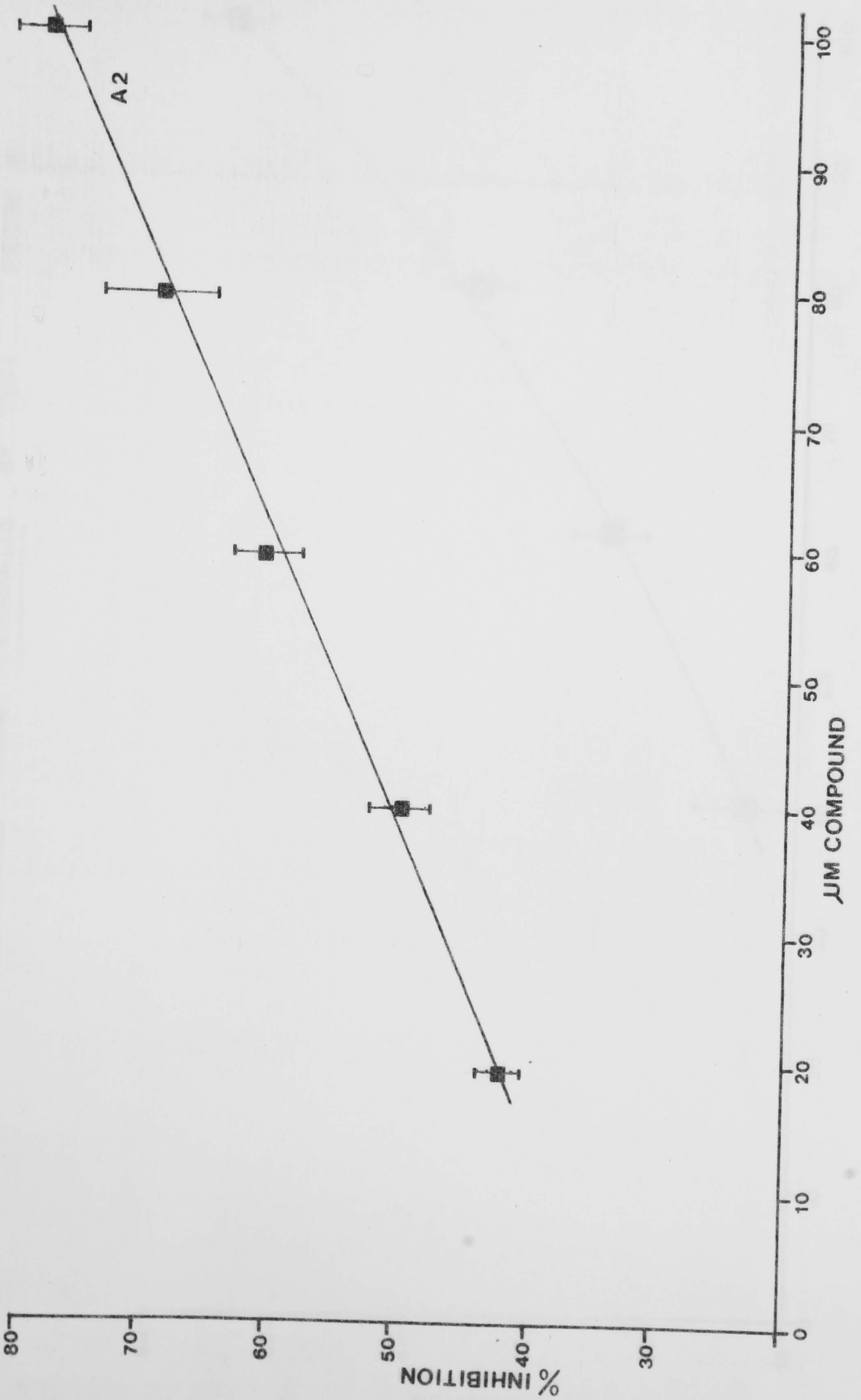

INHIBITION OF HEXOKINASE FROM MOUSE LIVER
% INHIBITION
0
10
20
30
40
50
60
70
80
90
100
JUM COMPOUND
A2

FIGURE 15

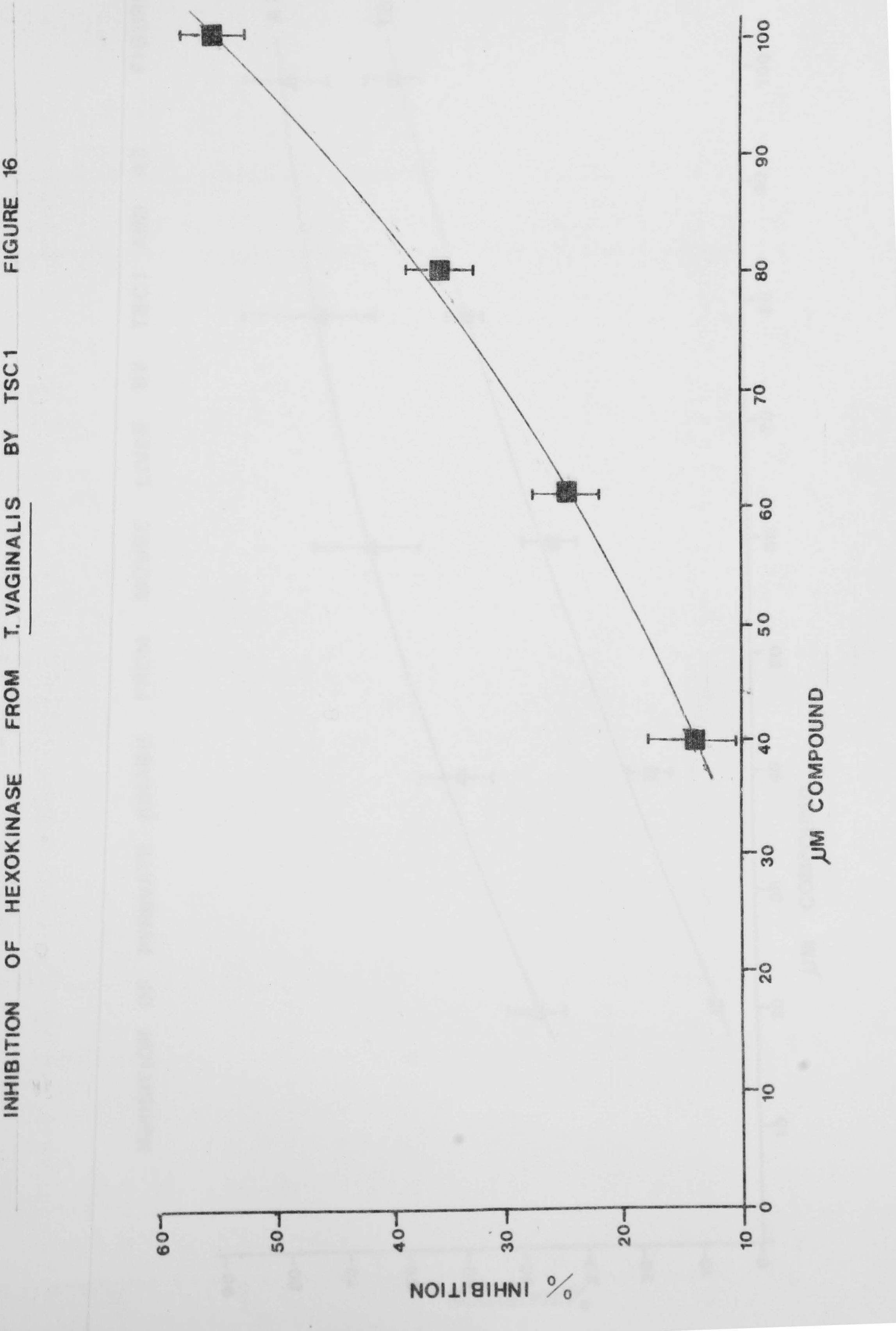
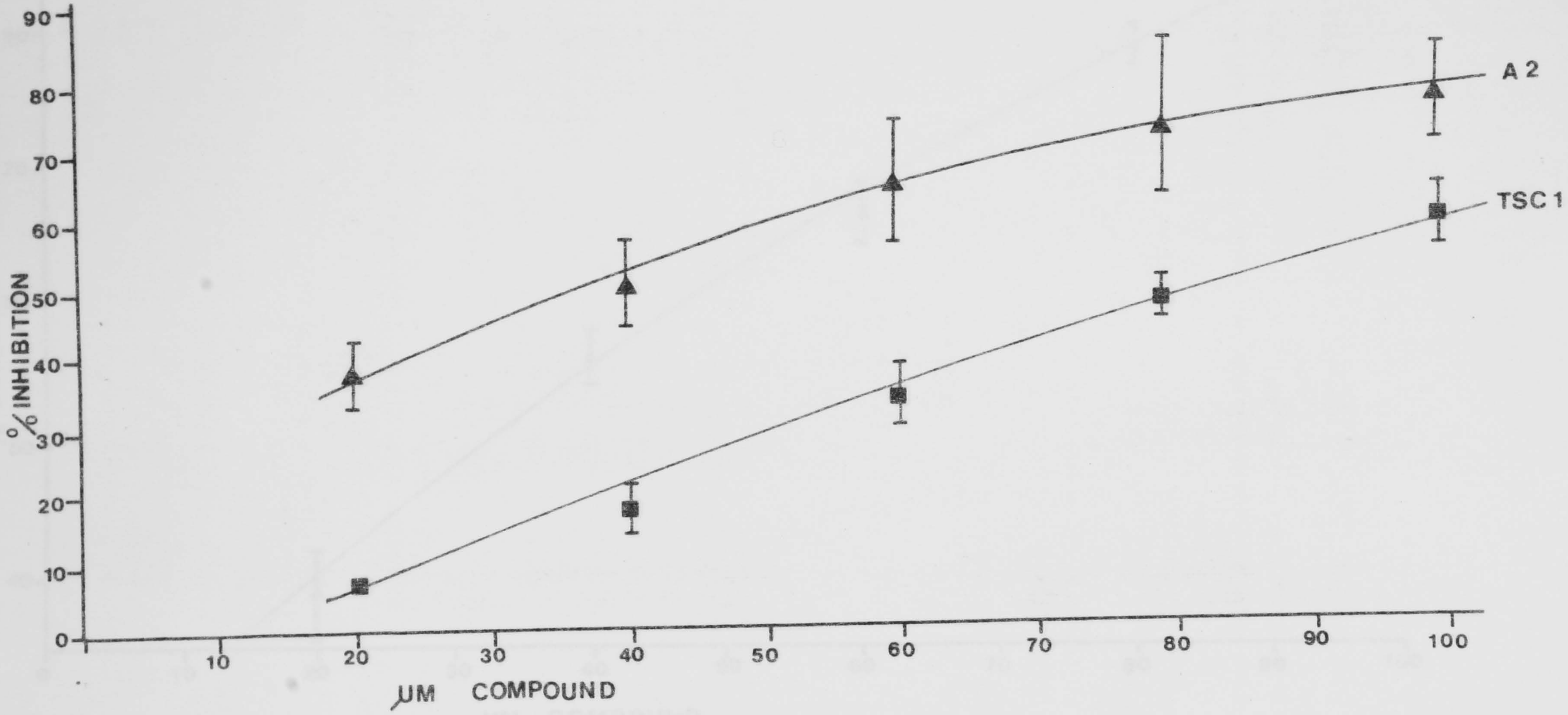

INHIBITION OF HEXOKINASE FROM T. VAGINALIS
BY TSC1
% INHIBITION
0
10
20
30
40
50
60
70
80
90
100
µM COMPOUND

FIGURE 16

INHIBITION OF PYRUVATE KINASE FROM MOUSE LIVER BY TSC1 AND A2 FIGURE 17




INHIBITION OF PYRUVATE KINASE FROM T. VAGINALIS BY TSC 2
FIGURE #8
% INHIBITION
90
80
70
60
50
40
0
10
20
30
40
50
60
70
80
90
100
µM COMPOUND

4. DISCUSSION

4.1. DIFFERENT METHODS OF ASSESSING ANTITRICHOMONAL ACTIVITY

Metronidazole has been used in the treatment of trichomonal vaginitis for over 20 years and is still the drug of choice. Unfortunately, resistance to the drug has been reported recently (Meingassner and Thurner, 1979; Müller, Meingassner, Miller and Ledger, 1980). Since resistance to metronidazole is likely to become more prevalent as the drug continues to be used on a wide scale, it is of importance to discover and develop new antitrichomonal compounds which may eventually replace metronidazole. An important step in this process is the design and use of a reliable and sensitive method to assess the antitrichomonal activity of potentially useful compounds.

Using the microtitre plate method developed, three lines of Trichomonas vaginalis were found to be sensitive to a range of compounds, including some known to have, and some similar to compounds which have, antitrichomonal activity (Table I). The 5-nitroimidazole metronidazole was first reported to have antitrichomonal activity in 1959 (Cosar and Julou, 1959), and its use as an antitrichomonal agent has been well documented (Rees, 1960; Porapakkham, 1967). Although metronidazole is the preferred compound in use against trichomonal vaginitis, a range of other nitroimidazole derivatives have been tested for antitrichomonal activity (Meingassner and Mieth, 1976; Ray, Chatterjee and Tendulkar, 1982). The anti-trichomonal activity of metronidazole against T. vaginalis clone G3

was similar to that reported by other authors (Jennison, Stenton and Watt, 1961; Edwards, Dye and Carne, 1975; Lindmark and Muller, 1976) for several other lines. The sensitivity of line IR78 to metronidazole, although similar to that of clone G3 under anaerobic conditions, was much less under aerobic conditions. This loss of sensitivity to metronidazole under aerobic conditions has been previously reported by Meingassner and Thurner (1979). This result suggested that oxygen is involved in the resistance of this line to metronidazole. The metronidazole sensitivity of line LRS (0.6), a laboratory produced strain grown under in vitro drug pressure until it was cultured in medium containing 0.6 µg metronidazole per ml, was slightly lower under both aerobic and anaerobic conditions than that of clone G3, and no effect of the presence of oxygen similar to that observed with line IR78 was found. These results suggested that lines IR78 and LRS (0.6) have different modes of resistance to metronidazole. The activity and mode of action of metronidazole to several lines of T. vaginalis is discussed more fully in section 4.2.

Meingassner and Mieth (1976) showed that a metronidazole-resistant line of Tritrichomonas foetus exhibited cross-resistance to a range of other nitroimidazole derivatives, both in vitro and in vivo. Although the activity of other nitroimidazoles was not tested in this study, the activity of other nitro-compounds (nitrodiazoles and nitrofurans) was investigated. The nitrodiazoles were less active than metronidazole against Trichomonas vaginalis

clone G3 and although line IR78 was relatively resistant to these compounds, this was so in both aerobic and anaerobic conditions suggesting that oxygen was not involved. These results suggest that cross-resistance between 5-nitroimidazoles and other nitro-cyclic compounds may not occur and that these different nitro-compounds act differently. No overall picture of the sensitivity of line LRS (0.6) to nitrodiazoles emerged from this study. Both nitrofurans were active against all three lines of T. vaginalis. This is of interest as it suggests that nitrofurans (such as compound 4) may be effective in treating infections resistant to metronidazole. This would, of course, need to be tested in vivo. In addition, it confirms that 5-nitroimidazoles and nitrofurans have different modes of action. Nitrofurans have previously been suggested as useful antitrichomonal compounds (Cavier, Chemtob, René and Royer, 1979) and have been shown to have antitrypanosomal (Gugliotta, Tanowitz, Wittner and Soeiro, 1980) and antibacterial (Ebringer, Jurášek, Kada, Kroška and Foltínóva, 1972) activity.

Of the three thiosemicarbazones tested using the microtitre plate method, none appeared to be of especial interest as an antitrichomonal compound. The one compound that was active (TSC3), was equally so against line IR78 and clone G3, suggesting that such compounds act in a different way to 5-nitroimidazoles. Drugs developed from them therefore should be useful against metronidazole-resistant lines, like IR78, when metronidazole treatment fails. A group of thiosemicarbazones (2-acetylpyridine thiosemicarbazones) have been shown to have antimalarial (Klayman, Bartosevich, Griffin,

Mason and Scovill, 1979) and antitrypanosome (Wilson, Revankar and Tolman, 1974) activity. The compounds tested in this study however, were not in this group of thiosemicarbazones.

It is of interest to note that apart from one thiosemicarbazone (TSC3), only nitro-compounds were found to be active against T. vaginalis at low concentrations using the microtitre plate method. Since it is known that the activity of 5-nitroimidazoles requires the reduction of the nitro-group (O'Brien and Morris, 1972) and this is also thought to be the case for nitrofurans, the activity of the nitrodiazoles may be dependant upon their reduction also. All these nitro-compounds may therefore be activated in a similar manner, although as the activity profiles of the drugs against the 3 lines of T. vaginalis differ markedly, it is probable that either different enzymes are involved in the nitro-reduction, or the subsequent targets are not the same.

The importance of the method of assessing antitrichomonal activity was illustrated when the activity of the same compounds was measured using a tube method (Table II). With most compounds, the results obtained using this method were similar to those obtained in the microtitre plate method, considering that a different cell line and medium was used. However, the thiosemicarbazones (TSC1 and TSC10), which were apparently inactive when assessed using the microtitre plate method, were found to be highly active using this method. These differences in observed activity could have been due to one or more of several factors: differences between the cell lines;

differences between the culture media; differences in other aspects of the two drug-screening methods. Upon testing, it was found that it was the culture media, and not the cell lines that was crucial. The main difference between the two media was the presence of high concentrations of cysteine (12.7 mM) and ascorbate (21.0 mM) in GTM medium, and their absence in Bushby's medium (See section 2.1.2.). The low activity of the thiosemicarbazone (TSC 10) in GTM medium was due, to a large extent, to the presence of cysteine. Ascorbate apparently had only a small effect. The antitrichomonal activity of thiosemicarbazones will be discussed in more detail in section 4.5. As well as the thiosemicarazones, a difference in activity of compound 7 (an isothiazole) was observed with the two methods. In this case the low activity of compound 7 in GTM medium may be due to the highly reducing conditions present, rather than to cysteine or ascorbate themselves.

The differences in the sensitivites of the two lines, using the two culture media, are of some importance, as they illustrate a shortcoming in the in vitro drug-screening methods used. If GTM medium alone had been used in these tests, then the thiosemicarbazones (TSC1 and TSC10) would not have been considered aspotentially useful antitrichomonal compounds, whereas by using Bushby's medium they were shown to be very highly active. This demonstrates that the type of drug screening method used can affect the results and in particular may produce falsely negative results, allowing potentially useful

antitrichomonal compounds to be passed over. This danger has been reported previously. In 1978, Meingassner, Mieth, Czok, Lindmark and Müller showed that the MLC (Minimal lethal concentration) of metronidazole against a line of Tritrichomonas foetus (Kv_1/M_{100}) differed markedly depending upon the medium in which it was grown and tested. This line of T. foetus was metronidazole resistant in an aerobic tray test if most culture media were used. If CACH (Müller and Gottschalk, 1970) or TTYS + 0.1% (w/v) ascorbic acid (Diamond, 1968) were used, however, then this line was normally sensitive, there being no difference in MLC when compared to a reference strain. The simplest method of circumventing the problem outlined above is to test the activity of each compound in a range of media.

4.2. SENSITIVITY OF TRICHOMONAS VAGINALIS TO METRONIDAZOLE

4.2.1. Mechanisms of resistance to metronidazole

The results given in Table V confirm the involvement of oxygen in the metronidazole-resistance of T. vaginalis lines IR78, Boston, Fall River and Albany, that has been previously reported (Meingassner and Thurner, 1979; Müller, Meingassner, Miller and Ledger, 1980; Müller, 1982), and show that the lower sensitivity of line LRS (2.0) is independant of oxygen. Although the mode of action of metronidazole is not fully understood, it has been shown that metronidazole inhibits hydrogen production (O'Brien and Morris, 1972; Coombs, 1976), not by directly inhibiting hydrogenase, but by

acting as an electron acceptor for the pyruvate:ferredoxin oxidoreductase system. This action involves the reduction of the nitro-group of metronidazole (Edwards, Dye and Carne, 1973; Coombs, 1976; Lindmark and Müller, 1976), a reduction that was inhibited *in vitro* by Oxygen (Müller, Lindmark and McLaughlin, 1976; Peterson, Mason, Hovespian and Holtzman, 1979).

Cytoplasmic NADH oxidase and NADPH oxidase (Müller and Lindmark, 1973; Tanabe, 1979) probably reduce the oxygen that enters *T. vaginalis* and so could be responsible for maintaining the anaerobic nature of the hydrogenosomes in living cells. It is in these organelles that metronidazole is reduced. The lower activities of NADH oxidase and NADPH oxidase in line IR78 compared with clone G3 (Table VI) suggests that drug resistance in this line may be due to significant amounts of the oxygen entering the parasite permeating unmetabolised to the hydrogenosomes, where it would interfere with the reductive activation of the drug. Beaulieu, McLafferty, Koch and Goldman (1981) reported that under aerobic, but not anaerobic, conditions line IR78 formed acetamide, a reduction product of metronidazole, at a lower rate than a metronidazole-sensitive line. This suggests that the oxygen present was inhibiting the reduction of metronidazole in line IR78 and this did not occur, or occurred to a much lesser extent, in the metronidazole-sensitive line. This correlates well with the findings presented above. However, Müller (1982) reported that the NADH oxidase activity of line IR78 was greater (830 nmoles/min/mg protein) than those of four metronidazole-sensitive reference lines,

ATC 30001, NYH 209, NYH 272 and NYH 286 (20, 370, 550 and 380 nmoles/min/mg protein) respectively. This is contrary to the findings of this study. The NADH oxidase activities of Müller's reference lines (except ATC 30001) were broadly similar to that of clone G3, indicating that the methods used by this author were comparable to the methods used in this study. There is, as yet, no explanation for the difference in NADH oxidase activities reported for line IR78. The low NADH oxidase activity reported for the line ATC 30001 suggests that the metronidazole activity exhibited by this line should be highly sensitive to oxygen, however Müller did not find this. This could perhaps be due to the protective effects of a high NADPH oxidase activity in this line. Clearly, further investigation will be necessary to determine the exact mechanism of metronidazole-resistance of these lines. It may be that the level of oxygen available is important and studies of enzyme activity under reduced oxygen tension could be a useful approach.

The growth of Trichomonas species (Mack and Müller, 1978) and another protozoan Entamoeba histolytica (Band and Cirrito, 1979; Gillin and Diamond, 1980) have been shown to be sensitive to oxygen and to the concentration of reducing agents in the medium (Gillin and Diamond, 1981 a, b). GTM medium contains high concentrations of cysteine (12.7 mM) and ascorbate (21.0 mM). These compounds produce a highly reducing environment in the medium and the lowering of their concentrations would result in the greater availability of oxygen. The observed adverse effects of such conditions upon the

growth of both clone G3 and line IR78 (Figures 2 and 4) support the suggestion that Trichomonas vaginalis is an anaerobie. Gillin and Diamond (1981b) showed that cysteine and ascorbate delayed the killing effect of oxygen on Entamoeba histolytica and Giardia lamblia. However, with Trichomonas vaginalis, although both cysteine and ascorbate protected the parasites from the killing effects of oxygen, cysteine gave markedly greater protection (Table VII). It was also shown with Entamoeba histolytica and Giardia lamblia that cysteine itself was a required nutrient. Other reducing agents did not support survival as well, and in the absence of cysteine the parasites did not grow, even under anaerobic conditions. This was not the case with Trichomonas vaginalis. Under anaerobic conditions, growth of T. vaginalis occurred in media lacking both cysteine and ascorbate, although at a reduced rate (Figures 1 and 3). The addition of other reducing agents as cysteine substitutes enhanced growth, but it was still less than when cysteine was present (Table VIII).

Gillin and Diamond (1981b) reported that l-cystine protected Entamoeba histolytica from oxygen. This was unexpected, as this amino acid lacks the thiol reducing group of cysteine. They suggested that E. histolytica may have the enzymatic capacity to reduce l-cystine to cysteine. Cystine reductase has been demonstrated in Histoplasma capsulatum (Maresca, Jacobson, Madoff and Kobajashi, 1978) and glutathione reductase in Entamoeba histolytica (Weinbach, Claggett and Singer (unpublished data). L-cystine was not tested

with Trichomonas vaginalis as a suitable solvent could not be found. Attempts were made to dissolve this compound in distilled water, ethanol, methanol and ethylene glycol, but only low micromolar concentrations could be obtained. The attempt to investigate this compound was hindered by the lack of experimental methods provided by Gillin and Diamond.

The greater effect of lowering cysteine and ascorbate concentrations in the medium upon the growth under aerobic conditions of line IR78 compared with clone G3, suggested that the former line was less capable of handling oxygen entering the cell than was clone G3. This correlated well with line IR78's lower NADH oxidase and NADPH oxidase activities. The low activities of these enzymes may also account for the relatively poor growth, even in GTM medium, of this line under aerobic conditions (Figures 5 and 6).

Mack and Müller (1978) showed that the presence of CO_2 did not affect the generation time of T. vaginalis in an anaerobic environment. In this study, however, growth of clone G3 and line IR78 were markedly enhanced by the addition of 5% CO_2 to anaerobic cultures (Figures 5 and 6). CO_2 is an endproduct of the parasite's metabolism and carboxylation reactions are not thought to be of major significance to T. vaginalis unlike Tritrichomonas foetus. The enhanced growth was not due to a lowering of the pH of the medium by CO_2 in solution, as such a change did not enhance growth. The presence of high CO_2 concentrations in the medium may have inhibited the activity of pyruvate:ferredoxin oxidoreductase and therefore of

acetate formation. There could have been a concomitant increase in the production of malate from pyruvate and phosphoenol pyruvate, and of pyruvate excretion. It is difficult to see how this would increase parasite growth, however, as the changes outlined would tend to lower the total amount of high energy phosphates produced per molecule of glucose. It would be of interest to investigate endproduct production in the presence and absence of CO_2, to determine whether acetate production was lowered and malate and pyruvate production and excretion increased. However, the differences in the findings given by Mack and Müller (1978) and those presented in this study may be related to the different media and parasite lines used.

In contrast to line IR78, line LRS (2.0) possessed similar levels of NADH oxidase and NADPH oxidase to clone G3. Cell-free homogenates of line LRS (2.0), however, anaerobically reduced the nitro-group of metronidazole at a significantly lower rate than similar homogenates of clone G3. This may be an expression of lower pyruvate:ferredoxin oxidoreductase activity. Such a difference *in vivo* could result in a slower rate of activation of the drug. This could account for the lower sensitivity of this line, compared with clone G3, to metronidazole. A similar mechanism of resistance has been reported with *Bacteroides fragilis* (Britz, 1981; Müller, 1982). Since a slower rate of drug activation would tend to lessen the concentration gradient of unreduced drug entering the parasite, it would be of interest to see if metronidazole uptake by *Trichomonas vaginalis*

line LRS (2.0) and these Bacteroides fragilis lines was less than uptake in metronidazole-sensitive reference lines.

In conclusion, two mechanisms of resistance to metronidazole have been postulated. It is suggested that the relatively low NADH oxidase and NADPH oxidase activities of Trichomonas vaginalis line IR78 may allow molecular oxygen to permeate unmetabolised to the hydrogenosomes in which it inhibits the reductive activation of metronidazole. The reduced drug sensitivity of line LRS (2.0) may be due to this line's diminished level of the enzyme activity responsible for reductively activating metronidazole.

4.2.2. The antagonistic effects of lactate and acetate

In 1981, Mahood and Wilson reported that lactate enhanced the killing activity of metronidazole against hypoxic Chinese hamster ovary cells. They postulated that this effect may be due to one of several possibilities: an increased activation of the drug through the formation of NADH and a general raising of the reduction potential of the cell; an increase in the levels of lactate dehydrogenase (LDH) and its related enzyme complex sensitive to attack by drug free radicals; or a decrease in the intracellular pH at critical sites.

The antagonism of lactate to the trichomonacidal activity of metronidazole (Table IX) contrasts with the above result. None of the mechanisms suggested for the effect reported by Mahood and Wilson (1981) appear to be involved in this phenomenon, as the

antagonism was not simply a result of the low pH of the medium produced by the acids (Table IX). It is possibly significant that of the acids tested, only those that were end products of T. vaginalis metabolism had an antagonistic effect. It was shown to be unlikely that either lactate or acetate were antagonistic to the trichomonacidal activity of metronidazole through a direct effect, either inhibition or repression, of parasite pyruvate: ferredoxin oxidoreductase (Table XI). It is this enzyme that is thought to be mainly responsible, through its generation of reduced ferredoxin, for the reductive activation of metronidazole (Coombs, 1973; Edwards, Dye and Carne, 1973). Acetate could affect the enzyme indirectly by decreasing, through product inhibition, the rate of catabolism of acetyl CoA in the parasite. This could cause an accumulation of acetyl CoA which would be likely to inhibit pyruvate:ferredoxin oxidoreductase, for which it is one endproduct. It would be anticipated, however, that such an effect would occur under both aerobic and anaerobic conditions, whereas the antagonism was dependant upon the presence of oxygen.

The enhancement of the parasite lactate dehydrogenase (LDH) activity, assayed in the direction of lactate production, upon the addition of lactate to the growth medium was surprising. The explanation of this higher activity, whether enzyme activation or synthesis, is unclear. This result may be significant, however, as it is feasible that the antagonistic effect of lactate could be due to stimulation of LDH activity resulting in a greater percentage of the pyruvate produced by the glycolytic pathway being metabolised to

lactate, and so less being available to generate reduced ferredoxin and so lead to the activation of metronidazole. Again, however, it would be expected that this would occur under both aerobic and anaerobic conditions. The lower concentration of lactate produced by parasites grown in medium containing lactate (Table XII) is in contrast to the enhancement of LDH activity by lactate. The decrease in lactate production, however, may have been due to the inhibition of one or more enzymes prior to LDH in the glycolytic pathway, although this might have been expected to cause growth inhibition, which did not occur. Thus, the significance of the enhancement of LDH by lactate to the growing parasite is unclear.

The dependance upon oxygen for the antagonistic effect of acetate and lactate on the toxic effect of metronidazole, suggested that the acids may have impaired, in some way, the parasites ability to metabolise oxygen. If some of the gas permeated unreduced to the hydrogenosomes it could have inhibited the reductive activation of the drug (Müller and Lindmark, 1976). As discussed previously, the cytosolic enzymes NADH oxidase and NADPH oxidase (Brugerolle and Metenier, 1973; Müller, 1973; Tanabe, 1979) are thought to be mainly responsible for metabolising the oxygen entering the cell, and may in this way keep the hydrogenosomes anaerobic. However, in this study it was shown that neither lactate nor acetate directly inhibited either of these enzymes (Table XI), nor did it affect the level of the enzymes in the cells. It is feasible, but not supported by data

on lactate production, that the increased levels of lactate dehydrogenase in parasites grown in the presence of lactate could have caused a depletion in the NADH concentration in the parasite and consequently a lower rate of oxygen reduction catalysed by NADH oxidase. It is likely, however, that a reduction in the activity of NADH oxidase (or NADPH oxidase or pyruvate:ferredoxin oxidoreductase) would have adversely affected the growth of the parasite, but neither acetate nor lactate had this effect (Figures 7 and 8) (Britz and Wilkinson, 1979).

Although the mechanisms whereby acetate and lactate exert their antagonistic effects are not understood, these pehonomena could have some bearing on the treatment of trichomoniasis. The failure of metronidazole treatment on T. vaginalis infections has been reported intermittantly during the two decades it has been in clinical use (Squires and McFadzean, 1962) and several causes of treatment failure, including metronidazole resistance and concomitent infection with 'metronidazole-inactivating' bacteria (Edwards, Dye and Carne, 1979), have been postulated. The results suggest a third possible cause, since not only will the excretions of T. vaginalis themselves tend to counter the drugs action, but other organisms present could have similar effects. The antagonistic action of acetate and lactate was observed only when the acids were present at relatively high concentrations (Table X), however, there is little information available on the levels of metabolites that can occur in the

microenvironment of T. vaginalis in the vagina. Lactate is present in the uninfected vagina (Stamey, Fair, Timothy and Chung, 1968). The predominant bacterial inhabitant of the uninfected vagina (Doderlein's bacilli) produces lactate in quantity such that the environmental pH is reduced to approximately pH 4.5 (Stamey and Kaufman, 1975). During infection with T. vaginalis, however, usually the pH rises to about pH 6.0 (Parsons, Lofland and Mulholland, 1977) and the Doderleins Bacilli are virtually eliminated (Pfau and Sacks, 1981). In the infected vagina, the predominant microbial flora is gram-negative, mainly Escherichia coli, often in conjunction with a heavy colonization by colonic organisms such as streptococcus faecalis (Parsons, Lofland and Mulholland, 1977). More information on the microenvironment of the parasite (and especially those causing infection insensitive to the drug) would be helpful in elucidating the relevance of the present findings to the causes of metronidazole-insensitive trichomoniasis.

4.3. ANTITRICHOMONAL ACTIVITY OF COMPOUNDS THAT AFFECT DNA AND ITS REPAIR.

The potentiation of the antitrichomonal activity of DMS by the four benzamides tested (Table XIII) suggested that poly (ADP-ribose) polymerase may required in this protozoan parasite in a

similar way to that reported for mammalian cells (Durkacz, Omidijii, Gray and Shall, 1980). There is at present no direct evidence on how the benzamides exerted this effect against Trichomonas vaginalis. Indeed, there is no explanation on how benzamides inhibit poly (ADP-ribose) polymerase in mammalian cells. However, as a product of the reaction catalysed by this enzyme in the mammal, is nicotinamide, and nicotinamide is a potent inhibitor of this enzyme (Clark, Ferris and Pinder, 1971), this compound could act by competitive inhibition. Benzamide is closely related to nicotinamide and it is feasible that it acts in a similar manner. The extent of the synergism of the benzamides with Dimethyl sulphate (DMS), which was much greater than that reported for mammalian cells (albeit from studies involving different experimental conditions), suggested that drug combinations acting in a similar way could have potential as antitrichomonal agents.

The lack of antitrichomonal activity and synergism of benzamides with ethidium bromide suggests two possibilities. Either the mechanism of action of this compound differs from that of DMS to such an extent that a different DNA repair mechanism is active, or that ethidium bromide was not able to enter the parasite in a concentration sufficiently high to do significant damage. The latter suggestion is perfectly feasible, as the anti-DNA activity of ethidium bromide has usually been studied using permeabilised cells (Mattern and Painter, 1979), and indeed probable, as no antitrichomonal activity from it was detectable.

The lack of synergism between metronidazole and the benzamides against T. vaginalis suggested that the mechanism of antitrichomonal activity of metronidazole and DMS were not identical. It does not exclude the possibility that activated metronidazole damages DNA, albeit in a different way to DMS.

The antitrichomonal activity of the thiosemicarbazone (TSC1) shows that this compound enters the cell without difficulty, but the lack of synergism between this compound and the benzamides gives no evidence that TSC1 affects DNA in a similar manner to DMS.

4.4. THE METABOLISM OF XENOBIOTICS BY TRICHOMONAS VAGINALIS

The metabolism of nitrobenzene and azobenzene in the mammal is now well documented (Robinson, Smith and Williams, 1951a; Parke, 1956). In the case of nitrobenzene, aniline is the only purely reductive metabolite, and in the case of azobenzene, aniline, hydraxobenzene and benzidine are purely reductive metabolites. All the other metabolites (See section 1.4.3.) involve oxidative steps in their production. As T. vaginalis is an anaerobe, it was of interest to investigate its ability to metabolise compounds which undergo oxidative metabolism in the mammal, with a view to elucidating some of the metabolic capabilities of the parasite. Nitrobenzene and azobenzene were investigated because of the wide range of metabolic pathways related to these compounds in the mammal and the depth of the literature concerning their metabolism.

The MIC of both compounds, and of aniline and p-nitrophenol (Table XIV), show that these compounds are toxic to T. vaginalis, but only at relatively high concentrations. This is interesting as nitrobenzene, azobenzene and aniline are toxic to mammals. Nitrobenzene was used in shoe polish, perfume, dyes, inks and furniture polish, and there were many cases of poisoning due to the nitrobenzene in these products (Steens, 1928; Chapman and Fox, 1945; Nabarro, 1948; Parkes and Neill, 1953). Nitrobenezene poisoning causes nausea, vomiting, cyanosis, coma and in some cases, death. Other nitrobenzene derivatives, such as dinitrobenzene, have also been shown to be toxic to mammals (Cody, Witherup, Hastings, Stemmar and Christian, 1981). The lack of toxicity of the compounds to T. vaginalis may be due to a lack of uptake of them by the parasite or to the different metabolism of them in T. vaginalis and man. It was important to assess the toxicity of nitrobenzene and azobenzene to T. vaginalis so that sub-inhibitory concentrations of each compound could be added to cell cultures in experiments to study the metabolism of these compounds by the parasite.

The reduction of the nitro-group of nitrobenzene and of the azo-group of azobenzene probably occurred by the same ferredoxin-linked system involved in the reduction of 5-nitroimidazoles such as metronidazole. The reduction of the nitro-group of nitrobenzene

and metronidazole, and of the azo-group of azobenzene, all were inhibited by oxygen, required pyruvate as a substrate, coenzyme A as a cofactor, and were enhanced by the addition of methyl viologen.

In 1975, Douch reported that the azoreductases of Moniezia expasa and Ascaris lumbricoides var suum were insensitive to oxygen, but that mammalian azo-reductase enzymes were oxygen-sensitive. The results presented in this study show that the azo reductase system found in Trichomonas vaginalis is not similar to that found in these helminths.

The lack of detection of any metabolites of either nitrobenzene or azobenzene by TLC could have been due to the solvents used in this method. Aniline, the only purely reductive metabolite of nitrobenzene in the mammal, and the most likely product of T. vaginalis, could not be detected using these solvents. Hydrazobenzene, the major reductive metabolite of azobenzene in the mammal, had a similar Rf value (Table XV) to that of azobenzene in each of the three solvents, making distinguishing the two compounds impossible. Thus it turned out that the reductive metabolites of each compound could not be studied using these solvents. The solvents used in this method clearly were not ideal for the investigation undertaken. Aminophenols and nitrophenols, mammalian metabolites produced in oxidative steps, could be separated from parent compounds and identified using these solvents. However, they were not detected,

suggesting they were not produced. It may well be that reductive pathways are the only ones available to T. vaginalis, and that they can occur was demonstrated by the observed reduction of the parent compounds using T. vaginalis cell-free homogenate.

The analytical methods available in the past for the detection and quantitation of nitrobenzene, azobenzene and their mammalian metabolites often required multiple extractions at alkaline pH, and subsequent chemical conversion to a more spectrophotometrically sensitive derivative (Boyland and Nery, 1964), fluorimetry (Sternson and Stewart, 1973) and isotopic techniques (Gang, Lieber and Rubin, 1972) at the final data, read out. The analysis of nitrobenzene, azobenzene and their mammalian metabolites by high-performance liquid chromatography (HPLC) was developed by Sternson and DeWitte (1977), who separated nanomole quantities of aniline, o- and p-aminophenol, nitrobenzene and azobenzene by reversed-phase chromatography. Methanol-water (15:85) or (50:50) was used as the mobile phase. This method is very similar to that used in the present study (section 2.4.3.) even including the mobile phase. The compound retention times are given in Table XIV. The method used in the present study was equally sensitive to that used by Sternson and DeWitte (1977), detecting nanomole quantities of the compounds, and was a marked improvement over earlier methods, such as the TLC method used in this study. Despite the greater sensitivity of the method, no metabolites of nitrobenzene

was detected using HPLC. However, in control samples, lacking T. vaginalis cell-free homogenate, parent compound (nitrobenzene) recovery was 70% whereas in experimental samples, with homogenate, only 30% of the parent compound was recovered. The only difference between these samples was the presence of homogenate. These results suggest that nitrobenzene was metabolised but that the metabolite (or metabolites) were undetected by this method. The loss of parent compound occurred only if pyruvate, coenzyme A and methyl viologen were included in the incubation mix along with nitrobenzene and extract. These are the compounds required for nitrobenzene reduction to take place in cell-free homogenates of T. vaginalis. Thus it appears that the reduction of nitrobenzene was occurring, but that the metabolite (or metabolites) were not being detected. The extraction and subsequent detection of nitrobenzene standards showed that 70% of the nitrobenzene was detected, the same amount as was recovered in control samples. This suggested that the smaller recovery of nitrobenzene in experimental samples was not due to the method used, but to its disappearance during the incubation when 3-aminophenol and aniline standards were similarly tested, 70% of the former was detected, whilst no aniline was found. The inability to detect aniline after extraction correlates well with the thoery that the reduction of nitrobenzene is responsible for the lower recovery of parent compound in experimental samples, and that its major product (which can't be detected) was aniline. The lack of 3-aminophenol

suggests that none was produced, that is, hydroxylation did not take place.

Hydrazobenzene was identified by cochromatography as a metabolite of azobenzene. This only occurred however, if pyruvate, coenzyme A and methyl viologen were present in the incubation mix along with azobenzene and cell-free homogenate. The lower azobenzene recovery rate in experimental samples (40%) compared with that in control samples (75%) could not all be accounted for by the hydrazobenzene present (15%). There was approximately 20% of the parent compound unaccounted for. This could have been converted to aniline which was not detected due to its loss in the extraction procedure.

The loss of aniline in the evaporation of the extract during the extraction procedure was a major drawback of this HPLC method. Aniline is a highly volatile substance and the combination of low pressure and high temperature (50°C) may have caused the loss of this compound. An improvement to this procedure would be the evaporation of the extract under vacuum at room temperature, which should improve aniline recovery.

The lack of direct evidence for nitrobenzene metabolites and the inability of the techniques used to detect aniline after extraction, means that further investigation is required to quantify the reductive metabolites of nitrobenzene and azobenzene.

The lack of any metabolites, of nitrobenzene or azobenzene, whose production involves oxidative steps, however, confirms the

anaerobic nature of T. vaginalis, and suggests that the ability of this organism to utilise oxygen, even when available, is limited. In addition, this may also explain the low toxicity of nitrobenzene and azobenzene to T. vaginalis, as the oxidative metabolism of these compounds is involved in their toxicity to mammals. The lack of oxidative metabolism of xenobiotics may provide a possible target for chemotherapeutic attack. Compounds normally detoxified by Man by oxidation may be selectively toxic to T. vaginalis.

4.5. ANTITRICHOMONAL ACTIVITY OF THIOSEMICARBAZONES

4.5.1. Involvement of chelates

The trichomonacidal activity of a range of thiosemicarbazones and some of their chelates are given in Table XVIII. Compounds unable to form chelates were much less active than compounds able to form chelates. Chelates were slightly more active than their parent compounds. In particular, TSC1 and TSC2, a thiosemicarbazone and its copper chelate, were highly active against T. vaginalis.

The ability of thiosemicarbazones to form chelates with transition metals has been known for some time, and metal ions have often been implicated in the activity of thiosemicarbazones (Venton, Chan, Passo, Racine and Morris, 1977; Ankel and Petering, 1980; Stunzi, 1982). Although thiosemicarbazones of the type used in this study have not been investigated previously, these results (Figures 9, 10 and 11) show that TSC1 is also an avid chelator of transition metals, which may be of importance to the mode of action

of this compound. The copper chelate (TSC2) was stable in the presence of excess transition metals. This suggests that chelation is not the mode of action of the killing activity of these compounds. Since only chelates, and compounds with the ability to form chelates, were highly active, and chelation itself was not the cause of cell death, then the chelate or a metabolic product of the chelate must be the forms active against the parasite.

4.5.2. Antagonism by thiol agents

The antagonistic effect of cysteine upon the trichomonacidal activity of a thiosemicarbazone (TSC10) has been reported (section 3.2.) and discussed (section 4.1.) in the context of the alteration of killing activity by culture medium components. This effect was also found with two other thiosemicarbazones (TSC1 and TSC2) but did not occur with a third (TSC3) (Table XVII). It is possible that TSC3 was not affected by cysteine because it lacks the C=S group in the lower chain to which the cysteine could bind (See diagram 1 for this mechanism.) Alternatively, the lack of antagonism by cysteine upon the action of TSC3 may be related to this thiosemicarbozone being unable to form a chelate. In either case, the results suggest that this thiosemicarbazone exerts its (albeit rather limited) antitrichomonal activity in a different way from thiosemicarbazones TSC1 and TSC2.

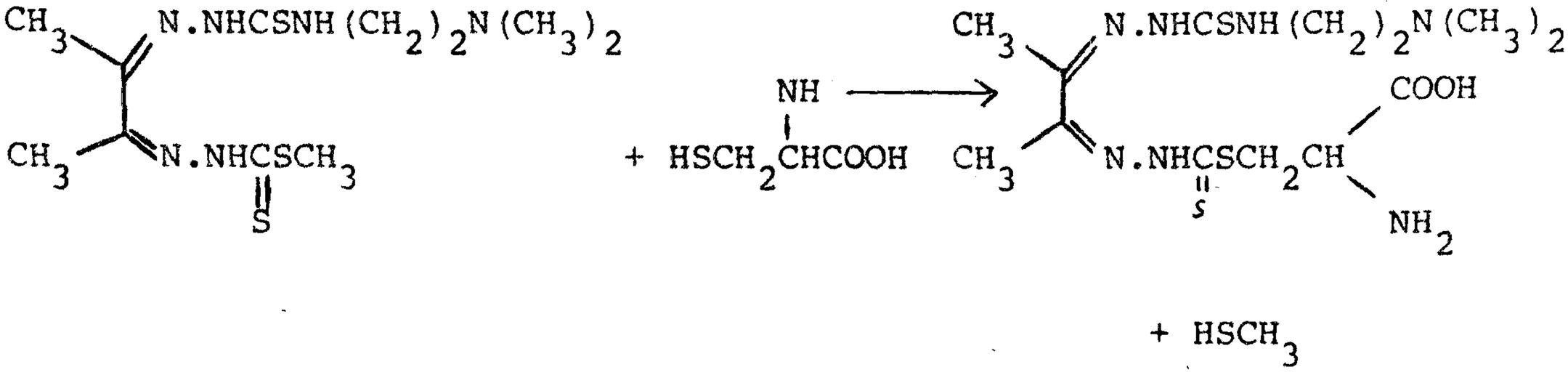
The antagonistic activity of some thiol-containing compounds against the killing activity of TSC1 and TSC2 with T. vaginalis, and of TSC1 with Leishmania mexicana mexicana is of interest, as it may help to elucidate the mode of action of these compounds.

All the thiol-containing compounds found to antagonise thiosemicarbazones had an -SH group and an -NH or $-NH_2$ group (e.g. cysteine: $HSCH_2\underset{\displaystyle NH_2}{\underset{|}{C}}HCOOH$), except for dithiothreitol (DTT) ($HSCH_2(CHOH)_2CH_2SH$) which has two -SH groups. The two compounds tested which did not exhibit antagonistic activity (methionine and thioglycolate) lack an -SH group ($HOOC\underset{\displaystyle NH_2}{\underset{|}{C}}HCH_2CH_2SCH_3$) and an -NH group ($HSCH_3COOH$) respectively.

The thiol compounds interfered with the antitrichomonal activities of both chelate and non-chelate, suggesting that they do not act by inhibiting chelation, although this only holds if both thiosemicarbazone and chelate act by the same mechanism.

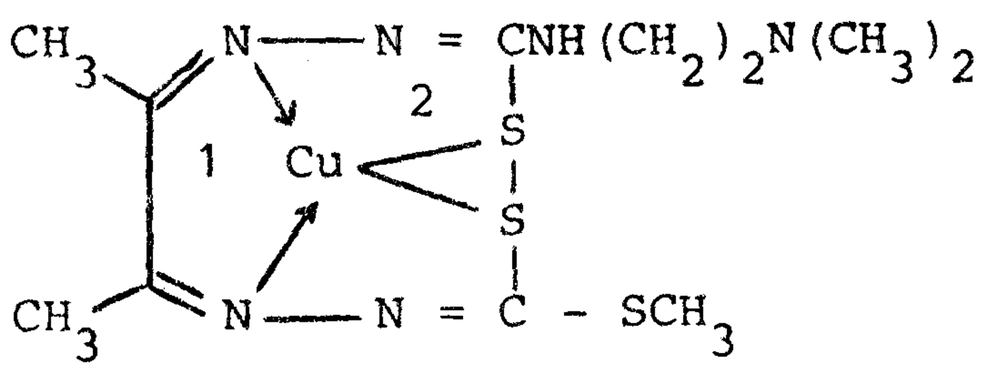
There are several ways in which thiol compounds could act with thiosemicarbazones and chelates. Cysteine might react with TSC1 to give a compound unable to form a chelate (Diagram 1). However, if this did occur, then the antitrichomonal activity of the chelate should not be affected, which it is. In addition, the lack of antagonistic activity of thioglycolate on the killing activity of TSC1 cannot be explained by this process, for it too should react with TSC1 in a similar way to cysteine.

Diagram 1. The possible effect of cysteine upon the structure of a thiosemicarbazone (TSC1).



Chelates could react with cysteine in three possible ways (see diagram 2): The dative bonds between the copper and the nitrogen (position 1) could be broken; the covalent bonds between

Diagram 2. The possible effect of cysteine upon the structure of a thiosemicarbazone: copper chelate (TSC2).



the copper and the sulphur atoms (position 2) could be broken; both the above reactions could occur, probably resulting in the breakdown of the chelate. Thioglycolate is a non-nitrogen thiol, and therefore may be expected to breakdown the covalent bonds between the sulphur and copper of TSC2 (position 2). Thioglycolate does not exhibit

antagonistic activity to the trichomonacidal activity of TSC2, suggesting that breakage of these bonds alone is not sufficient for antagonistic activity. Methionine has no -SH group, but contains an -NH group, and is likely to affect the chelate at position (1). It also does not exhibit antagonism against the antitrichomonal activity of TSC2 however, suggesting that breakage of the dative bonds to the nitrogens is not sufficient for antagonistic activity. All the other thiol-containing compounds (except DTT) contain both a thiol group and an -NH group, and have antagonistic activity. This suggests that only compounds able to hydrolyse at positions (1) and (2) antagonise the activity of TSC2 and that they do so by interfering with the structure of the chelate. The results obtained with DTT do not fit in with this hypothesis. Unlike thioglycolate however, DTT contains two thiol groups and this may affect its reaction with TSC2, so that it has the same effect as thiol- and amine-containing compounds such as cysteine.

These interactions of thiols with thiosemicarbazones and chelates however are only theoretical, and whether they occur has not been experimentally studied in detail. Attempts were made to see if the chelate was broken down upon the addition of cysteine, by looking for changes in the absorption spectrum, but none was observed. Thus it is feasible that the thiols do not react directly on the chelate but rather exert their effect by interferance with its action against cellular components. It is possible that it is the

effectiveness of the compounds at maintaining host enzyme thiol groups reduced that is the most important characteristic of the antagonists.

The antagonistic effect of cysteine upon the killing activity of TSC1 with Leishmania mexicana mexicana shows that the thiol-antagonism exhibited with Trichomonas vaginalis is not a function of this parasite, and that the mode of action of TSC1 in Leishmania mexicana mexicana may be the same as in Trichomonas vaginalis, and probably involves chelation of TSC1 to a transition metal chelate. However, there is no evidence to suggest whether the chelate itself is the active agent or whether the metabolic action of the parasite upon the chelate produces a toxic substance.

In conclusion, the antitrichomonal activity of thiosemicarbazones appears to involve the formation of chelates. Thiol- and amine-containing compounds antagonise the antitrichomonal activity of the thiosemicarbazones and chelates. It is unlikely that this is due to them interfering with the formation of the chelate, but it is possible that they affect the structural integrity of the chelate itself. Structure-activity analysis of the antagonists suggests that the ability to break the dative bonds from the nitrogens of the chelate and of the bonds from the metal to the disulphide bridge between the upper and lower chains of the thiosemicarbazone: copper chelate appears to be necessary for antagonistic activity. There is no experimental evidence that the thiol compounds cause the

breakdown of the chelate however, and it is quite possible that they antagonise its activity in some other way, such as interference with its action against cellular components.

4.5.3. Effect on proteinase activity

Proteinases are highly important enzymes, whose presence has been noted in a wide variety of prokaryotic and eukaryotic organisms (North, 1983). Proteinases are implicated in protein degradation and cell turnover.

Thiol proteinase activity has been previously reported in T. vaginalis (Coombs, 1982) and Tritrichomonas foetus (McLaughlin and Müller, 1979). The proteinase activity of Trichomonas vaginalis clone G3 is given in Table XIX. The enhancement of activity on the addition of dithiothreitol (DTT) has been previously reported (Coombs, 1982; Coombs and North, 1983) and confirms the thiol-dependant nature of at least some of the enzymes. The proteinase activity was not maximal between pH 5.0 and pH 8.0 (Table XX), but the activity increased with increasing acidity. This is unlike the enzyme found in Tritrichomonas foetus, which had a pH 7.0 optimum, but is more like the mammalian thiol proteinase cathepsin B, which has a pH optimum between pH 3.5 and pH 5.0 (Otto, 1971). It must be remembered however, that it is likely that there were several trichomonal proteinases detected using azocasein, and so the results reflect their combined activities.

Proteinase assays were carried out at pH 6.0 and pH 7.5 (Table XIX). These pH's were used for several reasons:

1. The proteinase activity did not have an optimum between pH 5.0 and pH 8.0.
2. pH 6.0 is the initial pH of Trichomonas vaginalis culture medium and may be similar to its normal environment.
3. pH 7.5 is likely to be similar to the physiological pH within the cytosol of the parasite.
4. Coombs (1982) used similar conditions and a comparison could be made with the results of that study.

The results obtained for T. vaginalis proteinase activity in this study were similar to those reported by Coombs (1982) and confirm that the proteinase activity of this species is very high compared with those of many other flagellate protozoa. The exception is the proteinase activity of Leishmania mexicana mexicana amastigotes, which at pH 6.0 was approximately five-fold greater than that of Trichomonas vaginalis (Coombs, 1982). Leishmania mexicana mexicana however, lives in the potentially lethal environment of the parasitrophorous vacuole of the host macrophage, and the high proteinase activity of this parasite may reflect the role these enzymes play in the survival of this parasite. A thiol-proteinase similar to cathepsin B also has been found in the anaerobe Entamoeba histolytica (McLaughlin and Faubert, 1977), although this enzyme was found to have an optimum pH of 6.0-6.3.

The function of the proteinase found in Trichomonas vaginalis is not yet understood. In other organisms, such as Schistosoma mansoni, proteolytic activity is believed to be associated with the parasites ability to penetrate the skin of the definative host (Dresden and Asch, 1972), and a thiol proteinase found in Trypanosoma cruzi may assist this organism to penetrate host cells (Bongeretz and Hungerer, 1978). Since Trichomonas vaginalis is not an intracellular parasite, cell penetration is unlikely to be a function of the thiol proteinase reported here. The proteinase reported in Tritrichomonas foetus (McLaughlin and Muller, 1979) was associated with a population of lysosome-like, posteriorly positional particles, and it was suggested that this proteinase is involved in the breakdown of macromolecules or organisms by the parasite, and perhaps assists in autophagy. Trichomonas vaginalis infections are associated with inflammation of the vaginal mucosa, and in culture T. vaginalis causes degenerative changes in Hela cells and inhibits cell division in fibroblasts (Honigberg and King, 1964). Proteinases secreted by the parasite may be responsible for these manifestations, although there is, as yet, no direct evidence for this.

Leupeptin is a proteinase inhibitor which was first isolated from Streptomyces spp. in culture (Aoyagi, Takeuchi, Matsuzaki, Kawamura, Kondo, Hamada, Maeda and Umezawa, 1969). Leupeptin is now well known to inhibit thiol proteinases, and has been shown to inhibit protein degredation (Ward, Chua, Li, Morgan and Mortimore,

1979; Hopgood, Clark and Ballard, 1977). The screening of actinomyces culture filtrates for papain inhibition led to the discovery of another compound, antipain (Suda, Aoyagi, Hamada, Takeuchi and Umezawa, 1972) which is structurally related to leupeptin. Antipain has been shown to inhibit cathepsin A and B, and through this proteolytic degredation (Leake and Peters, 1981). Leupeptin and antipain were potent inhibitors of Trichomonas vaginalis proteinases (Figure 13), but neither exhibited great trichomonacidal activity, even at concentrations which inhibited proteinase activity by 90% (Table XXI). The insensitivity of T. vaginalis to these compounds suggests that they do not reach the enzymes within the cell.

As the trichomonacidal activity of TSC1 and TSC2 was inhibited by compounds containing thiol groups, and thiol-containing compounds could bind to these drugs (section 4.5.2.), and thiol proteinases are dependant upon thiol groups for activity and exhibit enhanced activity in the presence of thiol-containing compounds such as DTT, it was considered that TSC1 and TSC2 could be interfering with thiol proteinases in the parasite. If this occurred, the lack of thiol proteinase activity may well kill the parasite, or at least be a contributing factor in its death. The chelate (TSC2) was found to inhibit trichomonal proteinases (Table XXI). The concentration of the drug required to give 90% proteinase inhibition, however, was approximately 250 fold greater than that required to kill the parasite. It is possible that the chelate is only poorly taken in to

the parasite, nevertheless these results suggest that thiol proteinase inhibition is unlikely to be the mode of action of the trichomonacidal activity of this compound.

The lack of inhibitory activity by TSC1 against the proteinases reinforces the suggestion given in section 4.5.2. that TSC1 does not bind to thiol-containing compounds.

Since thiol compounds affect the trichomonacidal activity of these thiosemicarbazones, the anatagonism of thiol-containing compounds upon the inhibition of thiol proteinase activity by TSC2 was a distinct possibility. All the thiol-containing compounds tested were found to be antagonistic in this way except for thioglycolate (Table XXII). Methionine also did not antagonise the inhibition. It is of interest to note that all the thiol-containing compounds and methionine, enhanced the thiol-proteinase activity, although thioglycolate and methionine had the least effect. This fits in with the previous observation (section 4.5.2.) that both on -NH and -SH group are necessary for maximal antagonism of the chelate, but in addition does show that compounds such as thioglycolate and methionine have some effect upon the chelate. This was not detected using trichomonacidal activity as a parameter (Table XVII), presumably because growth inhibition was a less sensitive measure and minor effects were less readily detected than with the method involving enzyme inhibition. The results do not distinguish between whether the thiol reagents exert their effect by directly reacting

with the chelate or by binding to the enzymes and so protecting them from interferance by the chelate. Clearly however, reaction with the enzymes is possible, as is shown by the stimulating affect of the thiols on the enzyme activity.

4.5.4. Effect on glycolytic kinases

Thiosemicarbazones have not previously been tested for activity against glycolytic kinases. Most reported work is on the effect of thiosemicarbazones on ribonucleoside diphosphate reductase (Sartorelli, Agrawal and Moore, 1971). The antagonistic activity of thiols on the inhibition of this enzyme from Sarcoma 180 ascites cells as reported by Sartorelli, Agrawal, Tsiftsoglou and Moore (1977) is analagous to the antagonistic effect of thiols upon the trichomonacidal activity of the thiosemicarbazones (TSC1 and TSC2) observed in this study. In 1977, Sartorelli et al., noted that ribonucleoside diphosphate reductase inhibition was more pronounced at low thiol levels and suggested that the thiosemicarbazone binds at, or near to, the site on the enzyme normally occupied by the thiol. This seems a reasonable suggestion, but many enzymes have associated thiol groups and the thiosemicarbazone may have some effect on these also.

Thy glycolytic kinases have been known to have associated thiol groups for some time (Peters and Wakelin, 1946; Ernest and Drobnica, 1980), the thiol groups being essential for these enzymes to function. It has been shown that arsenical compounds inhibit

pyruvate kinase both in vitro and in vivo (Peters, Sinclair and Thompson, 1946) and it has been suggested that the arsenical combines with the enzyme by reacting with its thiol groups (Stocken and Thompson, 1946a). It has also been shown that the addition of thiol compounds protects the pyruvate kinase system from the effect of arsenicals (Stocken and Thompson, 1946b). Antitrypanosomal arsenicals are thought to act through inhibition of the trypanosome pyruvate kinase (Flynn and Bowman, 1974). It therefore seemed reasonable to postulate that thiosemicarbazones, which react with thiol groups, may have a similar effect to arsenicals on the activity of pyruvate kinase and other glycolytic kinases.

The specific activities of hexokinase, pyruvate kinase and phosphofructokinase from T. vaginalis clone G3 cell-free homogenates are given in Table XXIII. All three enzymes were assayed under anaerobic conditions in order to avoid interference from the parasite NADH oxidase activity. The activities of all three kinases were of the same order and greater than the metronidazole reduction rate of this parasite line (Table VI). This was not unexpected as the metronidazole reduction activity is a measure of the parasite pyruvate:ferredoxin oxidoreductase activity. The flux through this enzyme must be lower than, or equal to, that through the glycolytic pathway if carbohydrate is the major energy substrate. Although enzyme specific activities are only general indications of metabolic fluxes in vivo, in this case they apparently correlate quite well with the relative fluxes as judged from considerations of the fate of catabolised glucose.

The glycolytic kinase activities observed in this study were somewhat lower than those reported by Arese and Cappuccinelli (1974). However, these authors assessed enzyme activity in 100,000 g supernatants of T. vaginalis homogenates, under anaerobic conditions. This procedure would remove a lot of the protein from the parasite extract, but as these enzymes are soluble, they would remain in the supernatant. Thus the apparent specific activity in such samples would be greater than that in the homogenate. No reference to the specific activities of these enzymes in T. vaginalis cell-free homogenate could be found in the literature, and so the results presented constitute the first report of T. vaginalis glycolytic kinase specific activities.

The inhibition of pyruvate kinase by arsenical compounds reported by Peters, Sinclair and Thompson (1946) suggested that these compounds may be of interest as antitrichomonal agents. Arsenical compounds have been used in the treatment of trichomoniasis in the past (Michaels, 1968). However, the three arsenical compounds, melarsen (A1), melarsen oxide (A2) and Mel B. (A3) tested in this study, showed poor trichomonacidal activity (Table XXIV). The likely explanations were that either these compounds were non-inhibitory to the parasite kinases or they did not freely enter the cell. A low permeability to arsenicals appears to be the mechanism responsible for several microorganisms being non-susceptible to

those compounds, as well as accounting for resistance of normally sensitive lines e.g. Escherichia coli mutants resistant to arsenate (Bennett and Malamy, 1970) and arsenite resistant Pseudomonas pseudomollei (Arima and Beppu, 1964). In addition, an arsenite-resistance mechanism involving increased thiol-group concentrations was reported by Herington (1959) in the blue tick, and although this method is not postulated here, it again serves to confirm the antagonistic effect of thiols on arsenical activity, and the apparent similarity of the toxic effects of thiosemicarbazones and arsenicals.

Arsenicals are known to have a wide spectrum of activity against glycolytic kinases, from microorganisms to man, thus the lack of inhibition of any of the glycolytic kinases of Trichomonas vaginalis by the arsenicals used was unexpected, but did explain the low toxicity of these compounds to this parasite. This lack of sensitivity of trichomonal enzymes to trivalent arsenicals makes them unusual. Although the molecular basis of the different sensitivities is not known, it is feasible that other inhibitors could be specific for the trichomonal enzymes and so have potential as antitrichomonal drugs.

In contrast to T. vaginalis enzymes, the trivalent arsenical melarsen oxide (A2) inhibited hexokinase and pyruvate kinase from mouse liver supernatant, but had no activity on purified pyruvate kinase from rabbit muscle, confirming the finding of Flynn and Bowman (1974). The lack of activity of the pentavalent arsenical

(compound A1), which did not inhibit any of the glycolytic kinases tested, can be explained as pentavalent arsenicals have no affinity with thiols. Flynn and Bowman (1974) suggested that the in vivo trypanocidal effects of pentavalent arsenicals lies in their activation by reduction, as originally postulated by Crawford (1947). This reductive activation does not take place in cell-free homogenates, which accounts for the lack of inhibitory activity exhibited by melarsen against the glycolytic kinases, as found in this study and by Flynn and Bowman (1974).

The difference in the inhibitory activity of mel B. (A3) between yeast and mammalian hexokinase suggests differences between the enzymes from the two sources, although similar degrees of inhibition of these enzymes by melarsen oxide (A2) would suggest that these differences may not be due simply to the thiols associated with the enzymes. It is possible that the two arsenicals require slightly different conditions at their enzyme binding sites and that these sites differ in the enzymes from the two sources.

The inhibitory activity of the thiosemicarbazone TSC1 on hexokinase from yeast and T. vaginalis (Table XXV and Figures 14 and 16) is not surprising, but the lack of inhibitory activity of the thiosemicarbazone:copper chelate (TSC2) suggests that the trichomonacidal activity of these thiosemicarbazones as described in section 4.5.1., (that is, TSC1 forms a chelate with a transition metal and it is the chelate that is active) is not mediated through

inhibition of this enzyme. The action of TSC1 against hexokinase probably is due to binding of the drug to the thiols associated with this enzyme. The lack of inhibitory activity of TSC1 with mouse liver hexokinase (Table XXVI) suggests that hexokinase from T. vaginalis and from yeast are more similar (at least at their thiol binding sites) to each other than either is to the mammalian enzyme. This confirms the suggestions made above, when discussing lack of activity of arsenicals. The inhibition by TSC2 of T. vaginalis pyruvate kinase and by TSC1 of mouse liver pyruvate kinase, whereas neither affected the other enzyme nor rabbit muscle pyruvate kinase, also indicates differences between trichomonal and mammalian pyruvate kinases. However, the differences in the inhibitors activities against the two mammalian pyruvate kinases suggests that the activity of TSC1 and TSC2 is highly dependant upon the structural conformation of the thiol binding site. If this is so, minor alterations in this site may change dramatically the effectiveness of an inhibitor. Such a situation holds promise for the design of specific antiparasite enzyme inhibitors.

The lack of inhibitory activity of both thiosemicarbazones against phosphofructokinase is similar to the situation with arsenicals. This suggests that either this enzyme is less dependant upon thiol groups for activity, although the low activity of TSC2 with T. vaginalis phosphofructokinase suggests that this enzyme requires thiols, or that stereo hinderance at the thiol binding site

stops these compounds inhibiting the enzyme.

The antagonism of thiol compounds to the inhibitory activity of TSC1 and TSC2 on T. vaginalis hexokinase and pyruvate kinase respectively (Table XXVIII), confirms the mode of action of these compounds with the kinases. The only compound tested that did not antagonise the inhibitory activity was methionine, which does not have a free -SH group.

The speed of the cell death of T. vaginalis in the presence of low concentrations of the thiosemicarbazones (Table XXIX) indicates that they may be inhibiting a system upon which the cell depends on a minute to minute basis. It seems unlikely that inhibition of DNA synthesis, although fatal in the longer term, would kill so rapidly.

The concentration of thiosemicarbazone required to achieve a significant level of kinase inhibition was high, although a similar concentration of the trivalent arsenical melarsen oxide (A2) was required for the inhibition of mammalian hexokinase (Figure 15) and pyruvate kinase (Figure 17), in addition, Flynn and Bowman (1974) reported that similarly high concentrations of melarsen oxide and other trivalent arsenicals were required to inhibit Trypanosoma brucei and Trypanosoma rhodesiense pyruvate kinase, which is thought to be the primary target of arsenicals in trypanosomes. Quite high concentrations of A2 are required to kill tryponosomes in vitro however (Flynn and Bowman, 1974). Nevertheless, recently it has been postulated that thiosemicarbazones suppress energy production (Emery, Stancato, Brown, Prichard and Wolfe, 1982) and glycolytic

kinase inhibition would fulfill this role. However, the MIC of TSC1 and TSC2 were approximately 100 fold lower than the I 50% of TSC1 for hexokinase and of the I 90% of TSC2 for pyruvate kinase, which indicates that inhibition of glycolytic kinases are unlikely to be the primary site of action of these thiosemicarbazones.

4.5.5. Effect of thiol content

The involvement of thiol groups with the activity of thiosemicarbazones and their possible interactions was discussed in sections 4.4.2. and 4.4.4. The total soluble thiol content of T. vaginalis was similar to that found in various bacteria (Fahey, Brown, Adams and Worsham, 1978). The affinity of thiosemicarbazones for thiol groups was illustrated by the drop in thiol content of T. vaginalis cultures incubated with TSC1 and TSC2 (Table XXX). This drop in soluble thiol was transient, however, with the thiol level returning to the control level after 210 minutes in the presence of 50 nmoles TSC1 or TSC2. That this loss was similar to the concentration of TSC1 or TSC2 added to the culture could have been significant, suggesting a 1:1 relationship between thiol and thiosemicarbazone. The initial loss of thiol however, was approximately similar irrespective of whether 50 nmoles or 250 nmoles of TSC1 or TSC2 was added to the culture. In each case thiol levels recovered after this initial loss. Thus, the apparent 1:1 relationship between thiol drop and thiosemicarbazone concentration may simply have been a coincidence.

The transient nature of the loss in thiol content suggests that the parasite can overcome this aspect of the activity of thiosemicarbazones if the drug is at a low concentration. The thiol lost presumably reflects the time taken for the cell to react to the presence of thiosemicarbazone, and produce more thiols. In the experiments carried out, significant numbers of parasites were killed within 210 minutes at the higher (5 μM) drug concentration. However, it was not possible to show a direct relationship between cell death and thiol level. Indeed, it is of interest to note (Table XXX) that after this time period the thiol content of one of the parasite cultures had increased significantly (by 58%) compared to the control culture, perhaps on indication of a parasite defence mechanism against the activity of the thiosemicarbazone. It has been shown that thiols protect the parasite itself from the effects of the thiosemicarbazone, as well as antagonising their effect on proteinase (section 4.5.3.) and glycolytic kinase (section 4.5.4.) activities.

Thus the loss of thiol probably does not in itself kill the parasite. Nevertheless it may play a part in the toxic action of thiosemicarbazones. It has been shown that glutathione deficient mutants of Escherichia coli are more susceptible to a wide range of chemical agents than their parent strains (Apontoweil and Berends, 1975) and these authors postulated that glutathione plays a part in the integrity of the cell membrane. Lowering the soluble thiol content of Trichomonas vaginalis may similarly make the parasite more

susceptible to the toxicity of thiosemicarbazones towards other aspects of cellular metabolism.

4.5.6. Conclusions on the modes of action of antitrichomonal thiosemicarbazones

The antitrichomonal activity of thiosemicarbazones appear to be associated with chelation, as only chelates and compounds capable of forming chelates are highly toxic to T. vaginalis. Thiol compounds antagonise the toxic activity of the thiosemicarbazones, but whether they act by directly interfering with the chelate or by interfering with its action against cellular components have not been elucidated.

The chelate inhibited thiol proteinase activity; and thiol compounds antagonise the inhibition. The anti-proteinase activity of the chelate (TSC2) was observed only at concentrations significantly greater than those required to kill the parasite, and this suggests that thiol proteinase inhibition is unlikely to be responsible for the trichomonacidal effect.

Thiosemicarbazones also inhibited glycolytic kinases and again their activity was antagonised by thiol compounds. The concentrations of the thiosemicarbazones necessary to kill T. vaginalis were significantly lower than those required to inhibit the glycolytic kinases however, suggesting that the inhibition of glycolytic kinase is not the primary mode of action of these compounds.

Incubation of the parasite cultures with both thiosemicarbazones and chelate caused a transient drop in the total soluble thiol level in the parasites, but this could not be correlated to parasite death.

Thiosemicarbazones and their chelates are highly toxic to T. vaginalis, and exert their effect rapidly. The primary mode of action of these compounds appears to involve thiol groups, but is still to be fully elucidated. Nevertheless, the results indicate that they have potential as antitrichomonal compounds.

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