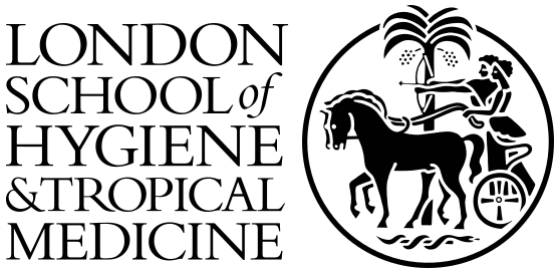

TRANSPORT AND METABOLISM OF POLYAMINES IN *TRYPANOSOMA CRUZI*

A thesis submitted to the Board of Studies in Biochemistry,
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ABSTRACT

The epimastigotes (insect stage) of *Trypanosoma cruzi*, are unable to synthesize *de novo* the diamines putrescine and its analogue cadaverine, from their amino acid precursors. Therefore the metabolic pathways to polyamines (aliphatic bases) in *T.cruzi*, clone X10/6 epimastigotes, have been studied by *in vitro* radiolabelling using these diamine precursors. [^{3}H]Putrescine was rapidly taken up from the medium and incorporated into the polyamines spermidine, spermine and the glutathione-polyamine conjugate N^1,N^8-*bis*(glutathionyl)spermidine (trypanothione). Likewise [^{3}H]cadaverine was rapidly taken up by *T.cruzi* and converted into the analogous polyamines aminopropylcadaverine and *bis*(aminopropyl)cadaverine and the glutathione-polyamine conjugates glutathionylaminopropylcadaverine and N^1,N^9*bis*(glutathionyl)-aminopropylcadaverine (homotrypanothione). Detailed analysis has revealed that *T.cruzi* epimastigotes (clone X10/6) transport exogenous [^{3}H]putrescine and [^{3}H]cadaverine by a rapid, high affinity, temperature dependent, diamine transport system which exhibits saturable kinetics (putrescine K_m = 2.0 μM, V_{max} = 3.3 nmol min^{-1} (10^8 cells)$^{-1}$; cadaverine K_m = 13.4 μM, V_{max} = 3.9 nmol min^{-1} (10^8 cells)$^{-1}$). Diamine transport requires the presence of a proton gradient and thiol groups, does not utilise an amino acid transporter and its activity is altered as the cells proceed through the growth cycle. This transporter shows high specificity for the diamines, putrescine and cadaverine, but low specificity for the polyamines, spermidine and spermine. Hence polyamine metabolism in *T.cruzi* epimastigotes differs from other trypanosomatids (*Trypanosoma brucei*, *Leishmania* and *Crithidia fasciculata*) in three ways. Firstly *T.cruzi* lacks the ability to synthesize diamines *de novo*. Secondly both putrescine and cadaverine are rapidly taken up and can be converted into significant

amounts of spermine and *bis*(aminopropyl)cadaverine respectively. Thirdly *T.cruzi* is able to synthesize homotrypanothione in addition to trypanothione. If the pattern of polyamine metabolism in the mammalian stages of *T.cruzi* is similar to that observed with the epimastigotes, these findings will have important implications with respect to future developmental strategies for the chemotherapy of Chagas' disease.

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TABLE OF CONTENTS

PAGE

LIST OF FIGURES

LIST OF TABLES

FACING PAGE

LIST OF ABBREVIATIONS

Abbreviation	Name in full
ADC	arginine decarboxylase
AdoMet	*S*-adenosylmethionine
AdoMetDC	*S*-adenosylmethionine decarboxylase
ATP	adenosine triphosphate
cAMP	cyclic adenosine monophosphate
CBSS	Carter's basic salts solution
CHOMG	methylglyoxal *bis*(guanylhydrazone) resistant Chinese hamster ovary cells
c-SAT	acetyl-CoA:spermidine/spermine N^1-acetyltransferase
DFMA	DL-α-difluoromethylarginine
DFMO	DL-α-difluoromethylornithine
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
EGF	epidermal growth factor
GTP	guanosine triphosphate
HPLC	high-performance liquid chromatography
IGF	insulin-like growth factor
MDL73811	5'-{[(Z)-4-amino-2-butenyl]methylamino}-5'-deoxyadenosine
MGBG	methylglyoxal *bis*(guanylhydrazone)
mRNA	messenger ribonucleic acid
ODC	ornithine decarboxylase
PSG-BSA	phosphate saline glucose plus 1% bovine serum albumin (fraction V)
RTH+CS	RPMI/Trypticase/haemin plus 10% chicken serum
RTH+FCS	RPMI/Trypticase/haemin plus 10% foetal calf serum
RNA	ribonucleic acid
TCA	trichloroacetic acid
V_{max}app.	the apparent maximum velocity

CHAPTER 1 : INTRODUCTION

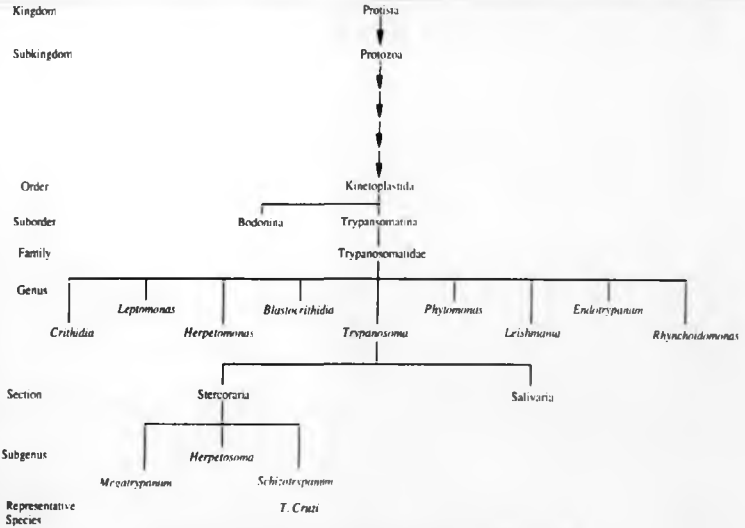


Figure 1.1 Classification of Kinetoplastida with special reference to *Trypanosoma Cruzi*

Table 1.1 Prevalence of some human diseases caused by parasitic protozoa

Disease	Distribution (continents)	Incidence of infection[1] (millions)	People at risk of infection (millions)	Deaths per annum (thousands)	References
Malaria	South and Central America, Asia, Africa, Europe	120	500-2200	500-1200	(WHO, 1992) (WHO, 1993)
Leishmaniasis	South and Central America, Asia, Africa, Europe	12	350	75	(Desjeux, 1992) (WHO, 1993)
Trypanosomiasis:					
South American (*T.cruzi*)	South and Central America	16-18	90	45	(Moncayo, 1992) (WHO, 1993)
African (*T.brucei* group)	Africa	0.015-0.020[2]	50	?	(WHO, 1993) (Kuzoe, 1993)

[1] Number of people infected with the parasite.
[2] Number of reported cases per annum. However the actual number of cases is more likely to be in the region of 200 000 to 300 000 per annum.

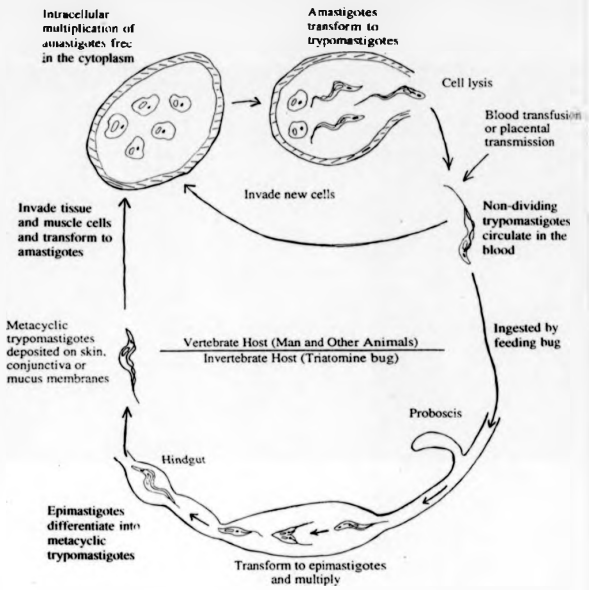


Figure 1.2 The life cycle of *Trypanosoma cruzi*

CHAPTER 1 : INTRODUCTION

1.1 Chagas' disease

1.1.1 Disease burden

Chagas' disease or South American trypanosomiasis is found only in the Americas. It is caused by the protozoan parasite *Trypanosoma cruzi*, which is a member of the Kinetoplastidia (Figure 1.1). According to the 1991-92 World Health Organization report, *T.cruzi* currently infects an estimated 16 to 18 million people in South and Central America with a further 90 million people (some 25% of the population) at risk of infection. The incidence of infection is probably close to one million new cases per year. Mortality due to Chagas' disease is difficult to estimate but is probably responsible for over 45,000 deaths per annum (WHO, 1993). It is a major public health problem in Latin America with the risk of infection being directly associated with socio-economic factors such as poor quality housing in rural areas and unplanned urban development (WHO, 1993), costing governments millions of dollars per annum in both health care costs and lost productivity (Kingman, 1991). In fact in many countries across large parts of Asia, Africa and South America, human diseases caused by parasitic protozoa (Table 1.1), continue to place an enormous burden on the health of the people and hamper development.

1.1.2 Life cycle of *Trypanosoma cruzi*

The life cycle of *T.cruzi* (Figure 1.2) involves the obligatory passage through both vertebrate (man and other animals) and invertebrate triatomine hosts, in a series of different developmental stages. Essentially then *T.cruzi* can be carried by a wide variety of domestic and wild animals, 'reservoir hosts', and is transmitted to humans

by the blood sucking triatomine or 'kissing' bugs through faecal contamination of the bite site or mucous membranes and increasingly via the transfusion of infected blood (Van-Voorhis, 1990; Richman & Kerdel, 1989; Docampo *et al.* 1991; de Castro, 1993; Dusanic, 1991).

1.1.3 Clinical symptoms

About 1-3 weeks after infection with *T.cruzi*, acute symptoms are observed in about 5% of patients, particularly children. These can involve local inflammation, for example a Chagoma or unilateral conjunctivitis (Romaña's sign), and a flu-like illness associated with the initial parasitaemia. These manifestations, which persist for about 1 to 3 months in the absence of treatment, are generally mild except in very young children where fatal myocarditis and meningoencephalitis can occur. Following a latent period which may last 10-40 years, about 10-30% of individuals go on to develop the clinical symptoms characteristic of chronic Chagas' disease, namely cardiac (cardiomyopathy), digestive (megacolon and mega-oesophagus) and neurological disturbances. Patients with severe chronic symptoms become progressively sick and commonly die as a result of heart failure (Van-Voorhis, 1990; Richman & Kerdel, 1989; de Castro, 1993; WHO, 1993).

1.1.4 Existing chemotherapy for Chagas' disease

The present state of chemotherapy is highly unsatisfactory, as no cheap and safe drug exists for the cure of Chagas' disease. Two drugs, the nitroheterocyclic compounds, nifurtimox (3-methyl-4-(5'-nitrofurfurylideneamino)tetrahydro-4*H*-1,4-thiazine-1,1-dioxide) sold under the name Lampit (Bayer company) and benznidazole

(*N*-benzyl-2-nitroimidazole acetamide) sold as Rochagan or Radinil (Roche company) were introduced in the mid 1970's for the treatment of patients with Chagas' disease (Van-Voorhis, 1990; de Castro, 1993). Both drugs are orally active (Gutteridge, 1987) making their administration easier. However long treatment regimes are required of up to 120 days, they are both extremely toxic and they are only of use in the control of acute symptoms (Van-Voorhis, 1990; de Castro, 1993; Gutteridge, 1987). No drugs are available for prophylaxis or the treatment of chronic patients. Furthermore, there is increasing evidence to suggest that autoimmunity, induced by the parasitic infection, is involved in the pathophysiology of the chronic phase (Petry & Eisen, 1989). This makes the urgent need for the development of a cheap and safe chemotherapy for Chagas' disease even more challenging.

1.1.5 An integrated approach to the control of Chagas' disease

In order to achieve the successful control of Chagas' disease an integrated approach is required. Control of the insect vector by use of residual pyrethroid insecticides should be coupled with the testing of all blood prior to transfusion, education, housing improvements and the development of a cheaper and more effective chemotherapy to cut the misery caused by the disease and eliminate the human reservoir of *T.cruzi*.

1.1.5.1 Transmission prevention:

1) Vector control *T.cruzi* is carried by triatomine bugs (Hemiptera, Reduviidae, Triatominae). The most important vectors are *Triatoma infestans*, *Panstrongylus megistus*, *Rodnius prolixus*, *Triatoma brasiliensis* and *Triatoma dimidiata*. The

triatomine bugs have a much longer life cycle and slower rate of reproduction than most insects making them more amenable to control by insecticides (Schofield *et al.* 1987). In the Northern Cone countries (those which lie North of the Amazon) transmission of *T.cruzi* is predominantly by the sylvatic (woodland) vectors *R.prolixus* and *T.dimidiata* making total eradication not feasible. In the Southern Cone countries (Argentina, Brazil, Bolivia, Chile, Paraguay and Uruguay) *T.cruzi* is mainly transmitted by the intra-domiciliary vector, *T.infestans* (with some transmission by sylvatic / domestic vectors *P.megistus* and *T.brasiliensis*) (Schofield *et al.* 1987). Therefore apart from an area of Bolivia where it lives in the wild, *T.infestans* lives almost exclusively in people's houses, making it an easy target for insecticides and total eradication a real possibility (Expanded Program on Immunisation, 1992; Kingman, 1991; WHO, 1993).

A joint initiative has been launched by the Southern Cone countries to eliminate Chagas' disease as a public health problem from these countries by the year 2000 (Expanded Program on Immunisation, 1992). This involves public health education, a massive house and outbuildings insecticide (synthetic pyrethroids) spray program with four years of follow-up entomological surveillance and measures to prevent transmission by blood transfusion (Expanded Program on Immunisation, 1992; Kingman, 1991). It is hoped that sufficient money will be made available to finance the project and that all the countries will be fully commited to the program, otherwise reinfection by migration across borders of neighbouring countries could pose a serious threat to the overall success of the program.

ii) Blood transfusion In endemic areas, infection via blood transfusions can be

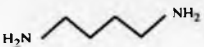
prevented by prior treatment of potentially infected blood with Gentian violet (Van-Voorhis, 1990; Docampo *et al.* 1991). This therapy is not ideal as a frequent blue discolouration of the skin is observed after transfusion making it difficult to spot early signs of anoxia and the gentian violet itself may be mutagenic, carcinogenic or teratogenic (Van-Voorhis, 1990; Richman & Kerdel, 1989). Diagnostic kits are also under development to screen blood for the presence of *T.cruzi* parasites (WHO, 1993).

iii) Placental transmission *T.cruzi* is known to be able to pass across the placenta from mother to child and may occasionally be transmitted through the mother's milk (Schofield *et al.* 1987).

1.1.5.2 Future prospects for chemotherapy

In 1991 a new steering committee on Integrated Chemotherapy for African trypanosomiasis, Chagas' disease and Leishmaniasis (I-CHEM) was established. This resulted in the creation of a Drug Development Group whose aim is to expedite compound development and to identify possible new leads for the chemotherapy of the three diseases (WHO, 1993). The purine analogue allopurinol (Marr *et al.* 1978) and antifungal azoles which inhibit sterol biosynthesis are now undergoing clinical trials for use in treatment of Chagas' disease (WHO, 1993).

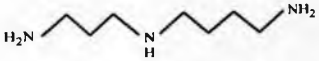
Not surprisingly the pharmaceutical industry has little interest in this area as any research programme would be expensive, time consuming, speculative and unjustifiable on a commercial basis (Gutteridge, 1987). Hence a well managed and efficiently run I-CHEM programme (or a similar type of scheme) may provide the only immediate hope for the development of new drugs to combat Chagas' disease.



Putrescine
(1,4-diaminobutane)

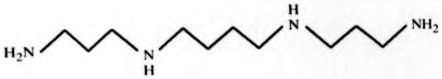
Diamines

Cadaverine
(1,5-diaminopentane)



Spermidine

Polyamines



Spermine

Figure 1.3 Structure of some naturally occurring diamines and polyamines.

1.1.5.3 Rational drug design

Rational drug design involves the identification of an essential enzyme or metabolite, that is present in the parasite, but either absent or sufficiently different in the human host to enable the design of inhibitors which will selectively block this parasitic target molecule. Ideally this will lead to the death of the parasite while at the same time not producing too many unwanted side-effects in the host. This has prompted investigations on the biosynthesis of polyamines in *T.cruzi*, as the polyamine spermidine is used in the production of trypanothione, a novel metabolite of spermidine covalently linked to two glutathiones, which so far appears to be unique to the Kinetoplastidia (Fairlamb & Cerami, 1992). In fact interference with polyamine metabolism has already been identified as a target for drugs in the chemotherapy of protozoal infections (Bacchi *et al.* 1980b; Schechter & Sjoerdsma, 1986). This is discussed in section 1.4 after a general introduction to the topic of polyamines.

1.2 Polyamines: Structure and function

The polyamines are small, nitrogenous aliphatic molecules. The most common naturally occurring polyamines are putrescine, spermidine and spermine (strictly speaking putrescine is a diamine but it is often placed under the general heading of 'polyamines') (Tabor & Tabor, 1984) (Figure 1.3). However many other polyamines and polyamine derivatives can also be formed within certain cells and polyamines are also present in some alkaloids, antibiotics and steroids (Yamakawa, 1986).

The polyamine, spermine (phosphate), was first observed as a constituent of seminal fluid over 300 years ago (Lewenhoeck, 1678), yet despite this and their

ubiquitous distribution among cells (Tabor & Tabor, 1984; Tabor & Tabor, 1985) relatively little is still known about their functions at the biochemical level. However, through the design of specific inhibitors of polyamine biosynthesis and the use of polyamine deficient mutants there is general agreement that polyamines are essential for normal cell proliferation, differentiation and macromolecular synthesis in both prokaryotic and eukaryotic organisms (Tabor & Tabor, 1984; Pegg & McCann, 1988; Marton & Morris, 1987; Pegg, 1986; Tabor & Tabor, 1985). The effects they exert on the cell are influenced by the fact that at a physiological pH the nitrogens of the primary and secondary amine groups are protonated, unlike the point charges of inorganic cations such as Ca^{2+} and Mg^{2+}. Hence polyamines are essentially organic polycations and as such will interact with anionic components of the cell in particular ribonucleic acid (RNA), deoxyribonucleic acid (DNA), phospholipid and adenosine triphosphate (ATP), with most of the polyamines existing as a polyamine-RNA complex in the cells (Watanabe *et al.* 1991). Polyamines mainly associate with these macromolecules (generally in the order spermine > spermidine >> putrescine) through non-covalent electrostatic forces but other interactions (hydrogen bonding and Van der Waals forces) are important for the specificity of binding of polyamines to macromolecules such as transfer RNA (Marton & Morris, 1987; Frydman *et al.* 1992). The binding of polyamines to nucleic acid both stabilises their tertiary structure (Brunton *et al.* 1991) and has a general stimulatory effect on macromolecular (DNA, RNA and protein) synthesis (Tabor & Tabor, 1984; Brunton *et al.* 1991). In particular a specific biochemical role for spermidine in protein synthesis has been identified as the eukaryotic translation initiation factor 5A (eIF-5A) appears to require the amino acid hypusine, formed at a specific lysine residue on the protein through the

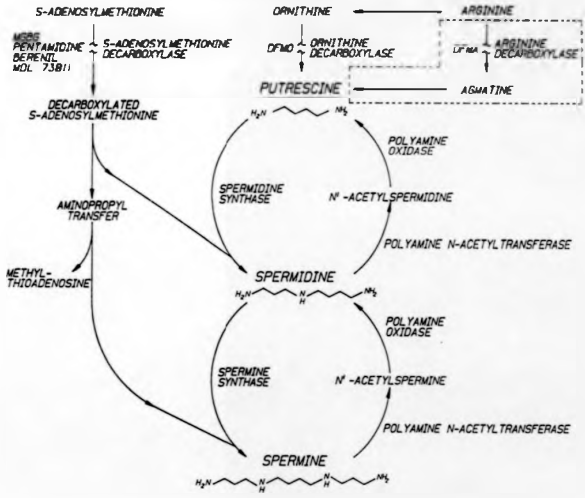


Figure 1.4 A generalised diagram showing the polyamine biosynthetic and retroconversion pathway. Adapted from Pegg and McCann 1988.

contribution of the 4-aminobutyl moiety of spermidine, for its translation initiation activity (Park *et al.* 1991; Park *et al.* 1993). Polyamines can also be covalently cross-linked to proteins at glutamine residues by the action of transglutaminases (Greenberg *et al.* 1991; Folk, 1980). In addition, polyamines, especially spermine, decrease membrane deformability and stabilize the membrane skeleton (Ballas *et al.* 1983), with the omission of polyamines from the growth media of a polyamine-deficient mutant of Chinese hamster ovary cells causing a loss of the actin filaments and microtubule components of the cells cytoskeleton (Pohjanpelto *et al.* 1981). In addition polyamines, in particular spermine, could possibly play a role in inter- and intra-cell communications, as they can modulate the activity of *N*-Methyl-D-Aspartate receptors (Williams, 1994) and appear to stimulate the GTPase activity of purified GTP-binding proteins (Bueb *et al.* 1992).

For most of the functions described here it appears that it is mainly the polyamines spermidine and spermine which are required with relatively little involvement from putrescine. However there are some specific roles for putrescine in bacteria (Munro *et al.* 1972) and mammalian cells (Poulin *et al.* 1991) where an expanded putrescine pool is required for adaption to growth under hypo-osmotic growth conditions.

1.3 Polyamine biosynthesis, retroconversion and regulation

1.3.1 General characteristics

A general scheme is shown (Figure 1.4) depicting the polyamine biosynthetic and retroconversion pathway, together with the enzymes catalysing each reaction and

the sites of action of some inhibitors of the polyamine pathway. Not all organisms carry out all the steps in the pathway. Some common differences that exist are:

1) The reactions converting arginine to putrescine via agmatine (surrounded by a dashed line in Figure 1.4) only occur in plants, bacteria (Kallio *et al.* 1981) and possibly *T.cruzi* (Kierszenbaum *et al.* 1987a; Majumder *et al.* 1992; Yakubu *et al.* 1992).

2) Eukaryotic protozoans such as *Trypanosoma brucei* and *Crithidia fasciculata* (Bacchi *et al.* 1977) and most bacteria (Tabor & Tabor, 1985), with the exception of the acetobacteria (Paulin *et al.* 1983), are unable to synthesize spermine under normal conditions.

3) The retroconversion pathway which converts spermine back to spermidine, and spermidine back to putrescine via the appropriate acetylated intermediates, is found mainly in vertebrates (Seiler, 1987a; Seiler, 1988; Bolkenius & Seiler, 1989; Mondovi *et al.* 1988) although it also occurs in some plants and microbial systems (Tabor & Tabor, 1985; Morgan, 1985; Smith, 1985). Members of the trypanosomatidae do not appear to contain a retroconversion pathway (Bacchi & McCann, 1987; Bacchi & Yarlett, 1993; Majumder & Kierszenbaum, 1993a).

The rest of this section will concentrate in more detail on the pathways to polyamines and their regulation in mammalian cells and the trypanosomatidae.

1.3.2 Polyamines in mammalian cells

1.3.2.1 An overview

A detailed analysis of each of the enzymes in the polyamine pathway is not dealt with here and readers are referred to several reviews which cover this area in

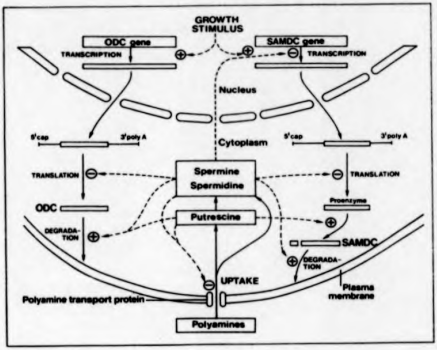


Figure 1.5 Regulation of the polyamine content in a mammalian cell. Taken from Heby and Persson 1990.

some detail (Tabor & Tabor, 1984; Pegg, 1986; Bolkenius & Seiler, 1989; Pegg & McCann, 1992; McCann & Pegg, 1992; Seiler, 1987b). The main aim here is to explore the way in which mammalian cells are able to regulate their polyamine content.

1.3.2.2 Regulation

The widely held view is that the polyamine content of mammalian cells is highly regulated. This appears to be largely achieved by alteration of the activity of three of the enzymes involved in polyamine biosynthesis and retroconversion namely ornithine decarboxylase, *S*-adenosylmethionine decarboxylase and spermidine/spermine N^1-acetyltransferase which all have very short half-lives of less than 1 hour in many cells (Pegg & McCann, 1988). Polyamine uptake, excretion, derivatisation and interconversion also serve to adjust intracellular polyamine levels.

Stimulation of cell growth by a wide variety of growth-promoting stimuli including hormones, growth factors and other drugs, leads to a rapid induction of ornithine decarboxylase (ODC) (Bachrach, 1984) and *S*-adenosylmethionine decarboxylase (AdoMetDC) (Pegg, 1988) gene expression. This, combined with the fast turnover rates of these enzymes, provides the cell with the means of regulating its polyamine content. Polyamines also exert feedback control of their own synthesis at the transcriptional, translational and post-translational levels (Figure 1.5).

Specific regulation of ODC is thought to involve PEST sequences (regions which are rich in the amino acids proline (P), glutamic acid (E), serine (S) and threonine (T)). Pest sequences were originally identified by computer algorithms of proteins which exhibit short (<2h) half-lives (Rogers *et al.* 1986). The most C-terminal

PEST region may in part account for the intracellular instability of mouse ODC as the trypanosomal ODC protein which lacks this PEST region is a much more stable protein (Ghoda *et al.* 1989; Ghoda *et al.* 1990). Another factor in the degradation of the ODC protein is the polyamine induced synthesis and release of a non-covalently bound 22 kDa ODC-inhibitory protein, named antizyme (Fong *et al.* 1976; Heller *et al.* 1976). Antizyme binds to the ODC protein in a region near the N-terminus (Li & Coffino, 1992) and causes a conformational change making the C-terminus more accessible to degradation (Li & Coffino, 1993) by the 26S proteosome (Murakami *et al.* 1992) or the 20S proteosome (Carrera *et al.* 1994). Furthermore distinct regions of the mouse ODC protein are required for constitutive degradation and polyamine-dependent regulation (Ghoda *et al.* 1992).

The AdoMetDC protein, which has a half life of under one hour in mammalian cells (Pegg, 1988; Heby & Persson, 1990), also contains a PEST region, but it is unclear what role it plays in the rapid turnover of this protein (Pegg & McCann, 1992). However the degradation of AdoMetDC is influenced by polyamines (reviewed in (Pegg & McCann, 1992; Pegg, 1986; Pegg, 1988; Heby & Persson, 1990)), with a rise in the intracellular levels of spermidine and spermine leading to an increase in its breakdown whilst putrescine has no effect and may even stabilize the protein. Conversely agents that cause a decrease in polyamines lead to an increase in the amount of active AdoMetDC protein (Pegg, 1984) through increases in the cellular level of AdoMetDC mRNA, in its translation efficiency (ratio of polysomes to monoribosomes goes up) and in the half-life of the mature enzyme (Pegg, 1988; Autelli *et al.* 1991; Persson *et al.* 1989; Pajunen *et al.* 1988; White *et al.* 1990). Spermine primarily affects the translation where as spermidine affects the levels of

AdoMetDC mRNA (Shantz *et al.* 1992). Finally putrescine accelerates the rate of cleavage of the pre-protein to the mature active enzyme (Kameji & Pegg, 1987) and also allosterically stimulates the activity of the mature AdoMetDC protein (Pegg & McCann, 1992), suggesting that ODC is the dominant controlling factor of the whole pathway.

The levels of rat cytoplasmic acetyl-CoA:spermidine/spermine N^1-acetyltransferase (c-SAT) are normally very low but can be induced by treatment of mammalian cells with the polyamines spermidine and spermine, hormones and drugs such as carbon tetrachloride, thioacetamide and methylglyoxal *bis*(guanylhydrazone) (Persson & Pegg, 1984; Seiler, 1987b). The acetylated polyamines formed can then either be removed from the cells by transport and catabolism, or oxidised back to shorter chain diamines and polyamines (Seiler, 1987b).

During this discussion on the regulation of the cells polyamine content, an area which has thus far been totally neglected is the fact that the polyamines spermidine and spermine are the most cationic molecules in the cell (Igarashi *et al.* 1982) and hence most of the polyamines will be bound to (sequestered by) the cells anionic constituents (nucleic acids and phospholipids) (Watanabe *et al.* 1991). Therefore the possibility arises that the polyamine content characteristic of various cell types might largely reflect the constancy of the macromolecules that are titrated by these basic amines.

However, some control of polyamine synthesis is vital, as if the polyamine levels drop too low normal cell growth will be halted (Pohjanpelto *et al.* 1985a; Steglich & Scheffler, 1982) and conversely the presence of too high a levels of spermine is directly or indirectly toxic to the cell (Morris, 1991; Brunton *et al.* 1991).

Table 1.2 The polyamine content of selected trypanosomatidae (Genus: *Leishmania*, *Trypanosoma* and *Crithidia*)

Organism	Stage	Polyamine content nmol (10^8cells)$^{-1}$ PUT	[1]SPD	SPN	Reference
Leishmania					
L.mexicana	P	82	20	[2]-	(Algranati *et al.* 1989)
L.m.amazonensis	P	9	19	3	(Keithly & Fairlamb, 1989)
L.m.mexicana	P	155	70	[3]T	(Bachrach *et al.* 1979)
	P	*120	38	2	(Coombs & Sanderson, 1985)
	A	*13	23	-	
L.braziliensis guyanensis	P	8	11	3	(Keithly & Fairlamb, 1989)
L.major	P	14	16	3	(Keithly & Fairlamb, 1989)
L.tropica major	P	50	70	T	(Bachrach *et al.* 1979)
L.donovani	P	*35	37	-	(Morrow *et al.* 1980)
	A	*2	19	4	(Balana-Fouce *et al.* 1991)
	P	6	13	-	(Coons *et al.* 1990)
		30	50	T	(Bachrach *et al.* 1979)
L.infantum	P	43	11	-	
L.sp.	P	40	190	T	(Bachrach *et al.* 1979)
Trypanosoma					
T.cruzi	E	1(3)	9	3	(Algranati *et al.* 1989)
		7(3)	13	18	(Schwarcz de Tarlovsky *et al.* 1993)
T.brucei	BT	*2	31	-	(Bacchi *et al.* 1977)
		*4	25	-	(Bacchi *et al.* 1979)
		3	12	-	(Fairlamb *et al.* 1987)
		4	17	-	(Berger *et al.* 1993)
T.mega		5	8	-	(Bacchi *et al.* 1977)
Crithidia					
C.fasciculata		59	47	-	(Shim & Fairlamb, 1988)
		29	22	-	(Hunter *et al.* 1991)
		*11	13	-	(Bacchi *et al.* 1977)

Most cells were assayed in mid to late exponential phase of growth. PUT = putrescine, SPD = spermidine, SPN = spermine, P=promastigote, A=amastigote, E=epimastigote, BT=bloodstream trypomastigote. *Polyamine content measured in nmol (mg protein)$^{-1}$; ( ) cadaverine concentration in nmol (10^8cells)$^{-1}$; [1]free spermidine concentration (not bound to glutathione); [2]not detectable; [3]spermine present but in trace amounts only.

In the case of ODC its regulation is both complex and unusual with a universal lack of allosteric (fast) feedback inhibition. Instead slower control mechanisms have been developed, which, with the appropriate modifications, are compatible with periods of rapid polyamine accumulation that may be necessary during growth, development or unusual environmental conditions (Davis *et al.* 1992). All this information highlights the complexity of the polyamine pathway and increases our need to understand how polyamines actually contribute to the growth and general well-being of a cell.

1.3.3 Polyamines in the Trypanosomatidae

1.3.3.1 Polyamine content

In most trypanosomatidae the major diamines and polyamines present are putrescine and spermidine respectively (Bacchi, 1981; Bacchi *et al.* 1977) (Table 1.2). Many of the *Leishmania* species studied also appear to contain trace amounts of spermine (Table 1.2). Some of this could be taken up from the medium but radiolabelling from putrescine indicates that they are able to *de novo* synthesize small amounts of spermine (Bachrach *et al.* 1979). However only in *T.cruzi* epimastigotes does spermine account for a substantial amount of their total intracellular polyamine content (19-44%) (Table 1.2). *T.cruzi* is also unusual as cadaverine (diaminopentane), is present at higher levels than putrescine at all stages of the epimastigote growth cycle (Algranati *et al.* 1989). Cadaverine is also found in certain bacteria, where it is synthesized from lysine via a lysine decarboxylase (Fecker *et al.* 1986; Meng & Bennett, 1992; Yamamoto *et al.* 1991). In fact many microorganisms, for example *Euglena* (Villanueva *et al.* 1980), *Acanthamoeba* (Kim *et al.* 1987) and the fast growing root nodule bacteria (Fujihara & Harada, 1989) contain functional polyamine

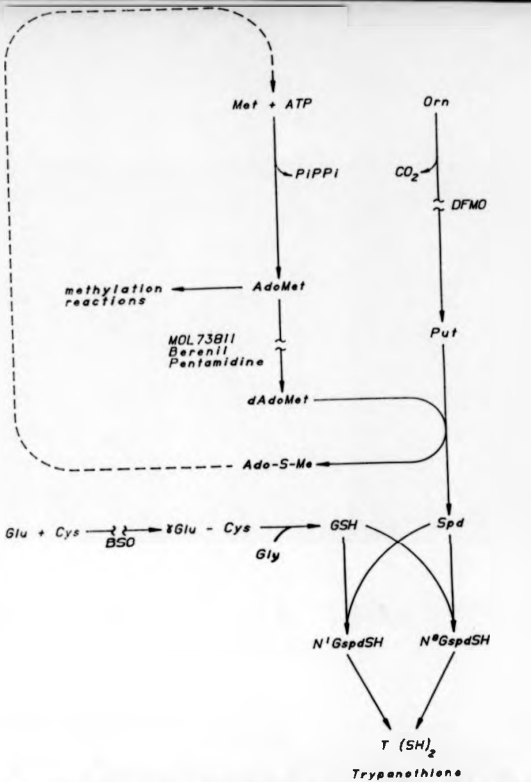
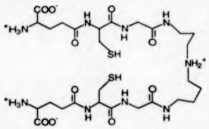
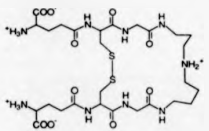


Figure 1.6 The biosynthesis of trypanothione. Adapted from Fairlamb 1989a. Met = methionine, ATP = adenosine triphosphate, Ornithine = 5-adenosylmethionine, dAdoMet = decarboxylated S-adenosylmethionine, Ado-S-Me = methylthioadenosine, Orn = ornithine, Put = putrescine, Spd = spermidine, Glu = glutamic acid, cys = cysteine, γGlu-Cys = γ-glutamyl cysteine, Gly = glycine, GSH = glutathione, N^1GspdSH = N^1-glutathionylspermidine, N^8GspdSH = N^8-glutathionylspermidine, BSO = buthionine sulphoximine, DL-α-difluoromethylornithine = DFMO.



Trypanothione ($T[SH]_2$)



Trypanothione disulphide ($T[S]_2$)

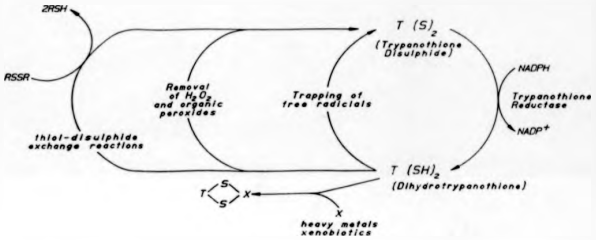


Figure 1.7 The structure and some possible functions of trypanothione.

analogues of putrescine, spermidine and spermine. These either replace or supplement the usual polyamines.

1.3.3.2 Trypanothione

In addition to the common polyamines (and cadaverine in *T.cruzi*), members of the trypanosomatidae contain (N^1,N^8-*bis*(glutathionyl)spermidine) commonly called trypanothione, consisting of two glutathione residues which are covalently linked via the carboxyl groups of the glycines to the terminal amino groups of a molecule of spermidine (Fairlamb *et al.* 1985; Fairlamb *et al.* 1986). Trypanothione is maintained in the reduced state by the action of the NADPH-dependent flavoprotein, trypanothione reductase (Shames *et al.* 1986). Together they preserve an intracellular reducing environment in the cell and act as a defence against damage by oxidants and heavy metals (Fairlamb & Cerami, 1992), replacing the functions of glutathione and glutathione reductase which are found in mammalian cells (Walsh *et al.* 1991; Zappia & Pegg, 1988; Fairlamb, 1991; Fairlamb, 1988) (Figures 1.6 and 1.7). Furthermore, several known trypanocidal drugs appear to inhibit various steps in the pathway (refer to section 1.4.3). Coupled with the fact that the trypanothione system is absent in human cells this makes it a good potential target for rational drug design. However it still needs to be shown (for example by knocking out the trypanothione synthetase or reductase gene) that the trypanothione system is essential for the parasites survival.

1.3.3.3 Regulation

Trypanosomatid ODC and AdoMetDC which lack PEST sequences do not turnover rapidly (Wang, 1991). Generally the polyamine levels in the

trypanosomatidae do not appear to be under such tight regulation as found in mammalian cells. For example, *T.b.brucei* ODC unlike its mammalian counterpart is unresponsive to polyamine levels. This could be because it lacks a polyamine-dependent regulatory site found in mouse ODC (Ghoda *et al.* 1992). However the possibility arises in the trypanosomatidae that excess spermidine could be stored in the form of glutathionylspermidine, ready for conversion back to free spermidine when required by the cell, for example when the organism encounters favourable growth conditions. So far this has only been found to be the case in *C.fasciculata* (Shim & Fairlamb, 1988).

1.4 Prospects for chemotherapy using agents which interfere with polyamine metabolism

As polyamines are required for cell growth, interference with polyamine metabolism has been identified as a potential anticancer (Pegg, 1988; Porter & Sufrin, 1986) and antiprotozoal (Bacchi *et al.* 1980b; Schechter & Sjoerdsma, 1986) chemotherapeutic strategy. Over the past 15 years a lot of effort has gone into the design of specific inhibitors of the polyamine pathway, initially as potential anticancer agents. This work has been summarized in several reviews and will not be dealt with in any detail here (Pegg, 1988; Porter & Sufrin, 1986; McCann & Pegg, 1992). Instead, in this review I will deal mainly with the effects of inhibitors of polyamine and trypanothione metabolism on the growth and polyamine content of the trypanosomatidae.

1.4.1 Inhibitors of ornithine and arginine decarboxylase

Since ODC is seen as a key enzyme in polyamine biosynthesis, much effort has been focused on the design of specific inhibitors of this enzyme. The most successful and widely used is the irreversible enzyme-activated inhibitor DL-α-difluoromethylornithine (DFMO) (Bey *et al.* 1987). Clinically DFMO was initially tested against a wide variety of tumours, with generally very disappointing results (Porter & Janne, 1987; McCann & Pegg, 1992). Subsequently it was found to be curative in the treatment of late stage, arsenical refractory, West African trypanosomiasis (Gambian sleeping sickness) (Van Nieuwenhove *et al.* 1985). DFMO represents the first new drug licenced in over 40 years for the treatment of West African sleeping sickness, which is caused by infection with the trypanosome *T.b.gambiense* (Schechter & Sjoerdsma, 1986; Schechter *et al.* 1987). In a summary of 711 cases treated with DFMO (to March 1991), the overall efficacy of the drug was 85-90%, with a relapse rate of 5.3% and deaths in 7% of patients during treatment (Van Nieuwenhove, 1992).

The exact basis for DFMO's selective toxicity towards *T.b.gambiense* is uncertain as DFMO affects many of the same aspects of parasite and host metabolism including bringing about the inactivation of ODC, a reduction in the levels of putrescine and spermidine , leading to an overall decrease in macromolecular synthesis (Bacchi & McCann, 1987; Bitonti *et al.* 1988). However many differences also exist in the influence DFMO exerts over host and parasite metabolism. Some reasons why DFMO may be effective against *T.b.gambiense* are:

1) The long half-life and slow turnover of African trypanosomal ODC compared to mammalian ODC (Ghoda *et al.* 1990).

2) A large increase in *S*-adenosylmethionine and decarboxylated *S*-adenosylmethionine concentrations, resulting in the potential for hypermethylation (Bacchi & McCann, 1987; Fairlamb *et al.* 1987; Yarlett & Bacchi, 1987; Byers *et al.* 1991).

3) A reduction in the content of glutathionylspermidine and trypanothione, which may compromise the parasites ability to cope with oxidative stress (Fairlamb *et al.* 1987).

4) In contrast to mammalian cells (Pegg, 1988), the African trypanosomes have a limited ability to transport putrescine and spermidine, a lack of spermine synthesis and an apparent absence of the retroconversion pathway (Bacchi & McCann, 1987; Bacchi & Yarlett, 1993). This may contribute to DFMO's selectivity, as the failure to deplete spermine in mammalian cells appears to be a major reason for the lack of success of DFMO in cancer chemotherapy (Pegg, 1988; Porter & Sufrin, 1986; Janne *et al.* 1991).

5) Leads to a general decrease in protein synthesis, including a concomitant reduction in the synthesis of variant-specific glycoprotein. This may then prevent the trypanosomes from undergoing antigenic variation, enabling the immune system to mount a better antibody response against the trypanosome, thus rendering DFMO more effective in clearing the infection (Bitonti *et al.* 1988).

6) Morphological changes also take place including the development of multinucleate or multikinetoplastic forms and the production of non-replicating stumpy forms (Bacchi & McCann, 1987; Giffin & McCann, 1989).

In contrast, DFMO monotherapy is not effective clinically against East African (Rhodesian) sleeping sickness caused by *T.b.rhodiense* (Van Nieuwenhove, 1992) and has shown only limited promise against some *Leishmania* species in *in vivo* model systems (Keithly & Fairlamb, 1989). *T.cruzi* is relatively insensitive *in vitro* or *in vivo*

to DFMO (Schwarcz de Tarlovsky *et al.* 1993; Hunter *et al.* 1994; Hanson *et al.* 1982), but pre-treatment of macrophages with DFMO does impair their ability to ingest *T.cruzi* (Kierszenbaum *et al.* 1987b). However, inhibitors of arginine decarboxylase, for example DL-α-difluoromethylarginine (DFMA), at very high concentrations (12-50mM), appear to decrease the capacity of *T.cruzi* to infect and multiply within mammalian cells (Kierszenbaum *et al.* 1987a) but do not affect trypomastigote-amastigote transformation (Yakubu *et al.* 1992). DFMA also decreases the growth of *T.cruzi* epimastigotes (Schwarcz de Tarlovsky *et al.* 1993). These effects can be overcome by the addition of exogenous agmatine or putrescine (refer to Figure 1.4 for pathway details). Although this strongly suggests the presence of ADC rather than ODC as a source of polyamines precursors in *T.cruzi*, initially neither ADC nor ODC activity could be detected in cell extacts containing up to 2.4 x 10^9 trypanosomes (McCann *et al.* 1988) and radiolabelling epimastigotes with [^{14}C]ornithine or [^{14}C]arginine also failed to demonstrate any putrescine formation (Algranati *et al.* 1989). Recently trace amounts of ADC activity (11.5 pmol CO_2 mg protein^{-1} h^{-1}) and slight (<0.1%) conversion of radiolabelled arginine to agmatine, putrescine and spermidine was observed in mixed trypomastigote / amastigote preperations but this required high cell numbers, (0.3 x 10^9 ml^{-1} for radiolabelling and 3 x 10^9 ml^{-1} for ADC activity), and long, 6-7h, incubation times (Majumder *et al.* 1992). Thus ADC, which is normally only found in prokaryotic and plant cells (Tabor & Tabor, 1984), could represent a selective target for chemotherapy. However the millimolar concentrations of ADC inhibitors (DFMA) required to slow *T.cruzi's* growth, compared to the very low (10nM-10μM) concentrations of DFMA needed to completely inhibit bacterial ADC (Kallio *et al.* 1981), coupled with the enormous

difficulty involved in detecting even trace amounts of ADC activity in *T.cruzi* cells, leads one to question the use of ADC as a target enzyme for the chemotherapy of *T.cruzi* infections. Furthermore it opens up the possibility that inhibition of ADC may not be DFMA's primary mode of action in *T.cruzi*.

1.4.2 Inhibitors of *S*-Adenosylmethionine biosynthesis and metabolism

The trypanosomal AdoMetDC, which decarboxylates *S*-adenosylmethionine (AdoMet) and so commits it to use in polyamine biosynthesis, differs from its mammalian counterpart in solubility, ionic properties, inability to cross react with human AdoMetDC antiserum and a different subunit structure (Tekwani *et al.* 1992) making it a potential target for trypanocidal agents. In fact trypanosomal AdoMetDC is reversibly inhibited by the anti-leukaemic agent methylglyoxal *bis*(guanylhydrazone) (MGBG) and the trypanocidal agent pentamidine, and irreversibly by the trypanocide Berenil (Bitonti *et al.* 1986). However it has not yet been established to what extent the inhibition of AdoMetDC contributes to their antiparasitic actions since all three have been found to interfere with many other cell functions (Williams-Ashman & Seidenfeld, 1986; Newton & Le Page, 1968; Bacchi *et al.* 1980a). The design of specific inhibitors of AdoMetDC has lagged behind that of ODC, but recently a group of AdoMet analogues have been synthesized which are enzyme-activated inhibitors of AdoMetDC (Casara *et al.* 1989). One of these agents, 5'-{[(Z)-4-amino-2-butenyl]methylamino}-5'-deoxyadenosine (MDL 73811) proved active at low doses against model *T.b.brucei* and *T.b.rhodesiense* infections (Bitonti *et al.* 1990; Bacchi *et al.* 1992) and also reduced the capacity of *T.cruzi* to infect and multiply within rat heart myoblasts (Yakubu *et al.* 1993). MDL 73811 blocked *T.b.brucei* AdoMetDC

activity (K_i 1.5μM), leading to a reduction in spermidine (50%) and increase in putreacine levels (Bitonti *et al.* 1990). MDL 73811 also causes a 20-fold increase in AdoMet levels in *T.b.brucei*, after just 1h of exposure *in vivo*, but produces less than a 2-fold rise in AdoMet levels in mammalian cells which have been cultured with the drug for 6h (Byers *et al.* 1991). This could be due to the fact that *S*-adenosylmethionine synthetase is much less sensitive to inhibition by the product (AdoMet) than its mammalian counterpart (Yarlett *et al.* 1993). Other trypanocidal agents such as DFMO, also bring about large increases in AdoMet levels (Byers *et al.* 1991) and the addition of exogenous AdoMet in place of MDL 73811 also reduces the ability of *T.cruzi* to infect mammalian cells (Yakubu *et al.* 1993). AdoMet, in addition to its use as an aminopropyl group donor in polyamine biosynthesis, is utilized as a methyl group donor in cellular methylation reactions (Ueland, 1982; Yarlett & Bacchi, 1987; Avila & Polegre, 1993). These large increases in AdoMet, for example on DFMO treatment of *T.b.brucei*, lead to an increase in the cell's methylation index (ratio of AdoMet:*S*-Adenosylhomocysseine) (Yarlett & Bacchi, 1987) and an accompanying rise in protein methylation (Yarlett *et al.* 1991; Bacchi *et al.* 1992; Bacchi *et al.* 1992) which in turn may lead to abberent methylation reactions within the cell (Ueland, 1982). It is possible then that major changes in AdoMet levels, rather than changes in polyamine levels, are responsible for the antitrypanosomal effects of these drugs.

1.4.3 Interference with trypanothione metabolism

The importance of the trypanothione system is underlined by the fact that a number of existing drugs interfere with trypanothione metabolism (Figure 1.6). In

addition to the inhibitors of polyamine biosynthesis outlined above (sections 1.4.1 and 1.4.2) which disrupt spermidine and hence trypanothione formation, buthionine sulphoximine inhibits glutathione formation (Arrick *et al.* 1981), redox-cycling compounds such as nifurtimox probably swamp the parasite's ability to deal with oxidative stress (Henderson *et al.* 1988) and aromatic trivalent arsenicals sequester dihydrotrypanothione as the dithioarsane adduct MelT (Fairlamb *et al.* 1989b), which in turn is a competitive inhibitor of trypanothione reductase (Fairlamb *et al.* 1989b; Fairlamb *et al.* 1992). Thus the marked synergism seen between DFMO and trivalent arsenicals (Jennings, 1990) could result from their combined action in lowering trypanothione levels.

1.4.4 Interference with polyamine regulation

N,N'-Di-substituted tetraamines with the general formula $RNH(CH_2)_xNH(CH_2)_yNH(CH_2)_xNHR$ have been designed with the aim of interfering with the biosynthesis and function of natural polyamines whilst not substituting for their proliferative functions (Porter & Bergeron, 1988; Baumann *et al.* 1990). In the trypanosomatidae, *bis*(benzyl)- and *bis*(thiophene)- substituted polyamine analogues have been shown to have anti-*T.cruzi* activity (Majumder & Kierszenbaum, 1993b; Majumder & Kierszenbaum, 1993a) whilst the former exhibit anti-leishmanial activity (Baumann *et al.* 1990). Relatively little is known about the mode of action of these analogues but work in mammalian cells with the *bis*(ethyl)-substituted polyamine analogues, suggests that not only polyamine depletion but also the accumulation of these polyamine analogues, leading to both structural and functional alterations in the mitochondrion, is involved in their ability to inhibit cell growth (Fukuchi *et al.* 1992;

Table 1.3 Characteristics of the different polyamine uptake systems found in mammalian cell lines.

Cell type	K_m(μM) Put	Spd	Spn	Transporter	Energy dependence	Other characteristics [1]Na+	[2]SH	[3]AA	Reference
Normal									
Fibroblast (human)	1.1	[4]nd	nd	Single	Yes	nd	nd	No	(Pohjanpelto, 1976) (DiPasquale *et al.* 1978)
(Swiss 3T3)	10-14	nd	nd	nd	nd	nd	nd	nd	(Bethell & Pegg, 1981)
Hepatocyte (mouse)	8-11	nd	nd	nd	nd	nd	nd	nd	(Martin *et al.* 1990)
(rat)	nd	nd	20	nd	Yes	Yes	nd	nd	(Auberger *et al.* 1983)
Mammary gland (mouse)	nd	47.6	nd	Single	Yes	nd	nd	No	(Kano & Oka, 1976)
Enterocyte (rat)	12.3	2.51	nd	Multiple	Yes	No	nd	No	(Kumagai *et al.* 1989) (Kumagai & Johnson, 1988)
Adrenocortical (bovine)	10	nd	nd	Multiple	Yes	Yes	No	No	(Feige & Chambaz, 1985)
Embryonic palate mesenchymal (mouse)	5.8	nd	nd	Single	Yes	Yes	Yes	No	(Gawel-Thompson & Greene, 1988)
Endothelial, aortic (pig)	9	0.6	nd	Multiple	Yes	Yes	Yes	No	(Bogle *et al.* 1994)
Lymphocytes (bovine)	3.7	0.4	0.2	Single	Yes	No	Yes	No	(Kakinuma *et al.* 1988)
Endothelial, umbilical-vein (human)	3.0	0.8	0.5	Multiple	Yes	Yes	Yes	nd	(Rajanayagam *et al.* 1992)
	3.0	0.7	1.0	Multiple	Yes	Yes	Yes	nd	(Morgan, 1992)
Macrophage, pulmonary alveolar (rabbit)	2.1	0.2	nd	Single	Yes	nd	nd	nd	(Saunders *et al.* 1989)
Pulmonary, epithelial Type II (rat)	nd	0.5	nd	Single	Yes	Yes	nd	No	(Kameji *et al.* 1989)
	nd	0.6	nd	Multiple	nd	Yes	nd	nd	(Ranneis *et al.* 1989)
Perfused lung (rat)	14	nd	nd	nd	nd	nd	nd	nd	(Wyatt *et al.* 1988)

Ovary (Chinese hamster)	6.2	6.3	1.0
Erythrocyte (human)	37	1.6	nd
(human)	nd	nd	nd
Transformed			
ADJ/PC6 Plasmacytoma (mouse)	nd	0.3	nd
NB15 Neuroblastoma (mouse)	2.8	nd	nd
AR4-2J Pancreatic acinar (rat)	3.1	0.4	nd
PC-3 Prostatic carcinoma (human)	3.3	nd	nd
L1210 Leukemia (mouse)	nd	1.6	0.7
(mouse)	8.5	2.2	1.6
(mouse)	nd	2.5	nd
P388 Leukaemia (mouse)	nd	0.9	nd
K562 Leukaemia (human)	6.9	8.0	nd
C6 Glioma (mouse)	nd	1.2	nd
U251 Glioma (mouse)	nd	1.0	nd
Balb/c 3T3 Normal embryonic (mouse)	nd	0.9	nd
SV40 Transformed Balb/C 3T3 (mouse)	nd	2.6	nd

[1]Na^+-dependent; [2]requires thiol groups for maximal activity;

Multiple	Yes	nd	nd	No	(Byers *et al.* 1987)
nd	Yes	nd	nd	nd	(Moulinoux *et al.* 1984)
Single	Yes	Yes	Yes	No	(Khan *et al.* 1989a)
nd	nd	nd	nd	nd	(Holley *et al.* 1992)
Single	Yes	Yes	Yes	Yes	(Rinehart & Chen, 1984) (Chen & Rinehart, 1981)
Multiple	Yes	No	nd	No	(Nicolet *et al.* 1990)
nd	nd	nd	nd	nd	(Heston *et al.* 1987)
nd	nd	nd	nd	nd	(Kramer *et al.* 1993)
Single	nd	nd	nd	nd	(Porter *et al.* 1984)
Single	nd	Yes	No	Yes	(Khan *et al.* 1990)
Single	nd	Yes	Yes	No	(Khan *et al.* 1990)
nd	nd	nd	nd	nd	(Khan *et al.* 1994)
Single	nd	Yes	Yes	Yes	(Khan *et al.* 1990)
Single	nd	Yes	Yes	No	(Khan *et al.* 1990)
Single	nd	Yes	Yes	No	(Khan *et al.* 1990)
Single	nd	Yes	Yes	No	(Khan *et al.* 1990)

[3]interaction with amino acid transport systems; [4]nd = not determined.

Table 1.4 Characteristics of some polyamine uptake systems found in fungi, bacteria and kinetoplastids

Organism	K_m(μM) Put	Spd	Spn	Transporter	Energy dependence	Other characteristics [1]Na^+	[2]SH	[3]AA	Reference
Saccharomyces cerevisiae (vacuolar membrane)	2000	700	2000	Single	Yes	No	[4]nd	No	(Kakinuma *et al.* 1992)
Aspergillus nidulans (mycelium)	1200	4000	1030	Multiple	Yes	nd	nd	nd	(Spathas *et al.* 1982)
Neurospora crassa (mycelium)	600	240	70	Single	Yes	No (inhibits)	nd	Yes	(Davis & Ristow, 1988)
Dictostelium discoideum (myxamoebae)	9.1	nd	nd	Multiple	Yes	nd	nd	nd	(Turner *et al.* 1979)
Escherichia coli	0.2	<0.8	>1	Multiple	Yes (partially)	nd No	nd	nd	(Tabor & Tabor, 1966) (Kashiwagi *et al.* 1986)
Leishmania infantum (promastigotes)	1.1	nd	nd	Multiple	Yes	nd	nd	Yes	(Balana-Fouce *et al.* 1989)
Leishmania mexicana mexicana (promastigotes)	10.7 (31)	nd	nd	nd	Yes	nd	Yes	nd	(Gonzalez *et al.* 1992) (Gonzalez *et al.* 1993)
Crithidia fasciculata	66 (23)	nd	nd	nd	Yes	nd	nd	nd	(Gonzalez *et al.* 1992) (Gonzalez *et al.* 1993)
Trypanosoma cruzi (epimastigotes)	5.7 (6.1)	nd	nd	Multiple	Yes	nd	Yes	Yes	(Gonzalez *et al.* 1992) (Gonzalez *et al.* 1993)

[1]Na^+-dependent; [2]requires thiol groups for maximal activity; [3]interaction with amino acid transport systems; [4]nd = not determined.

He *et al.* 1994; Snyder *et al.* 1994; Ghoda *et al.* 1992).

1.4.5 Potential problems with this approach

One of the major problems which has hampered the effectiveness of many inhibitors of polyamine biosynthesis is that many cells are able to get round the block by taking up polyamines they require for growth from their surrounding environment. In this respect African trypanosomes are somewhat of an exception as they are unable to take up significant exogenous polyamines (Bacchi & Yarlett, 1993) and so this might in part explain why DFMO is so effective against them but much less useful against many other cell types including *T.cruzi*. The next section examines in some detail how polyamines are taken up into cells.

1.5 Polyamine Uptake

1.5.1 General Characteristics

Polyamine uptake has been studied in numerous mammalian cell lines, both normal and transformed, and in a variety of other organisms. A summary is given of the main characteristics of polyamine uptake in mammalian cells (Table 1.3) some fungi, bacteria and trypanosomatids (Table 1.4). In most of the systems studied, polyamines can enter the cell via either single or multiple uptake systems which exhibit saturable, Michaelis-Menten-type kinetics with Michaelis constants (K_m's) ranging from 0.2 to 50 μM (Tables 1.3 and 1.4). Yeasts appear to be an exception with K_m values in the high micromolar to millimolar range (Kakinuma *et al.* 1992; Spathas *et al.* 1982). The word 'transport' has been avoided as many of the examples

detailed (Tables 1.3 and 1.4) have not considered the possibility of metabolism of the diamine or polyamine label once it is inside the cell. For example, with putrescine there was no appreciable metabolism (<5%) of the label taken up by fibroblasts (Pohjanpelto, 1976), adrenocortical cells (Feige & Chambaz, 1985) and lymphocytes (Kakinuma *et al.* 1988) where-as there was considerable metabolism (>30%) of the same label by *A.nidulans* (Spathas *et al.* 1982) and pancreatic acini (Alves *et al.* 1992) over the time course in which the measurements were made. Hence it might be more appropriate to talk about the 'uptake' of diamines and polyamines as this will encompass both the transport of these molecules into the cell and any subsequent metabolism components. Hence in cases where there is significant metabolism of the radiolabel the K_m values determined would not represent the true value for the transporter but also encompass the K_m's of the enzymes involved in the radiolabels subsequent metabolism. Some arguments concerning the transport versus metabolism of molecules in cultured cells are dealt with in some detail elsewhere (Wohlhueter & Plagemann, 1989) and will not be discussed any further here.

1.5.2 Energy dependency

Polyamine uptake appears to be an energy-dependent process as judged by one of three criteria. First the rate of polyamine uptake is temperature dependent in a wide range of organisms including various mammalian cell types (Pohjanpelto, 1976; Kumagai & Johnson, 1988; Bogle *et al.* 1994), yeast cells (Kakinuma *et al.* 1992), bacteria (Tabor & Tabor, 1966) and trypanosomes (González *et al.* 1993; Balana-Fouce *et al.* 1989). For example, in fibroblasts the rate of 100 μM putrescine uptake is 30-fold higher at 37°C than at 5°C (Pohjanpelto, 1976). Killing of the cells

by heating to 65°C or lysing them in water for 1 h prior to incubation with putrescine, reduced the amount of putrescine associated with the cell remnants to only 1-2% of the untreated controls. Therefore there appears to be relatively little non-specific binding of putrescine to the cells. In red blood cells (erythrocytes) the initial velocity of spermidine uptake is 15 times greater at 37°C than at 4°C (Khan *et al.* 1989a). In addition when erythrocytes are incubated at 37°C the polyamines are located mainly in the hemolysate with only about 5% associated with the stromata membranes, whilst at 4°C there is a greater than seven fold reduction in binding to the hemolysate with the amount bound to the stromata remaining constant. This suggests that it is the internalization process which is energy dependent rather than binding *per se* (Moulinoux *et al.* 1984).

Second polyamines appear to be concentrated several fold intracellularly in a wide range of organisms. However caution must be exercised here because in many cases this could at least in part be due to their sequestration by anionic molecules such as nucleic acids within the cell (Braunlin *et al.* 1982). Treatment with butanol or toluene disrupts permeability barriers without causing cell lysis, releasing free ionised polyamines but leaving those bound to intracellular sites such as nucleic acids (Tabor & Tabor, 1966; Kakinuma *et al.* 1988; Pohjanpelto, 1976). If one accepts that the quantity of polyamines released on butanol permeablisation is representative of the cells free polyamine content, then active transport is occurring if this exceeds the exogenous polyamine content. This has been demonstrated for *E.coli* (Tabor & Tabor, 1966), fibroblasts (Pohjanpelto, 1976), lymphocytes (Kakinuma *et al.* 1988) and *N.crassa* (Davis & Ristow, 1988). Another indication that active transport is occurring is that the incorporated polyamines are not effluxed (exchanged) on addition of a vast

excess of unlabelled polyamine, for example in human fibroblasts (DiPasquale *et al.* 1978), bovine lymphocytes (Kakinuma *et al.* 1988), mouse hepatocytes (Martin *et al.* 1990) and B16 melanoma cells (Minchin *et al.* 1991).

Third, there is a reduction in the rate of diamine and polyamine uptake in response to metabolic inhibitors such as 2,4-dinitrophenol and KCN (González *et al.* 1992; Kano & Oka, 1976; Kumagai *et al.* 1989; Kumagai & Johnson, 1988; Tabor & Tabor, 1966; González *et al.* 1993; Balana-Fouce *et al.* 1989; Bogle *et al.* 1994; Alves *et al.* 1992; Morgan, 1990a; Davis & Ristow, 1988; Munro *et al.* 1974). These metabolic inhibitors affect oxidative phosphorylation, leading to a reduction in cellular ATP which would in turn affect many cell processes, not just the transport of diamines and polyamines into the cell.

However the difficulty with these studies is that they do not indicate whether this energy dependence is exerted via a direct or indirect action on the transporter and they may be further complicated by metabolism or intracellular sequestration of the radiolabel.

1.5.3 Sodium dependency

As can be seen from Table 1.3 many cell types appear to exhibit sodium-dependent (Na^+-dependent) polyamine uptake. In these systems it is common for the uptake of putrescine and spermidine to be Na^+-dependent whilst spermine transport is often Na^+-independent (Feige & Chambaz, 1985; Rannels *et al.* 1989; Nuttall *et al.* 1990; Morgan, 1992). Also many cellular systems have a non-saturable Na^+-independent component to their polyamine transport (Feige & Chambaz, 1985; De Smedt *et al.* 1989; Auberger *et al.* 1983). However the criteria for Na^+-dependent

transport used in Table 1.3, which is only based on either the iso-osmotic replacement of sodium, Na^+, with choline, Ch^+, (or other monovalent cations such as lithium and *N*-methyl-D-glucamine) and / or the use of ionophores (gramicidin (Na^+/K^+), monensin (Na^+)) and the Na^+/K^+ pump inhibitor, ouabain (Rinehart & Chen, 1984; Khan *et al.* 1990; Feige & Chambaz, 1985; Gawel-Thompson & Greene, 1988; Kameji *et al.* 1989; De Smedt *et al.* 1989; Nuttall *et al.* 1990), does not give the full picture. Replacement of Na^+ with Ch^+ or other monovalent cations results in a reduction in the uptake of putrescine and spermidine in rat enterocytes (Kumagai *et al.* 1989; Kumagai & Johnson, 1988) or of putrescine but not spermidine in pancreatic acini (Nicolet *et al.* 1990). Whereas if Na^+ is replaced iso-osmotically with uncharged molecules such as mannitol or sucrose there is no reduction in the rate of polyamine uptake in either cell type. Furthermore, in *E.coli* putrescine, spermidine and spermine are readily taken up from a medium containing no exogenous Na^+ (Tabor & Tabor, 1966). This suggests that in *E.coli*, rat enterocytes and pancreatic acini polyamine uptake is not a Na^+-coupled process. Instead the inhibition of polyamine uptake in the presence of lithium or organic cations may be due to direct interaction of these cations with the carrier(s). However in Balb/c 3T3 cells spermidine is transported with Na^+ in a 1:1 stoichiometric relationship, suggesting that the spermidine is directly coupled in a ternary complex, Na^+-spermidine-carrier (Khan *et al.* 1990).

In addition some ionophores such as gramicidin (Na^+/K^+), carbonylcyanide *m*-chlorophenylhydrazone (H^+) and calcimycin (A23187, Ca^{2+},Mg^{2+}) can also disrupt the cell's membrane potential (Kakinuma *et al.* 1988). As only those ionophores which disrupt the membrane potential decrease putrescine uptake, this led to the conclusion that in bovine lymphocytes, *S.cerevisiae* (Kakinuma *et al.* 1992) and *E.coli* (Kashiwagi

et al. 1986) polyamine uptake is dependent on a membrane potential (Kakinuma *et al.* 1988). For these reasons any sodium-dependency denoted in Table 1.3, must be viewed with extreme caution. Thus, the true picture may be more complex, with different uptake systems having different ion and membrane potential requirements.

1.5.4 Requirement for sulphydryl groups

Many polyamine uptake systems appear to require the presence of sulphydryl (-SH) groups for maximal activity (see Tables 1.3 and 1.4). However caution must be exercised in the interpretation of any inhibition of polyamine uptake by *N*-ethylmaleimide, as its rather permeable nature makes it difficult to distinguish its interactions with membrane components from its effects on cell metabolism (Rothstein, 1970). In NB15 neuroblastoma cells the potent inhibition of putrescine uptake by the rather impermeable sulphydryl reagent *p*-chloromercuribenzene sulphonate (Rothstein, 1970) is reversed on addition of dithiothreitol (Rinehart & Chen, 1984). This suggests that the sulphydryl groups of certain membrane proteins, possibly on the transporter itself or perhaps on any ion channels driving uptake, could be involved in polyamine uptake.

1.5.5 Interaction with amino acid transport systems

As can be seen from tables 1.3 and 1.4, the vast majority of cell types do not take up polyamines on any of the amino acid transport systems. However polyamine uptake in NB15 neuroblastoma (Rinehart & Chen, 1984), embryonic palate mesenchymal (Gawel-Thompson & Greene, 1989), L1210 leukaemia and C6 glioma cells (Khan *et al.* 1990) can be stimulated by system A amino acids (asparagine or the

system A amino acid analogue 2-aminoisobutyric acid). Whereas in aortic endothelial cells pre-incubation with *L*-arginine (system Ly^+) has a slight stimulatory effect on spermidine uptake (Bogle *et al.* 1994). In contrast in the trypanosomatid *L.infantum* putrescine uptake can be partially inhibited by the amino acids lysine, arginine (system Ly^+) and aspartic acid (system β^+) (Balana-Fouce *et al.* 1989).

1.5.6 Other factors affecting polyamine uptake

1.5.6.1 Polyamine content of the cell's environment

In order for the polyamine uptake systems to be of any physiological significance then polyamines must be available for uptake from the cell's immediate surroundings. In mammalian cells this means polyamines must be present in the blood and tissue fluids. Whole blood contains <0.81μM putrescine, 6-34μM spermidine and 4-10μM spermine (Cooper *et al.* 1978; Claverie *et al.* 1987), whilst plasma and serum contain much lower levels of polyamines at 0.03-0.5μM (Morgan, 1990b). This is due to the cellular constituents of the blood containing micromolar levels of polyamines (Cooper *et al.* 1978) coupled with the fact that polyamines bind well to the negatively charged red blood cell membrane (Chun *et al.* 1977) predominantly by electrostatic interactions in the order spermine > spermidine > putrescine (Braunlin *et al.* 1982). However as most polyamine transporters have affinities for polyamines in the 0.1-10μM range (see Tables 1.3 and 1.4) they should still be able to take them up.

1.5.6.2 Intracellular polyamine content

Intracellular polyamine concentrations are normally in the mid-micromolar range with putrescine concentrations generally lower than those of spermidine and

spermine (Morgan, 1990b). Treatment of L1210 Leukemia cells with inhibitors which block polyamine biosynthesis such as DFMO and / or *S*-(5'-deoxy-5'-adenosyl)-methylthioethylhydroxylamine leads to a reduction of intracellular polyamine pools and an increase in the V_{max} of the polyamine uptake system(s) (Kramer *et al.* 1993; Byers & Pegg, 1989). DFMO treatment also increases the rate of polyamine uptake in, for example, neuroblastoma cells (Rinehart & Chen, 1984), embryonic palate mesenchymal cells (Gawel-Thompson & Greene, 1989), prostate tumour cells (Heston *et al.* 1984), Chinese hamster ovary cells (Byers & Pegg, 1990; Byers & Pegg, 1989) and the trypanosomatids *L.mexicana* and *C.fasciculata* (González *et al.* 1993; González *et al.* 1992). However DFMO does not induce putrescine uptake in the trypanosomatid *T.cruzi* (González *et al.* 1993; González *et al.* 1992). This may be related to either the apparent lack (Hunter *et al.* 1994) or presence of only trace levels (Algranati *et al.* 1989) of ODC activity in this organism. In contrast, DFMO has no effect on putrescine uptake in cultured mouse hepatocytes, despite lowering ornithine decarboxylase activity by >90% and causing a drop in the intracellular polyamine concentration (Martin *et al.* 1990). Conversely, incubation of the cells in exogenous polyamines or the polyamine analogue N^1,N^{12}-*bis*(ethyl)spermine (Kramer *et al.* 1993) leads to a reduction in polyamine uptake in most (Kramer *et al.* 1993; Rinehart & Chen, 1984; Gawel-Thompson & Greene, 1989) but not all cell types studied (Martin *et al.* 1990). This suggests that, in general, polyamine transport systems are responsive to modifications in intracellular polyamine concentrations.

1.5.4.3 Growth conditions

Polyamine uptake, like that of ODC (reviewed in (Bachrach,

1984)), can be stimulated by a wide variety of growth factors, hormones and other stimuli which provoke cell growth. In fibroblasts (Pohjanpelto, 1976; Bethell & Pegg, 1981; Miyahira & Dvorak, 1994), baby hamster kidney cells (Wallace & Keir, 1981), melanoma and macrophages (DiPasquale *et al.* 1978) polyamine uptake is stimulated by the addition of fresh serum (to serum starved cells). More specifically the hormone insulin alone is able to promote putrescine uptake in fibroblasts (Pohjanpelto, 1976; DiPasquale *et al.* 1978) and pancreatic acinar cells (Stüber *et al.* 1993), or polyamine uptake in combination with prolactin in mammary glands (Kano & Oka, 1976). Epidermal growth factor (EGF) and or insulin-like growth factor (IGF) stimulates putrescine uptake in pancreatic acini (Stüber *et al.* 1993), fibroblasts (DiPasquale *et al.* 1978) and embryonic palate mesenchymal cells (Gawel-Thompson & Greene, 1989), whilst trypsin stimulates putrescine uptake in fibroblasts (DiPasquale *et al.* 1978) and Concanavalin A activates putrescine uptake in lymphocytes (Kakinuma *et al.* 1988).

An increase in cell density coupled with the onset of confluence or entry into stationary phase causes a decrease in the rate of polyamine uptake (Pohjanpelto, 1976; DiPasquale *et al.* 1978; Nicolet *et al.* 1991; Gawel-Thompson & Greene, 1989). Cell differentiation brought about by the differentiating agents retinoic acid and dimethylsulphoxide (DMSO) in hepatocytes (DiPasquale *et al.* 1978) and dibutyryl cAMP and 3-isobutyl-1-methyl xanthine in NB-15 neuroblastoma cells (Chen & Rinehart, 1981) likewise causes a drop in the rate of putrescine uptake.

Alterations in the rate of polyamine uptake in response to growth stimuli are mainly associated with a 2-10 fold increase in the maximum rate of transport (V_{max}) whilst the affinity of the transporter (K_m) remains essentially unaltered (Martin *et al.* 1990; Bethell & Pegg, 1981; DiPasquale *et al.* 1978; Nicolet *et al.* 1991; Pohjanpelto,

1976). An exception to this is NB-15 neuroblastoma cells in which undifferentiated cells have 10-fold lower K_m than differentiated cells, with the V_{max} remaining the same (Chen & Rinehart, 1981). This may simply be due to the fact that fully differentiated (non-dividing) cells have a lower requirement for polyamines. Furthermore, in contrast to normal Swiss 3T3 fibroblasts the Simian virus 40 transformed cells show no regulation of V_{max} with growth state (Bethell & Pegg, 1981). These changes in the kinetics of polyamine uptake, in response to alterations in the growth conditions, appear to be relatively specific. For example, EGF stimulated putrescine uptake does not alter the transport of the amino acids leucine, ornithine and 2-aminoisobutyric acid or the nucleoside thymidine into fibroblast and mesenchymal cells (DiPasquale *et al.* 1978; Gawel-Thompson & Greene, 1989). Similarly, serum stimulated putrescine uptake to a much greater extent than the addition of uridine, thymidine, deoxyglucose or leucine to fibroblast cells (Pohjanpelto, 1976). Increases in the V_{max} of the uptake system in response to a growth stimulus requires *de novo* protein and RNA synthesis in some (Bethell & Pegg, 1981; Kakinuma *et al.* 1988; Byers & Pegg, 1990) but by no means all cases (Kano & Oka, 1976; Gawel-Thompson & Greene, 1989) with the carrier apparently having a fairly long half-life (Byers & Pegg, 1990). The increases in polyamine uptake observed can be reversed by the addition of exogenous polyamine (Kakinuma *et al.* 1988; Byers & Pegg, 1990). Finally, studies on cultured hepatocytes and B lymphocytes indicate that the induction of putrescine uptake occurs in G1 phase (Martin *et al.* 1991; DeBenedette *et al.* 1993) and is down regulated as the cells go from G1 to S phase (Martin *et al.* 1991).

1.5.7 Regulation of polyamine uptake

1.5.7.1 A role for antizyme

Antizyme was initially identified as a polyamine-induced labile protein which inhibited the ODC protein (Fong *et al.* 1976; Heller *et al.* 1976). Recent studies suggest that antizyme may also be responsible for mediating the rapid feedback inhibition of polyamine uptake observed when exogenous spermidine is added to cultured rat hepatoma or Chinese hamster ovary cells (Mitchell *et al.* 1992; Mitchell *et al.* 1994). In the simple eukaryote *N.crassa* polyamine uptake also seems to be regulated by a labile protein (Davis *et al.* 1991). More studies are required in different cell types to find out whether this type of regulation of polyamine uptake is a widespread phenomenon.

1.5.7.2 A possible role for the Na^+-K^+pump, protein kinase C and Ca^{2+}

In L1210 leukaemia cells protein kinase C activators such as the phorbol ester, phorbol myristic acid (PMA), increase spermidine, Na^+ and Rb^+ (a measure of the Na^+-K^+pump activity) uptake whereas inhibitors such as H-7 lower Na^+ and Rb^+ uptake (Khan *et al.* 1992). Likewise PMA stimulates putrescine uptake in pancreatic acini (Stiber *et al.* 1993). Trifluoroperazine, a calmodulin antagonist and an inhibitor of protein kinase C, also inhibits polyamine uptake (Khan *et al.* 1993; Khan *et al.* 1989b). If the transport of polyamines is directly coupled to Na^+ and Na^+-K^+ pump activity as in Balb/c 3T3 cells (Khan *et al.* 1990), then one possible model for the regulation of polyamine uptake involves phorbol esters activating the Na^+-K^+ pump perhaps via activation of protein kinase C, thereby stimulating the efflux of sodium from the cell and hence polyamine-Na^+ coupled entry into the cell (Khan *et al.* 1989b;

Khan *et al.* 1994). In addition an increase in free intracellular Ca^{2+}, may cause the translocation of cytosolic protein kinase C to the plasma membrane where it activates the Na^+-K^+pump and the activation of calmodulin (by translocation to the plasma membrane). It is possible that this calmodulin may then influence the efflux of Ca^{2+} from the cell (via the Ca^{2+}ATPase), which in turn might stimulate both the uptake of extracellular Ca^{2+} and the entry of polyamines in to cells (Khan *et al.* 1993; Khan *et al.* 1994). Although this provides us with an interesting model for the regulation of polyamine transport, the fact that some of the polyamine uptake systems studied do not appear to be either Na^+-dependent (reviewed in section 1.5.3) or stimulated by phorbol esters (Morgan, 1992), casts doubt on how universally applicable this model really is to the regulation of polyamine uptake systems.

1.5.8 Specificity of uptake

Polyamine transporters are not specific for just putrescine and / or spermidine and spermine. A wide range of other diamines and triamines are also taken up by L1210 leukemia cells (Porter & Bergeron, 1983; Porter *et al.* 1984). Generally the triamines of chain lengths similar to that of spermidine and spermine were taken up most effectively (i.e. were the best competitive inhibitors of polyamine uptake) with homospermidine showing the greatest specificity for the transporter (Porter *et al.* 1984). Most diamines were taken up with lower specificity than triamines. The highest affinity for the transporter was shown by the diamines whos chain lengths were similar to spermidine (1,7-diaminoheptane and 1,8-diaminooctane) and the least by those with chain lengths similar to putrescine (Porter & Bergeron, 1983; Porter *et al.* 1984). This suggests that the transporter contains at least three negatively charged

groups with the distance between them corresponding to the positvely charged nitrogens of spermidine. This is supported by work on B16 melanoma cells which indicates that an inter-nitrogen distance of 0.6-0.7 nm or 1.0-1.1 nm is optimal for uptake, corresponding to the inter-nitrogen distance of putrescine and the N^1N^8 bridge of spermidine (Minchin *et al.* 1989).

Following on from this, the terminal (primary) amino groups appear to be critical for uptake since *N*-alkyl substitutions at the terminal amino groups of putrescine (Heston *et al.* 1987; OSullivan *et al.* 1991) and spermidine (Porter & Sufrin, 1986; Khan *et al.* 1990) lower the ability of the analogue to compete for uptake. An increase in the *N*-methylation, for example, by methylating both rather than just one terminal amino group, or an increase in the size of the *N*-alkyl substituents in putrescine derivatives, further reduces their ability to inhibit putrescine uptake (Heston *et al.* 1987; OSullivan *et al.* 1991). Substitution with one or two fluoro- (negatively charged) groups in the 2 position of putrescine also decreases its (Dezeure *et al.* 1988) and spermidine's (Khan *et al.* 1990) uptake. Unsaturated derivatives of putrescine, 1,4-diaminobut-2-ene (both the (*E*) and (*Z*) isomers) and 1,4-diaminobut-2-yne, are poor inhibitors of putrescine uptake (Heston *et al.* 1984; OSullivan *et al.* 1991). Conversely addition of an aziridine moiety to putrescine to form *N*-(4-aminobutyl)aziridine (OSullivan *et al.* 1991; Heston *et al.* 1987) makes it a good substrate for the polyamine uptake system, whilst polypyridium quaternary salts (Minchin *et al.* 1989) are good inhibitors of putrescine uptake, although there is no evidence that they are substrates for uptake. However, other polyamine analogues, such as the *bis*(benzyl)polyamines, are transported into the cell by an uptake mechanism which appears to be distinct from the polyamine transport system (Byers

et al. 1990).

Two other compounds which structurally resemble polyamines, methylglyoxal-*bis*(guanylhydrazone) (MGBG, a potent inhibitor of *S*-adenosylmethionine decarboxylase (Williams-Ashman & Schenone, 1972) which has a structure that resembles that of spermidine), and the herbicide paraquat (*N,N'*-dimethyl-4,4'-bipyridium, which has similarities to a six carbon chain α,ω-diamine (OSullivan *et al.* 1991)), share (at least partially in the case of paraquat) the polyamine uptake system(s) of mammalian cells (Byers *et al.* 1987).

1.5.9 Molecular Characterisation

The genes for four polyamine transport systems have been identified in *E.coli*. This bacterium contains a spermidine-preferential uptake system which consists of potA, -B, -C, and -D proteins (Furuchi *et al.* 1991; Kashiwagi *et al.* 1993) and a putrescine uptake system which consists of potF, -G, -H and I proteins (Pistocchi *et al.* 1993). The potA and -G proteins are membrane associated proteins which have a nucleotide-binding site which shows the greatest affinity for ATP. The potB and -C, and potH and -I proteins each consist of six transmembrane-spanning segments linked by hydrophilic segments of variable length. Whereas the potD and -F proteins are periplasmic spermidine / putrescine (potD) and putrescine only (potF) binding proteins. *E.coli* also contains a putrecine-ornithine antiporter which consists of a single membrane protein, potE, which has 12 transmembrane segments (Kashiwagi *et al.* 1992) and a cadB membrane protein which acts as a lysine-cadaverine antiporter (Meng & Bennett, 1992). It has been proposed that these antiporters (potE and cadB proteins) act to reduce the acidity of the cell's external environment by taking up

ornithine and lysine (with a proton) from the medium, synthesizing putrescine and cadaverine respectively and then exporting these diamines from the cell (Meng & Bennett, 1992).

Our knowledge of mammalian polyamine uptake systems is much less advanced. However our understanding of the physiological importance of the polyamine uptake systems of mammalian cells has been greatly enhanced by use of these mutant Chinese hamster ovary cells, CHOMG, which lack a functional polyamine uptake system (Heaton & Flintoff, 1988; Byers & Pegg, 1989). By comparison with normal Chinese hamster ovary cells it has been found that, if exogenous polyamines are present, the polyamine uptake system(s), can be used to maintain normal intracellular polyamine concentrations when *de novo* synthesis is blocked by DFMO, prevent the increase in ODC activity usually observed on addition of serum after a period of serum deprivation and minimise polyamine loss or excretion from the cell. It has also been used to show that the cytostatic *bis*(ethyl)polyamine analogues enter the cell via the polyamine uptake system whereas the drug bleomycin does not (Byers & Pegg, 1989). These CHOMG cells have been used to express a human gene for polyamine uptake (Byers *et al.* 1989). Therefore, the use of polyamine uptake-deficient mutants could provide a useful tool in the future for the isolation of polyamine transport genes.

1.5.10 Therapeutic approaches

As fast growing cells, for example tumor cells, have an active polyamine uptake system (see Table 1.3), one approach is to use polyamines as vector molecules for (molecules or) chemical moieties which have biological activity. N^4-spermidine

derivatives are good potential canditates as they are good substrates for the polyamine uptake system (Porter *et al.* 1982). Chloroambucil has been conjugated to the N^4-position of spermidine, leading to it being 4-fold more potent *in vivo* than chloroambucil alone in inhibiting ADJ/PC6 tumor growth in Balb/c mice (Holley *et al.* 1992). Unfortunately the therapeutic index was not increased. Nevertheless, this provides an interesting lead which could be followed up in parasitic protozoa such *Trypanosoma cruzi* which possess polyamine uptake systems (González *et al.* 1993; González *et al.* 1992). As described previously, pretreatment with DFMO enhances polyamine uptake in mammalian cells and can be used to increase the amount of polyamine conjugate entering the cells (Holley *et al.* 1992). This could be potentially useful given that such polyamine conjugates were selectively toxic towards the parasitic protozoa.

As *Trypanosoma cruzi* is an intracellular parasite direct inhibition of polyamine uptake is unlikely to succeed because of the problems in attempting to design a compound which is a substrate for the mammalian system but an irreversible inhibitor for the parasite one. Hence I would favour pursuing the former option.

CHAPTER 2 : MATERIALS AND METHODS

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2.1 Metabolic labelling studies

2.1.1 Cell culture

Crithidia fasciculata cells (clone HS6), seeded at 1 x 10^6cells ml^{-1}, were grown at 28°C in a yeast tryptone broth as previously described (Pascal *et al.* 1983), only without shaking and subcultured every 2 days. *Trypanosoma cruzi* epimastigotes, line MHOM/BR/78/Silvio (clone X10/6), were seeded at a final concentration of 1 x 10^6cells ml^{-1} and maintained at 28°C by serial passage (every 6 days) in an RPMI 1640 based medium (Pereira & Hoff, 1986) using the modifications of Gibson and Miles (Gibson & Miles, 1986). This was produced as follows: to 500 ml RPMI 1640 (Life Technologies Ltd) was added 10 ml of 1 M Hepes, 14 ml Trypticase (Becton Dickinson Microbiology Systems) at 0.175 g ml^{-1}, 60 ml heat-inactivated (30 min at 56°C) foetal calf serum (FCS) (Life Technologies Ltd), 6 ml penicillin/streptomycin (5000 units ml^{-1} / 5 mg ml^{-1}) (Life Technologies Ltd) and 4ml haemin at 2.5 mg ml^{-1}. This was designated RTH+FCS. In some specified instances the FCS was replaced by an equal volume of chicken serum (CS) (Life Technologies Ltd) and this medium was designated RTH+CS.

2.1.2 Purification of the radiolabels

2.1.2.1 [^{3}H]Cadaverine

[^{3}H]Cadaverine was prepared from the diacetyl derivative (kindly provided by Dr. M.H.Park, National Institutes of Health, USA), by acid hydrolysis. This was carried out by the addition of 500 µl 6 M HCl and 25 µl (0.5mCi) of the crude [^{3}H]cadaverine diacetyl derivative to each of two 1 ml screw-capped reactivials. After

sealing, the samples hydrolysed overnight at 115 °C. The remaining HCl was removed by rotary evaporation under reduced pressure (Heto vacuum rotator (VR-1) and freeze dryer connected to a Javac high vacuum pump). The solid left in each vial was resuspended in 10 ml water and adjusted to pH 8 with 1 M NH_4OH. These samples were pooled and purified by application to a silica gel column (1 g activated gel (J.T.Baker B.V., Holland), transferred to a 2 ml plastic pipette) as described by Grettie (Grettie *et al.* 1972). Essentially the column was washed with water, then 0.002 M acetic acid (in both cases until radioactivity was <200 dpm / 0.1 ml) and the [^{3}H]cadaverine eluted in 10 mM HCl using pressure from a 50 ml syringe (Becton Dickinson) attached to the top of the pipette to force the liquid through the column. Fractions containing the purified [^{3}H] cadaverine were pooled, freeze-dried and resuspended in 1 ml 10 mM HCl yielding 263 µCi [^{3}H]cadaverine with a purity of >98% (26% recovery).

2.1.2.2 [^{14}C]Spermidine and [^{14}C]Spermine

The radiolabel was separated from unwanted contaminants by HPLC method 1 (section 2.1.6) with the post-column detection switched off. One minute fractions containing the pure radiolabel were pooled and diluted with two volumes of water (to lower the propan-1-ol concentration). A Waters C_{18} Sep-Pak cartridge (Millipore Ltd) was connected to a 5 ml plastic syringe and the resin prepared by washing with 2 ml propan-1-ol then 5 ml HPLC solvent A. The pooled sample was then added to the column, washed with 5 ml water, to remove the camphor sulphonate present in the HPLC solvents, and eluted in 5 ml propan-1-ol. The propan-1-ol fraction was dried down by rotary evaporation under reduced pressure and resuspended in a small volume

of 10 mM HCl.

2.1.3 Radiolabelling experiments

2.1.3.1 Long term labelling in cell culture medium

T.cruzi and *C.fasciculata* were set up at 1 x 10^6 cells ml^{-1} and incubated to late log phase in RTH+FCS (6 days) or yeast/tryptone broth (2 days) respectively in the presence of 1 µCi ml^{-1} [1,4<n>-^{3}H]putreacine.2HCl (11 Ci $mmol^{-1}$, Amersham International plc) or [1,5<n>-^{3}H]cadaverine.2HCl (18 Ci $mmol^{-1}$). *T.cruzi* cells were also cultured under identical conditions at 1 µCi ml^{-1} [tetramethylene 1,4-^{14}C]spermidine.3HCl (10.3 mCi $mmol^{-1}$, New England Nuclear) or [tetramethylene 1,4-^{14}C]spermine.4HCl (13.4 mCi $mmol^{-1}$, New England Nuclear) except that RTH+CS was used in place of RTH+FCS. At the end of this time cells were harvested by centrifugation (1500 × g, 10 min, 4 °C) and washed twice by resuspending then pelleting the cells in PSG-BSA (70 mM sodium phosphate pH 7.4, 0.9% NaCl, 1% glucose, 1% bovine serum albumin FractionV (BSA) and 2% penicillin/streptomycin solution).

2.1.3.2 Short term labelling in PSG-BSA

Late log phase cells (1.5 x 10^8) were pelleted by centrifugation (1500 × g, 10 min, 4 °C), the supernatant discarded and then resuspended in 2 ml of PSG-BSA containing 10 µCi of the [^{3}H]-diamines or 5 µCi of the [^{14}C]-polyamines. The cells were incubated at 28 °C for 2 h, pelleted by centrifugation and then washed twice in PSG-BSA as detailed in section 2.1.3.1.

2.1.3.3 Preparation of cell extracts

The cell pellets (sections 2.1.3.1 & 2) were lysed in 0.25 ml distilled water and the protein precipitated with 0.25 ml 20% (m/v) trichloroacetic acid (TCA). 1,7-Diaminoheptane was added as an internal standard and the suspensions left on ice for 30 min or frozen (-20 °C) awaiting analysis. The precipitated protein was removed by centrifugation in a Beckman microfuge E (15,800 × g, 2 min), the supernatants extracted five times with water-saturated ethyl acetate and then concentrated by rotary evaporation under reduced pressure. The residue which was left was resuspended in 50 µl 10 mM HCl and stored at -20 °C prior to analysis by HPLC method 1.

Performic acid oxidation was carried out by the method of Hirs (Hirs, 1967) and acid hydrolysis with propionic acid/HCl as described by Westhall and Hesser (Westhall & Hesser, 1974) on selected cell extracts, prior to analysis by HPLC method 1, using the modifications of Fairlamb *et al.* (Fairlamb *et al.* 1986).

2.1.4 Analysis of the polyamine content of the medium

To 1 ml of medium was added an equal volume of 20% (w/v) TCA. The samples were then prepared as detailed in section 2.1.3.3. Analysis of these samples was carried out using HPLC method 2.

2.1.5 Analysis of the polyamine content of the excreta of *Rhodnius prolixus* after a blood meal

Three adult *R.prolixus* bugs were fully fed through a piece of Parafilm placed on my right arm (takes about 15 min per bug). The excreta which they produced immediately after feeding and over the following 2 h was collected at regular intervals

and placed in Eppendorf tubes kept at 4 °C until all the samples had been gathered. The protein was then precipitated with an equal volume of 20% (w/v) TCA and the samples were prepared as detailed in section 2.1.3.3. The polyamine content of the excreta was analysed using HPLC method 2.

2.1.6 High-Performance Liquid Chromatography (HPLC) analysis.

All separations were carried out by reverse phase chromatography on a Beckman System Gold HPLC system (Beckman Instruments Ltd.) Samples were injected onto the system by a Beckman 507 (Method 1) or 506 (Method 2) autosampler and passed through an Brownlee ODS 7 µm guard column (Anachem) and onto a Beckman Ultrasphere Ion Pairing 5 µm C_{18} (ODS 2, 250 mm x 4.6 mm) column (method 1) or a Beckman C_{18} 5 µm (ODS 1, 250 mm x 4.6 mm) column (method 2) at room temperature.

Method 1. Standards were detected with a Gilson 121 fluoromonitor following post-column derivatisation with fluoreacamine (Fluram, Roche) (Weigele *et al.* 1973). The column was equilibrated with 100% Solvent A (0.25% (w/v) D-camphor sulphonate (Li salt, pH2, Aldrich)) for 40 min at a flow rate of 1 ml min^{-1}. Then at time zero the sample was injected onto the column and eluted by application of linear gradients of Solvent B (25% (v/v) propan-1-ol and 0.25% (w/v) D-camphor sulphonate (Li salt, pH2)), 0-20% over 60 min and 20-75% over the subsequent 40 min.

In radiolabelling experiments 1 min fractions were collected in plastic 6 ml mini-vials (Beckman) using a 95 place Frac-100 fraction collector (Pharmacia Ltd). Radioactivity was determined by addition of 5 ml Pico-Fluor 40 (Camberra Packard) and counting on a Beckman LS-6000LL series scintillation counter (Beckman

Instruments Ltd).

Method 2. 50 µl of sample was derivatized with dansyl chloride and analysed by HPLC, using a linear 10 mM phosphate / acetonitrile gradient, as detailed by Kabra (Kabra *et al.* 1986) except that the post column derivatization clean up procedure was not used. Fluorescence was detected using a Perkin-Elmer series 3000 fluorometer with a micro-flow cell. In radiolabelling experiments 0.25 min fractions were collected and the radioactivity determined as detailed in method 1.

Standards of dihydrotrypanothione, trypanothione disulphide, N^1-glutathionylspermidine and N^1-glutathionylspermidine disulphide were prepared as described previously (Fairlamb *et al.* 1986). Dihydrohomotrypanothione and homotrypanothione disulphide standards were prepared in the same way as the equivalent trypanothione standards.

2.1.7 Polyamine oxidation.

2.1.7.1 Assay for Polyamine Oxidase.

Polyamine oxidising activity in serum was measured fluorometrically by the standard method of Snyder and Hendley (Snyder & Hendley, 1968). This is based on the formation of H_2O_2 as a reaction product. The assay mixture contained 0.1 M potassium phosphate, pH 8, 0.04 mg horseradish peroxidase (175 U mg^{-1}), 150 µl of serum under test, 0.25 mg homovanillic acid, 0-100 nmol polyamine substrate and ±1 mM aminoguanidine bicarbonate (Aldrich) (added before pre-incubation) in a final volume of 3ml. The only alteration to the procedure was to reduce the incubation time from 1 h to 30 min.

2.1.7.2 Culture of *T.cruzi* in Spermidine or Spermine

Twelve well culture plates were prepared, containing 4×10^6 *T.cruzi* cells and 0-500 μM spermidine or spermine in 2 ml of RTH+FCS or RTH+CS medium. Aminoguanidine (1mM) was added to half the RTH+FCS samples and they were all incubated for 6 days (to late log). Growth under each condition was estimated microscopically by cell counting using a haemocytometer.

2.1.8 Measurement of the cell volume of *T.cruzi* epimastigotes by the Inulin exclusion method

A previously published method (Damper & Patton, 1976) was followed using inulin [^{14}C]carboxylic acid (11.9 mCi mmol^{-1}, Amersham International plc) which had previously been dialysed against 0.9% (w/v) NaCl. This gave a cell volume for *T.cruzi* epimastigotes of 5.5 ± 0.4 μl (10^8cells)$^{-1}$ (n = 4).

2.2 Transport of polyamines

2.2.1 Cell culture

T.cruzi epimastigotes (clone X10/6) and *C.fasciculata* were cultured as detailed in section 2.1.1 except that the *C.fasciculata* was also grown in RTH+FCS. *Leishmania donovani* promastigotes line MHOM/ET/67/HU3 (LV9), were seeded at about 1 x 10^6 cells ml^{-1} into Graces insect medium (Kelly *et al.* 1992) or RTH+FCS, incubated at either 22 °C or 28 °C and subcultured every 5-6 days. An arsenical sensitive clone of *Trypanosoma brucei brucei* (S427 c118) procyclics were seeded at about 1 x 10^5 cells ml^{-1} into SDM79+FCS (Brun & Schonenberger, 1979), incubated

at 28 °C and subcultured every 5-6 days. When the cells were required for uptake or transport studies, they were all seeded initially at 1 x 10^6 ml^{-1} in the appropriate medium. *T.b.brucei* bloodstream form were obtained by Dr.E.Akuffo from the blood of adult Sprague-Dawley rats (200-440g) 3 days after infection with 10^7 organisms and then purified free of contaminating blood elements by chromatography on DE-52 cellulose (Lanham, 1968).

2.2.2. General uptake/transport method for diamines and polyamines involving centrifugation through silicone oil

Transport was measured using a rapid sampling technique (Aronow *et al.* 1985; Carter & Fairlamb, 1993) involving centrifugation of the cells through silicone oil. All operations with *T.cruzi* and *L.donovani* were carried out in a Class II safety cabinet. Unless otherwise stated *T.cruzi* cells were always assayed on day 3 of their growth cycle for diamine and polyamine uptake/transport activity. Aliquots (0.1ml) of a basal salts solution [CBSS (Fairlamb *et al.* 1992) and 2% penicillin/streptomycin (5000 U ml^{-1} and 5mg ml^{-1} respectively) (CBSS+PS), 28 °C, 0.1 ml] containing [^{3}H]putrescine, [^{3}H]cadaverine or [terminal methylene^{3}H(N)-] spermidine.3HCl (15.6Ci $mmol^{-1}$, New England Nuclear) and unlabelled diamine / polyamine at 2 times the final concentration were overlaid on silicone oil (0.1 ml GE F-50; viscosity 75 centistokes; specific gravity 1.05 g ml^{-1}; Medford Silicones, New Jersey) in 1.5 ml Eppendorf tubes (Merck Ltd). These were then placed in the fixed angle rotor of an Eppendorf 5415C centrifuge. Cells were washed twice by centrifugation (1500xg, 10 min, 4 °C) and then resuspended to 2 x 10^8 cells ml^{-1} in CBSS+PS and prewarmed to 28 °C. At intervals, aliquots (0.1 ml) were added to the radioisotope-containing medium by

rapidly pipetting down the side of each tube (to ensure adequate mixing) and the tubes capped. After addition of the last sample the cells were separated from the radiolabel by centrifugation (16,000 × g, 1 min). Subsequently, the medium was aspirated, the region above the oil layer rinsed twice with phosphate buffered salts to remove any residual label before aspirating the silicone oil. The pellet was then extracted overnight with 0.1 ml 1 M NaOH before scintillation counting in 1ml Pico-fluor 40. The initial rates of uptake (Figures 4.3-4.6, 4.8, 4.10-4.12 and Tables 4.2-4.7) were determined by linear regression analysis on up to 5 time points spaced at 5 s intervals. All rates have a regression coefficient of $r > 0.95$ and the K_m and V_{max} values were determined using the Enzfitter software package (Elsevier Biosoft, Cambridge, UK).

2.2.3 A comparison of [^{3}H]putrescine and [^{3}H]cadaverine uptake in the trypanosomatids

Cells were grown for either 2 (*C.fasciculata*) or 3 days (*T.cruzi*, *L.donovani* or *T.b.brucei* procyclics). *T.b.brucei* bloodstream forms were isolated from Sprague-Dawley rats as detailed in section 2.2.1. Uptake was measured at intervals ranging from 10 s to 30 min using diamine concentrations of 1, 10 and 100 μM by a technique involving the centrifugation of the cells through silicone oil as detailed in section 2.2.2. For each cell type, a background count, which represents any non-specific binding of the label to the cells and tube, was obtained at each diamine concentration and then subtracted from each of the uptake measurements. This was achieved by adding the cells to the appropriate radiolabel at 4 °C and then centrifuging them immediately through the silicone oil.

2.2.4 Metabolism of the ^{3}[H]putrescine and ^{3}[H]cadaverine labels over 90 s

This was carried out essentially by the method of Damper and Patton (Damper & Patton, 1976). *T.cruzi* epimastigotes grown for 5 days in RTH+FCS were prepared as described in section 2.2.2 and then resuspended at 2 x 10^8 cells ml^{-1} in CBSS. The cells and label (both at 2x final concentration) were prewarmed to 28 °C for 10 min. At time zero an equal volume of cells were added to the label, mixed, and immediately 100µl aliquots were withdrawn with an Eppendorf automatic pipette and layered on top of 0.1 ml of silicone oil which was overlaid on 100 µl of 10% TCA containing 3.5 µM 1,7 DAH in 0.4 ml polyethylene Eppendorf tubes (Merck Ltd). The tubes were capped and placed in the fixed horizontal rotor of a Beckman microfuge E. After 90 s the tubes were spun for 1 min (12,500 × g) to pellet the cells through the oil. Metabolism ceases immediately upon reaching the TCA, since the cells are lysed and denatured protein precipitated. The tubes were left overnight at 4°C for full extraction of the polyamines and then the TCA layer was withdrawn using a fine bore microlance needle (26G 1/2 L.B.0.45x13B.L.; Becton Dickinson) attached to a 1ml syringe (Becton Dickinson). Pooled ^{3}[H]putrescine and ^{3}[H]cadaverine fractions were acid hydrolysed and dansylated prior to HPLC analysis by method 2 as described previously in section 2.1.6. Fractions (0.25 min) were collected on a Prac-100 in 6 ml mini scintillation vials, 5 ml of Pico-Fluor 40 was added, the tubes capped, mixed and counted in a Beckman liquid scintillation counter. Results were expressed as the percentage $\frac{\text{total}}{\int}$ ^{3}H]putrescine or [^{3}H]cadaverine label in putrescine or cadaverine equivalents respectively.

2.2.5 Determination of the maximum rate of 2 μM Putrescine transport within the 6.6-8.0 pH range

CBSS was made up to 80% of its final volume and then adjusted to the required pH with either 1 M NaOH in the pH range 6.8-7.0 or 1 M HCl for the pH 6.6 standard before making it up to the correct concentration. The transport of 2 μM putrescine into *T.cruzi* cells was measured as detailed in section 2.2.2.

2.2.6 Effect of cycloheximide, actinomycin D or putrescine on the rate of putrescine transport

Cycloheximide and Actinomycin D were made up as 50 times stock solutions in CBSS+PS and putrescine as a 100-400 times stock in 10 mM HCl, then filter sterilized through 0.22 μm hydrophilic syringe filters (Techmate Ltd). The experiment was initiated by the addition of these compounds, to cells in RTH+FCS medium, at a final concentration of 10 or 100 μM cycloheximide, 2 μM actinomycin D and 10 or 100 μM putrescine. Putrescine transport was measured under saturating conditions (10 μM) at 4-48 h intervals as detailed in section 2.2.2.

2.2.7 The effect of potential inhibitors on diamine transport

N-ethylmaleimide, paraquat, MGBG and the amino acids were added from a 2 to 100 times stock solutions in CBSS+PS. All other compounds were made up as 200 times stock solutions in various solvents and subsequently diluted in CBSS+PS. Oligomycin, ouabain and iodoacetic acid stock solutions were made up in 70% ethanol, gramicidin in absolute ethanol, valinomycin, carbonylcyanide *m*-chlorophenyl hydrazone and calcimycin in dimethyl sulphoxide (DMSO) and 2,4-dinitrophenol, *p*-

hydroxymercuribenzoate and *p*-chloromercuriphenyl sulphonate in 0.1M NaOH.

When pre-incubation was required the reagent was added (at 2 times the final concentration) to the cells (8×10^8 cells) in a 1:1 ratio, the cells pre-incubated for the indicated time (10-20 min) and then added to the putrescine label as detailed in section 2.2.2. Controls were carried out in which cells were pre-incubated in the same final concentration (0.5%) of DMSO, ethanol or 0.1 M NaOH alone, to ensure the solvents themselves were not adversely affecting putrescine transport. These values together with one for pre-incubation of the cells with CBSS+PS were taken to represent a control transport rate of 100%. The amino acids, MGBG and paraquat were added directly (no pre-incubation with the cells) at 2 times their final concentration to the putrescine label. A final concentration of 2 μM putrescine was used in all these experiments.

2.3 Protein assay

This was carried out with Sigma's protein assay kit (procedure No. p5656) using Peterson's modification of the micro-Lowry method. This method utilizes sodium dodecylsulphate included in the Lowry reagent to facilitate the dissolution of relatively insoluble membrane lipoproteins (Lowry *et al.* 1951; Peterson, 1977). It was carried out on exponentially growing *T.cruzi* epimastigotes using the procedure involving protein precipitation with deoxycholate and TCA. From this method a protein content of 233 ± 19 μg $(10^8 \text{ cells})^{-1}$ (n = 4) was determined for *T.cruzi*.

2.4 Statistical analysis

K_m and V_{max} values (± the standard error of means, SEM) were calculated by

a matrix inversion method using the enzfitter program (Elsevier Biosoft, Cambridge, UK.). Where appropriate, all the other data points shown are the arithmetical means ± the standard deviation (SD). In Table 4.2 the data was analysed by student's paired t-test. Confidence levels were set at 99.9% ($p > 0.001$ was not considered significant).

2.5 Chemicals

Aminopropylcadaverine was kindly provided by Drs. P.P.McCann and A.J.Bitonti of the Marion-Merrell-Dow Research institute (Cincinnati, USA). *Bis*(aminopropyl)cadaverine was generously provided by Dr.P.J.Rodrigues and Prof.M.Israel of the University of Tennessee College of Medicine, (USA). Trypanothione disulphide and N^1-glutathionylspermidine were purchased from, and homotrypanothione disulphide custom synthesised by Bachem Feinchemikalien AG (Switzerland). All other reagents were of the highest purity available and purchased from Sigma unless otherwise stated. All sterile tissue culture flasks and pipettes were puchased from Greiner Labortechnik.

CHAPTER 3 : RESULTS - PART 1

DIAMINE AND POLYAMINE METABOLISM IN *T.CRUZI*

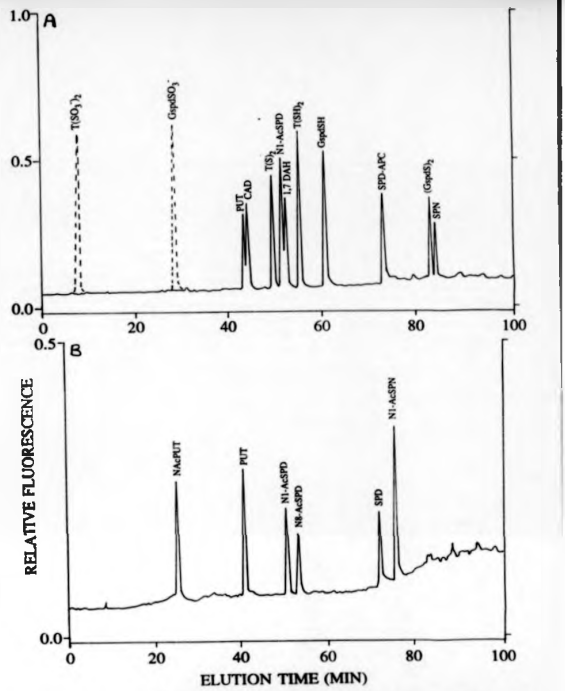


Figure 3.1 Representative HPLC traces depicting the elution times of polyamines, *N*-acetylpolyamines and polyamine-glutathione conjugates. A) Polyamines and polyamine-glutathione conjugates; B) *N*-Acetylpolyamines. 1,7-Diaminoheptane (1,7 DAH) is the internal standard. PUT = putrescine, CAD = cadaverine, SPD = spermidine, APC = aminopropylcadaverine, SPN = spermine, *N*AcPUT = *N*-Acetylputrescine, *N*1AcSPD = N^1-acetylspermidine, *N*8AcSPD = N^8-Acetylspermidine, *N*1AcSPN = N^1-Acetylspermine, GspdSH = glutathionylspermidine, $(GspdS)_2$ = glutathionylspermidine disulphide, $T(SH)_2$ = dihydrotrypanothione, $T(S)_2$ = trypanothione disulphide. Glutathionylspermidine sulphonate ($GspdSO_3^-$) and trypanothione *bis*(sulphonate) ($T(SO_3^-)_2$) are the performic acid oxidation products of glutathionylspermidine and trypanothione respectively. *Bis*(aminopropyl)cadaverine contains with spermine under this system.

CHAPTER 3 : RESULTS

PART 1 : DIAMINE AND POLYAMINE METABOLISM IN *T.CRUZI*

3.1 Uptake and metabolism of tritiated diamines

3.1.1 Long term labelling

Since *T.cruzi* epimastigotes do not appear to make the diamines putrescine and cadaverine *de novo* (Hunter *et al.* 1994; Algranati *et al.* 1989), *T.cruzi* X10/6 cells were cultured in RTH+FCS medium to late log phase (6 days) in the presence of [^{3}H]putrescine and [^{3}H]cadaverine to determine the fate of each compound. As a control, similar labelling experiments were carried out in the non-pathogenic trypanosomatid, *C.fasciculata*, except that the cells were cultured with the tritiated diamines in a yeast/tryptone broth to late log phase (2 days). TCA soluble cell extracts were prepared and the radiolabelled metabolites were separated by HPLC. Peak assignments were made on the basis of their coelution from the reverse phase ion paired HPLC column with the authentic standards (Figure 3.1). Under these conditions the greater the hydrophobicity and basicity of the compound, the more tightly it will bind to the column and hence the longer it will take to be eluted from the column. Elution times varied according to the age of the column (shortening as the column aged) and the ambient temperature on the day of analysis.

3.1.1.1 [^{3}H]Putrescine

These experiments showed that 75% of the total (label plus medium) exogenous putrescine (0.77 nmol ml^{-1}) was taken up by *T.cruzi*. Following HPLC separation, only 7% of the radioactivity taken up was recovered as putrescine. The majority was incorporated into spermidine (14%), spermine (37%),

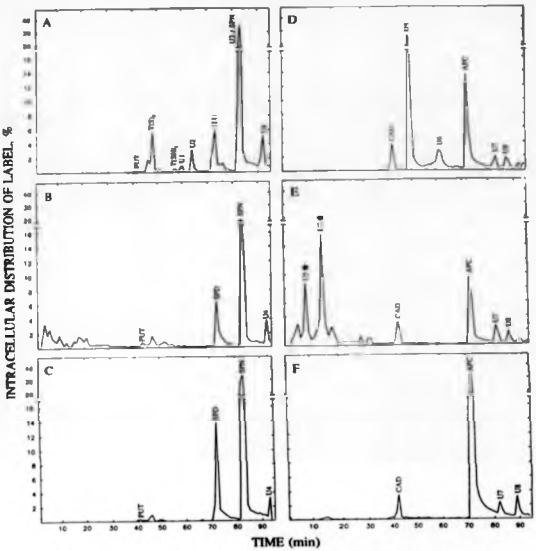
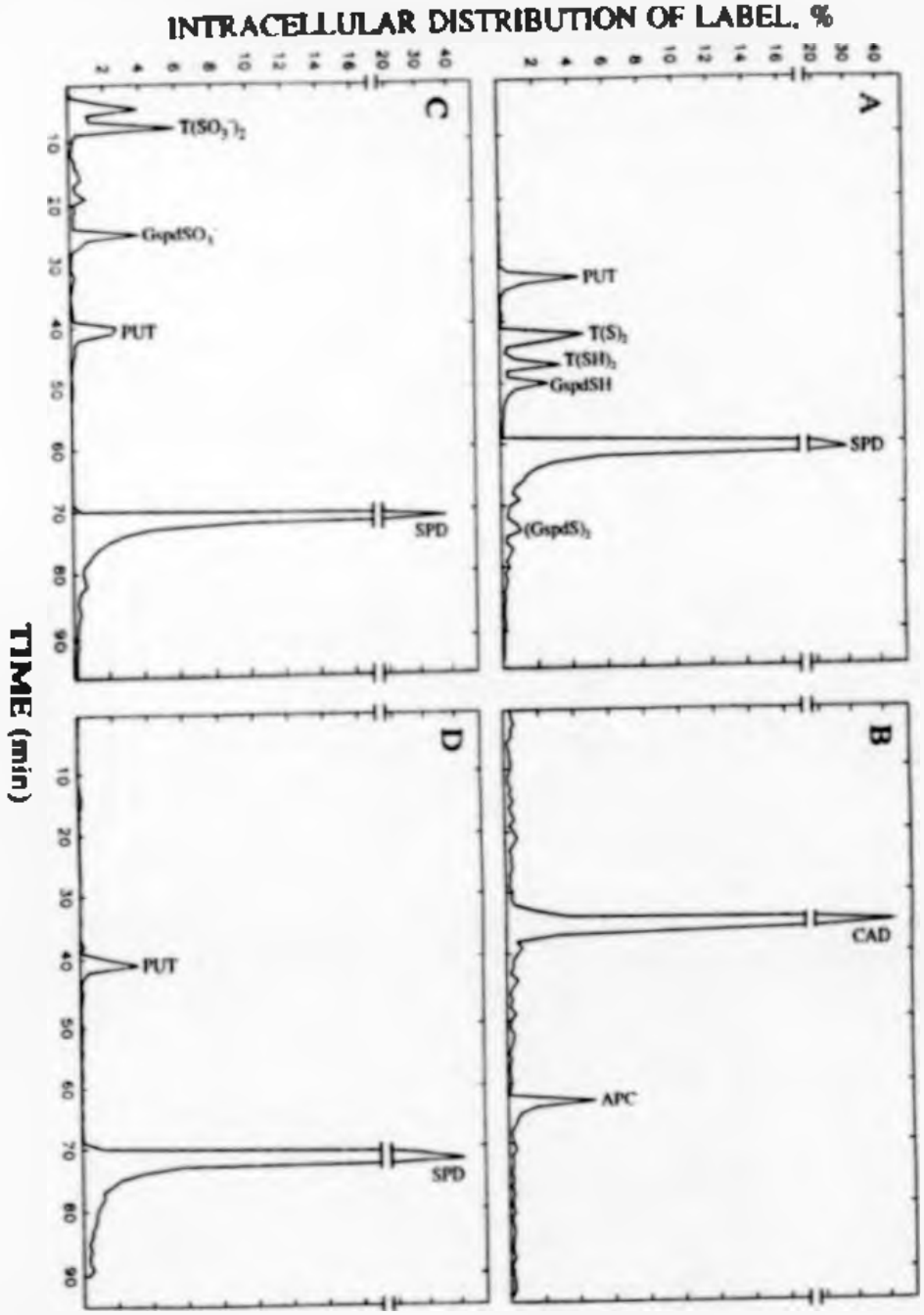


Figure 3.2 HPLC chromatograms of *T.cruzi* cells labelled with tritiated putrescine and cadaverine. Extracts were prepared as detailed in the Materials and Methods. Percentages represent the amount of the label in each fraction compared to the total recovered from the column. Mean recovery of the label from the column was 66%. The polyamine content of the RTH+FCS medium was 0.7μM putrescine, 1.0μM cadaverine, 1.1μM spermidine and 0.5μM spermine. A) [^{3}H]Putrescine-labelled *T.cruzi* cells, B) after performic acid oxidation, C) after acid hydrolysis, D) [^{3}H]cadaverine-labelled *T.cruzi* cells, E) after performic acid oxidation, F) after acid hydrolysis. PUT = putrescine, CAD = cadaverine, SPD = spermidine, APC = aminopropylcadaverine, SPN = spermine, $T(SH)_2$ = dihydrotrypanothione, T(S)$_2$ = trypanothione disulphide, U5 = homotrypanothione disulphide, U7 = *bis*(aminopropyl)cadaverine. U5* are formed following performic acid oxidation of authentic homotrypanothione disulphide. The chemical identities of U1, U3, U4, U6 and U8 are not known. See text for further details.

Figure 3.3 HPLC chromatograms of *C.fasciculata* cells labelled with tritiated putrescine and cadaverine. Extracts were prepared as detailed in the Materials and Methods. Percentages represent the amount of the label in each fraction compared to the total recovered from the column. Mean recovery of the label from the column was 85%. The polyamine content of the yeast/tryptone broth was 3.2μM putrescine, 0.5μM cadaverine and 1.2μM spermine. A) [^{3}H]Putrescine-labelled *C.fasciculata* cells, B) [^{3}H]cadaverine-labelled *C.fasciculata* cells, C) [^{3}H]Putrescine labelled cells after performic acid oxidation, D) after acid hydrolysis. PUT = putrescine, CAD = cadaverine, SPD = spermidine, APC = aminopropylcadaverine, GspdSH = glutathionylspermidine, $(GspdS)_2$ = glutathionylspermidine disulphide, $T(SH)_2$ = dihydrotrypanothione, $T(S)_2$ = trypanothione disulphide. Chromatograms A and B were run at a separate time from chromatograms C and D, using a different HPLC column, hence the difference in the observed retention times. See text for further details.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TIME (min)
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B
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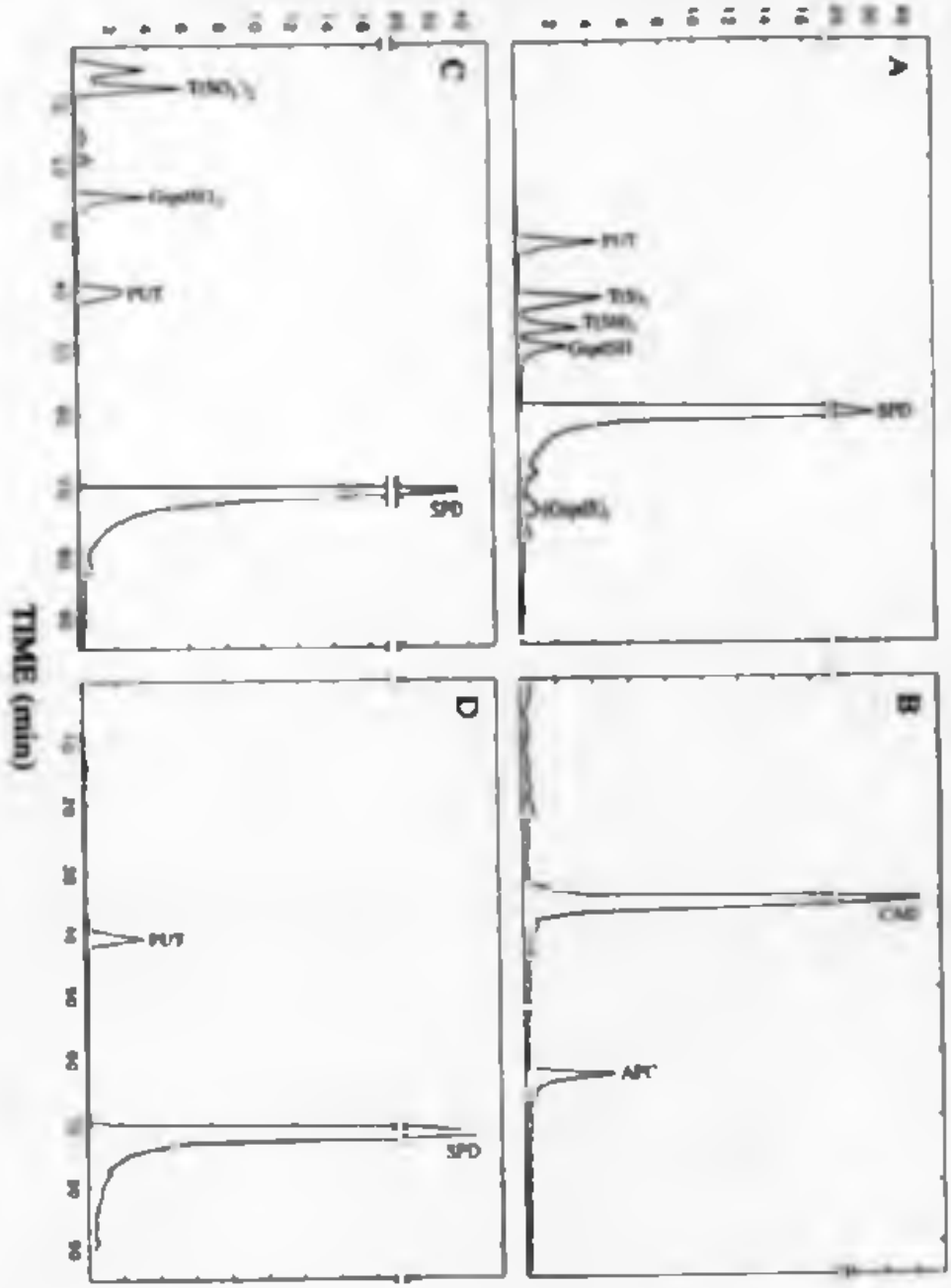
T(S)2
T(SH)2
GspdSH
SPD
(GspdS)2
CAD
APC
T(SO3-)2
GspdSO3-

Figure 3.3 HPLC chromatograms of *C.fasciculata* cells labelled with tritiated putrescine and cadaverine. Extracts were prepared as detailed in the Materials and Methods. Percentages represent the amount of the label in each fraction compared to the total recovered from the column. Mean recovery of the label from the column was 85%. The polyamine content of the yeast/tryptone broth was 3.2μM putrescine, 0.5μM cadaverine and 1.2μM spermine. A) [^{3}H]Putrescine-labelled *C fasciculata* cells, B) [^{3}H]cadaverine-labelled *C fasciculata* cells, C) [^{3}H]Putrescine labelled cells after performic acid oxidation, D) after acid hydrolysis. PUT = putrescine, CAD = cadaverine, SPD = spermidine, APC = aminopropylcadaverine, GspdSH = glutathionylspermidine, $(GspdS)_2$ = glutathionylspermidine disulphide, $T(SH)_2$ = dihydrotrypanothione, $T(S)_2$ = trypanothione disulphide. Chromatograms A and B were run at a separate time from chromatograms C and D, using a different HPLC column, hence the difference in the observed retention times. See text for further details.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dihydrotrypanothione (< 1%), trypanothione disulphide (9%) and four unidentified peaks, U1 (1%), U2 (4%), U3 (13%) and U4 (9%) (Figure 3.2A). Performic acid oxidation and acid hydrolysis confirmed the sulphur and polyamine content of these compounds. Following performic acid oxidation, the radioactivity associated with dihydrotrypanothione, trypanothione disulphide and compounds U1-3 (27% of the total recovered) disappeared, with the corresponding formation of a number of radioactive peaks with retention times of less than 30 min (28% of the total recovered, Figure 3.2B). Following acid hydrolysis there is an increase in the spermidine peak from 14 to 23% of the recovered label, which correlates with the loss of the radioactivity associated with dihydrotrypanothione and trypanothione disulphide (9%). There is also an increase in the spermine peak from 37 to 64% of the recovered label, suggesting that some of the unknown compounds (including U1 and U3) may be conjugates of spermine with other cellular components (Figure 3.2C). Subsequently, U2 was found to coelute with the mixed disulphide of glutathione and glutathionylspermidine, prepared by mixing glutathione and glutathionylspermidine disulphide in a molar ratio of 1:3 at pH 7.4 (data not shown). Compound U4 is stable to both performic acid oxidation and acid hydrolysis. Its chemical identity remains to be determined.

In comparison, 41% of the [^{3}H]putrescine in the medium that was taken up by *C.fasciculata*. Most of this radioactivity was recovered as putrescine (7%) spermidine (65%) and glutathionyl-spermidine conjugates (18%). No spermine or unidentified peaks were formed (Figure 3.3A). Performic acid oxidation (Figure 3.3C) and acid hydrolysis (Figure 3.3D) confirmed that putrescine was converted only into spermidine, glutathionylspermidine and trypanothione, in agreement with previous findings (Fairlamb *et al.* 1986).

3.1.1.2 [^{3}H]Cadaverine

With [^{3}H]cadaverine, 79% of the total (label plus medium) exogenous diamine (1.06 nmol.ml^{-1}) was incorporated into *T.crusi*. After separation by HPLC, only 6% of the radioactivity taken up was recovered as cadaverine. The majority was converted to aminopropylcadaverine (24%) and four major unidentified peaks, U5 (40%), U6 (11%), U7 (3%) and U8 (3%) (Figure 3.2D). Following performic acid oxidation, the radioactivity associated with peaks U5 and U6 disappeared (51% of the total recovered) and was associated with the appearance of two major new peaks eluting at less than 20 min (peaks U5^{ox}, Figure 3.2E). The sum of U5^{ox} (fractions 2-20) represents 51% of the radioactivity recovered, suggesting that U5 and U6 were converted to U5^{ox} products by performic acid oxidation. These suggest that U5 and U6 are sulphur-containing metabolites. In contrast, the amount of radioactivity recovered as cadaverine and aminopropylcadaverine was essentially unchanged by this treatment (6 *versus* 6% for cadaverine and 24 *versus* 20% for aminopropylcadaverine, respectively). Following acid hydrolysis, peaks U5 and U6 disappeared (Figure 3.2F). The radioactivity associated with U5 and U6 (51%) could be accounted for by the increase in aminopropylcadaverine from 24 to 77%, suggesting that these metabolites contained aminopropylcadaverine. Compounds U7 and U8 were stable to both performic acid oxidation and acid hydrolysis. U7 was tentatively identified as *bis*(aminopropyl)cadaverine based on the fact that it coeluted with the authentic standard (Figure 3.1); the identity of U8 remains to be determined.

In contrast, only 2% of the exogenous [^{3}H]cadaverine was taken up by *C.fasciculata*. A small amount of this was converted to aminopropylcadaverine (8%). No further metabolism was observed (Figure 3.3B).

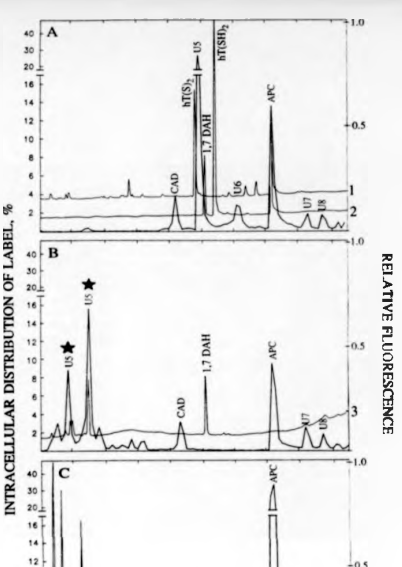

Figure 3.4 A comparison of the elution characteristics of authentic homotrypanothione disulphide with peak U5. A) [^{3}H]Cadaverine cell extracts and homotrypanothione standards, B) performic acid oxidation of [^{3}H]cadaverine cell extracts and homotrypanothione disulphide and C) acid hydrolysis of [^{3}H] cadaverine cell extracts and homotrypanothione disulphide. Base line traces in A, B and C corresponed to the equivalent chromatograms in Figure 3.2 and give the percent intracellular distribution of the label into other compounds. Traces 1-4 depict HPLC chromatograms of the authentic homotrypanothione standard in which peak areas are measured in terms of their relative fluorescence intensity. Trace 1: 0.5 nmol 1,7 DAH, 4 nmol dihydrohomotrypanothione ($hT(SH)_2$), 4 nmol spermidine (residues with monosulphonyl-anthracene under this system, see Figure 3.1). Trace 2: 2 nmol of homotrypanothione monoamide ($hT(SH)_2$) and 4 nmol of spermidine. Trace 3: 0.5 nmol 1,7 DAH and 2 nmol of performic acid oxidised homotrypanothione monoamide. Trace 4: 0.5 nmol 1,7 DAH and 4 nmol acid hydrolysed homotrypanothione disulphide. The full compound names of the other abbreviations used here can be found in the legends of Figures 3.1 and 3.2.

3.1.1.3 Identification of peak U5 as homotrypanothione

The above radiolabelling experiments suggest that *T.cruzi* cells grown in exogenous cadaverine can conjugate aminopropylcadaverine to sulphur-containing molecules (U5 and U6). The chromatographic behaviour of these unidentified peaks suggests that U5, which elutes just after the standard for trypanothione disulphide, could be the aminopropylcadaverine-containing equivalent N^1,N^8-*bis*(glutathionyl)-aminopropylcadaverine ("homotrypanothione") disulphide. Unsuccessful attempts were made to synthesize enough N^1,N^8-*bis*(glutathionyl)aminopropylcadaverine for formal identification, either enzymatically using glutathionylspermidine and trypanothione synthetases from *C.fasciculata* or by extracting it directly from *T.cruzi* cells (Hunter *et al.* 1994). Therefore based on the preceding evidence N^1,N^8-*bis*(glutathionyl)-aminopropylcadaverine disulphide was custom synthesized by Bachem Feinchemikalien AG. Using this compound, peak U5 was identified as homotrypanothione disulphide (Figure 3.4A). Furthermore peaks labelled U5^a were found to coelute with homotrypanothione disulphide treated with performic acid under identical conditions (Figure 3.4B). Presumably two peaks are found in the radiolabelled cells, and a doublet and separate peak which coelute with these in the standard (Figure 3.4B), due to incomplete oxidation to the *bis*(sulphonate) form. On acid hydrolysis aminopropylcadaverine and the component amino acids are formed (Figure 3.4C). The structure of homotrypanothione is depicted in Figure 3.5.

3.1.1.4 Possible identity of U6

The identity of U6 (Figure 3.2D) still needs to be confirmed. It could be glutathionylaminopropylcadaverine, as it elutes within 1-2 min of the equivalent

Table 3.1 Comparison of the pattern of uptake and incorporation of [^{3}H]putrescine and [^{3}H]cadaverine between *T.cruzi* grown in RTH medium which has been supplemented with either 10% foetal calf (FCS) or chicken serum (CS).

component detected	Radioactivity in acid extracts, % [^{3}H]putrescine RTH+FCS	[^{3}H]putrescine RTH+CS	[^{3}H]cadaverine RTH+FCS	[^{3}H]cadaverine RTH+CS
putrescine	0.7	1.5	-	-
spermidine	13.9	14.0	-	-
spermine / U3[1]	49.9	61.5	-	-
N^1-acetylspermine	nd[2]	2.9	-	-
glutathionylspermidine[3]	3.8	6.8	-	-
trypanothione	10.0	7.7	-	-
cadaverine	-	-	5.8	2.5
aminopropylcadaverine	-	-	24.5	32.1
bis(aminopropyl)cadaverine	-	-	3.3	2.5
glutathionyl-aminopropylcadaverine	-	-	<0.5	<0.5
homotrypanothione	-	-	39.7	36.7
Unassigned peaks	U1 1.3 U3 12.9 U4 9.1	U1 nd U3 ?%[4] U4 nd	U6 11.4 U8 3.4	U6 10.4 U8 nd
Label taken up by the cells, %	75	59	79	81
Recovery of the label from the column, %	46[5]	89	58[5]	113

Percentages represent the amount of the label in each fraction compared to the total recovered from the column. The polyamine content of: i) RTH+FCS was 0.7 μM putrescine, 1.0 μM cadaverine, 1.1 μM spermidine and 0.5 μM spermine; ii) RTH+CS was 2.3 μM putrescine, 0.8 μM spermidine and 0.5 μM spermine, prior to the addition of the radiolabel. Tentative assignments for some of these unidentified compounds (U1-8) are suggested in the accompanying text. [1]In *T.cruzi* some of the 'spermine' could be U3 which coelutes with it; [2]not detectable; [3]both the free form and the mixed disulphide of glutathionylspermidine and glutathione (U2 in Figure 3.2) are included in the percentages depicted here; [4]amount of spermine / U3 peak present as U3 not determined; [5]the low recoveries in these experiments are discussed in section 3.1.1.6.

glutathionylspermidine standard. However, this is unlikely since homotrypanothione is found almost exclusively as the disulphide under these extraction conditions and thus glutathionylaminopropylcadaverine should also be present as its disulphide or as the mixed disuphide with glutathione. Furthermore, no peak which could correspond to glutathionylaminopropylcadaverine disulphide can be identified in these cell extracts. Instead, considering the large quantities of glutathione present in these *T.cruzi* cells (approximately 2 nmol.10^8cells^{-1}) (Hunter *et al.* 1994) and the fact that U6 elutes close to the mixed disulphide of glutathione and glutathionylspermidine (U2, Figure 3.2A), it is more likely that U6 is the equivalent mixed disulphide of glutathione and glutathionylaminopropylcadaverine. Confirmation that U6 is the mixed disulphide of glutathione and glutathionylaminopropylcadaverine could be obtained by chemical synthesis of this compound (as detailed in section 3.1.1.1 for the mixed disulphide of glutathione and glutathionylspermidine) and then demonstrating that it coelutes with U6.

3.1.1.5 Use of RTH + 10% chicken serum

When, in a parallel experiment, *T.cruzi* cells were incubated with [^{3}H]putrescine and [^{3}H]cadaverine to late log phase in RTH medium containing 10% chicken serum (CS), in place of the FCS a similar pattern of uptake and incorporation of each label was observed (Table 3.1) confirming the initial findings with RTH+FCS (Figure 3.2).

3.1.1.6 Recoveries

The generally low recoveries of tritiated putrescine and cadaverine from the

column, with a mean of 67% and 85% for the *T.cruzi* and *C.fasciculata* labelling studies respectively, could indicate that there are still radiolabelled compounds adhering to the column especially as U4 and U8 elute right at the end of the chosen gradient. However on extending the gradient from 70 to 100% solvent B, no additional compounds were eluted from the column. Although the possibility of radiolabelled compounds still being retained on the column under these conditions can not be ruled out, it is more probable that it was due to an injector error, as in other experiments we experienced problems at that time with low volumes of sample being injected onto the column by the autosampler. The fact that the recoveries from *T.cruzi* cells labelled with tritiated diamines in RTH+CS (Table 3.1), which was carried out when the autosampler was working properly, are in the region of 89-113% favours this conclusion.

3.1.2 Short term labelling

In this study *T.cruzi* and *C.fasciculata* were incubated in PSG-BSA for 2 h in order to give an estimate of the relative rates of putrescine and cadaverine uptake and incorporation between the two organisms. BSA (1%) was added to the PSG since it improved cell viability and represented a possible 'polyamine-free' alternative to the RTH+FCS medium used in the long term labelling studies. Unfortunately, subsequent analysis revealed that the PSG-BSA contained 2.3μM putrescine and 0.2μM spermidine, so a direct comparison could not be made between rates of uptake of putrescine and cadaverine.

Table 3.2 Radiolabelling of *T.cruzi* and *C.fasciculata* with [^{3}H]putrescine and [^{3}H]cadaverine in PSG-BSA for 2 h.

component detected	Radioactivity in acid extracts, %			
	[^{3}H]putrescine		[^{3}H]cadaverine	
	T.cruzi	*C.fasciculata*	*T.cruzi*	*C.fasciculata*
putrescine	1.3	92.3	-	-
spermidine	34.5	4.1	-	-
N^8-acetylspermidine	2.37[1]	nd	-	-
spermine / U3[2]	16.8	nd.[3]	-	-
glutathionylspermidine	3.8	nd	-	-
trypanothione	26.4	0.23?	-	-
cadaverine	-	-	70.7	95.0
aminopropylcadaverine	-	-	14.4	1.2
bis(aminopropyl)cadaverine	-	-	0.4?	nd
glutathionyl-aminopropylcadaverine	-	-	nd	nd
homotrypanothione	-	-	4.0	nd
Unassigned peaks	U1 3.8 U4 1.8	0	U6 1.2	0
Label taken up by the cells, %	97	77	5.2	nd

Percentages represent the amount of the label in each fraction compared to the total recovered from the column. Mean recovery of the label from the column was 91%. The polyamine content of the PSG-BSA was 2.3 μM putrescine and 0.2 μM spermidine. [1]tentative assignment only; [2]In *T.cruzi* some of the 'spermine' could be U3 which coelutes with it; [3]not detectable.

3.1.2.1 [^{3}H]Putrescine

In *T.cruzi* 69% of the total exogenous [^{3}H]putrescine (2.8μM) was taken up from the PSG-BSA over 2 h, and of this only 1% was found as putrescine, the remainder having been converted to other putrescine containing metabolites (Table 3.2). In contrast, although a similar amount of exogenous [^{3}H]putrescine (60%) was taken up by *C.fasciculata* over the 2 h, 92% of this stayed as putrescine (Table 3.2). This suggests that *T.cruzi* is considerably faster than *C.fasciculata* in its ability to incorporate the [^{3}H]putrescine taken up by the cells into polyamines and then conjugate these polyamines to glutathione.

3.1.2.2 [^{3}H]Cadaverine

With [^{3}H]cadaverine (0.3μM), *T.cruzi* took up 80% of the label and of this 30% was converted to other cadaverine containing metabolites. In comparison, *C.fasciculata* only acquired 1% of the exogenous label and of this 95% remained as cadaverine (Table 3.2). As the initial concentration of cadaverine in the PSG-BSA was the same (0.3μM), this suggests that *T.cruzi* has a much greater ability than *C.fasciculata* to both take up and metabolise cadaverine.

3.2 Uptake and metabolism of [^{14}C]polyamines

Initial attempts to label *T.cruzi* cells with 1 μCi.ml^{-1} [^{14}C]spermidine and [^{14}C]spermine to late log phase (6 days) in RTH+FCS medium were unsuccessful as all the cells rounded up and in the case of spermine nearly half of them died. Others have reported that FCS contains the polyamine oxidising enzyme, serum amine oxidase (abundant in ruminant serum) capable of oxidising spermidine and spermine

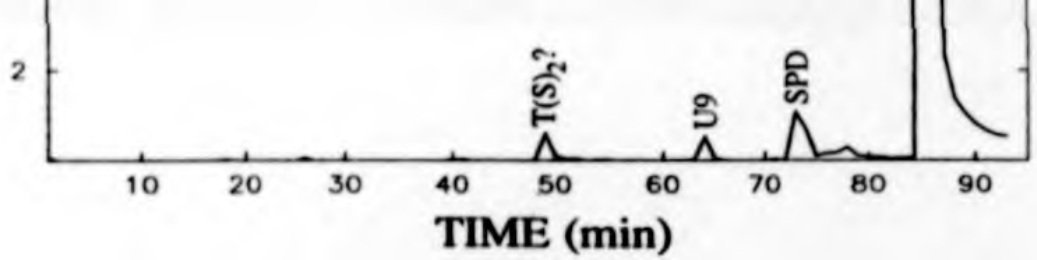

Figure 3.6 HPLC chromatograms of *T.cruzi* cells labelled with [^{14}C] spermidine and spermine. Extracts were prepared as detailed in the Materials and Methods. Percentages represent the amount of the label in each fraction compared to the total recovered from the column. Mean recovery of the label from the column was 44%. A) [^{14}C]Spermidine-labelled *T.cruzi* cells, B) [^{14}C]spermine-labelled *T.cruzi* cells. The full compound names of the other abbreviations used here can be found in the legend of Figure 3.2. The chemical identity of U9 is not known.

INTRACELLULAR DISTRIBUTION OF LABEL, %

to toxic aminoaldehydes (Ferrante *et al.* 1982; Ferrante *et al.* 1984; Morgan *et al.* 1986).

Assaying for a polyamine oxidase (Snyder & Hendley, 1968) revealed that heat inactivated FCS can slowly oxidise polyamines in the order spermine > spermidine >> putreacine = cadaverine. This oxidation can be inhibited by >90% if the FCS is pre-incubated with aminoguanidine, a known inhibitor of serum amine oxidase (data not shown). Furthermore *T.cruzi* can be grown in RTH supplemented with 10% chicken serum (RTH+CS) in place of the FCS (Ariyanayagam & Fairlamb, unpublished data). No polyamine oxidising activity was detected in the chicken serum when tested with up to 100 nmol / assay of spermidine or spermine.

Additional tests in 12 well plates revealed that all the *T.cruzi* epimastigotes grown for 6 days in FCS containing ≥100 μM spermidine or spermine alone died. If 1mM aminoguanidine was also added to the wells then the cells incubated in the presence of 100 μM spermidine grew normally whereas those in 100μM spermine still grew to a lower density than the control cells. *T.cruzi* incubated for 6 days in RTH+CS ± 1mM aminoguanidine grew normally up to spermidine and spermine concentrations of 500 μM (data not shown).

As aminoguanidine is a weak inhibitor of AdoMetDC and so could interfere with normal polyamine metabolism (Williams-Ashman & Seidenfeld, 1986), cells were incubated with spermidine and spermine in RTH+CS. These results indicate that of the total polyamines, 30 % of the total exogenous spermidine (98 nmol ml^{-1}) and 10% of the total spermine (75 nmol ml^{-1}) was taken up by the cells (Figure 3.6). However the growth of cells labelled with spermidine and spermine was only 60 and 17% respectively, of the control where no radiolabel was added. When labelled for 2 h in

Table 3.3 Radiolabelling of *T.cruzi* with [^{14}C]spermidine and [^{14}C]spermine in PSG-BSA for 2 h.

component detected	Radioactivity in acid extracts, % [^{14}C]spermidine	[^{14}C]spermine
putrescine	nd.[1]	nd
spermidine	85.7	0.7
spermine	4.5	92.4
N^1-acetylspermine	0.97[2]	1.47
glutathionylspermidine	nd	nd
trypanothione	3.7	nd
Unassigned peaks	0	U9[3] 1.7
Label taken up by the cells, %	12	17

Percentages represent the amount of the label in each fraction compared to the total recovered from the column. Mean recovery of the label from the column was 124%. The polyamine content of the PSG-BSA was 2.3 μM putrescine and 0.2 μM spermidine. [1]not detectable; [2]tentative assignment only; [3] for the position of U9 refer to Figure 3.6.

PSG-BSA, 12% of the total exogenous spermidine (243 nmol ml^{-1}) and 17% of the spermine (187 nmol ml^{-1}) was taken up by the cells (Table 3.3). However, the [^{14}C]spermidine and [^{14}C]spermine have an approximately 1000-fold lower specific activity than the tritiated putrescine and cadaverine labels, and so are present (even when taking into consideration the diamines found in the RTH+CS medium or PSG-BSA) at about a 100-fold greater concentration in the medium or PSG-BSA than the diamines. Hence the cells actually take up considerable quantities of these polyamines. For example, when labelled for 2 h in PSG-BSA this represents a very sizeable uptake of 39 nmol. spermidine $(10^8 cells)^{-1}$ and 42 nmol. spermine $(10^8 cells)^{-1}$. If these polyamines are toxic to the cell at high concentrations as other results indicate (Morris, 1991; Brunton *et al.* 1991), or if the CS still possesses a serum amine oxidase activity (which is below the detection limits of the assay), then this might in part explain the retarded growth rates observed in the long term (RTH+CS) labelling studies. In both the long and short term labelling with these [^{14}C]polyamines very little interconversion is observed with >86% of the spermidine and >92% of the spermine remaining as the parent compound (Figure 3.6 & Table 3.3). A small quantity of acetylated polyamines may be present (Figures 3.1 and Table 3.3) but less than 2% of the spermine was found as spermidine.

3.3 Determination of the cell volume of *T.cruzi*

Using the inulin exclusion method (Damper & Patton, 1976) the cell volume of *T.cruzi* epimastigotes was determined to be 5.5 ± 0.4 µl $(10^8 cells)^{-1}$ (n = 4), while the previously published value of 10.5 ± 0.6 µl $(10^8 cells)^{-1}$ was used for *C.fasciculata* (Fairlamb *et al.* 1986). From this it can be seen that over a 2 h labelling period in

Table 3.4 Amounts of the radiolabelled diamines and polyamines taken up by *T.cruzi* and *C.fasciculata* over 2 h from the PSG-BSA and the intracellular concentration that this represents.

Organism	Radiolabel	Amount taken up by cells (nmol $(10^8 cells)^{-1}$)	Cell volume (µl $(10^8 cells)^{-1}$)	Internal concentration of radiolabel and its metabolites (mM)
T.cruzi	putrescine	2.57	5.5±0.4	0.47
	cadaverine	0.32		0.06
	spermidine	38.91		7.08
	spermine	42.39		7.71
C.fasciculata	putrescine	2.24	10.5±0.6	0.21
	cadaverine	0.004		0.004

The total amount of each of the diamines and polyamines (radiolabelled and those present in the PSG-BSA) available for uptake from the PSG-BSA by a total of 1.5×10^8 cells was: 5.6 nmol putrescine, 0.6 nmol cadaverine, 486 nmol spermidine and 374 nmol spermine.

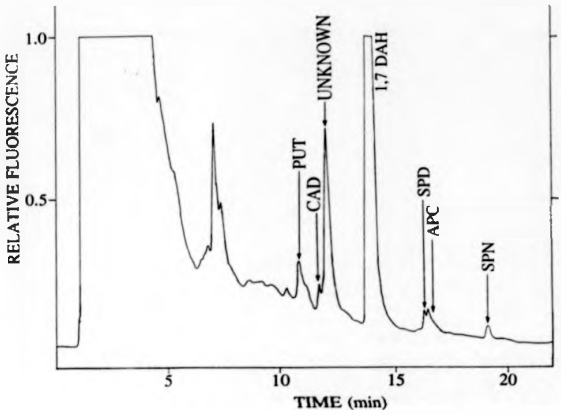

Figure 3.7 HPLC chromatogram depicting the polyamine content of *Rhodnius prolixus* excreta. The polyamines were identified by their coelution with authentic standards and the amount of each present was calculated from the appropriate standard's response factor (concentration / peak area). 1,7 DAH is the internal standard. The chemical identity of the 'unknown' still needs to be established, but it was not any of the compounds listed in Table 3.5. The full names of the compounds corresponding to the abbreviations used here can be found in the legends of Figure 3.1.

Table 3.5 Possible identity of the unknown peak found at 12 min on the HPLC trace of the excreta produced by *Rhodnius prolixus* immediately after a blood meal.

Compounds tested	Unknown compound	General comments
Nitrogenous excretion		
Uric acid	No	Main route for nitrogenous excretion in *R.prolixus*
Allantoin/Allantoic acid	No	Breakdown products of uric acid
Creatine	No	
Xanthine	No	Precursors to uric acid - possible
Hypoxanthine	No	excretory products
Urea	No	From the blood meal
Pigments		
Biliverdin	No	Derived from the breakdown of haemoglobin in the blood meal
3-hydroxykynurenine	No	*R.prolixus* contains the ommochrome rhodnitin, a fluorescing sulphuric ester ($-SO_3NH_4$) of 3-hydroxykynurenine

PSG-BSA *T.cruzi* accumulated micromolar concentrations of the tritiated diamines and their metabolites and millimolar concentrations of the ^{14}C-polyamines and their metabolites within the cell (Table 3.4). *C.fasciculata* has twice the intracellular volume of *T.cruzi* but the total extracellular concentration of diamines available for uptake from the PSG-BSA is the same for both organisms. Therefore in *C.fasciculata* there is a 2- and 150-fold lower intracellular accumulation of tritiated putrescine and cadaverine (and their metabolites) respectively than that observed for *T.cruzi* over the same 2 h time period (Table 3.4).

3.4 Polyamine content of the excreta from *Rhodnius prolixus* after a blood meal

As the present work uses the epimastigote form of *T.cruzi*, which normally resides in the mid- and hind-gut of the blood-sucking triatomine bugs, we thought that it would be interesting to measure the polyamine content of their excreta immediately after a blood meal (mine!). Using *Rhodnius prolixus*, one of the common vectors of Chagas' disease, it was found that their excreta contained 4.5 μM putrescine, 1.1 μM cadaverine, 0.9 μM spermidine and 0.5 μM spermine (Figure 3.7). A large unidentified peak eluted about 15 seconds after cadaverine on the DNS-CL HPLC system. This compound is probably amine positive and / or fluoresces at similar wavelengths to dansylated compounds. A number of different possible compounds were tried but none of these gave a peak which coeluted with the unknown peak at 12.0 min (Table 3.5) and so its identity still remains a mystery.

3.5 Summary

The results obtained so far indicate that diamine and polyamine uptake in

T.cruzi epimastigotes is quantitatively more important than *de novo* synthesis. Therefore the kinetics, specificity and regulation of diamine uptake in *T.cruzi* is investigated in Chapter 4.

CHAPTER 4 : RESULTS - PART 2

DIAMINE UPTAKE IN *T.CRUZI*

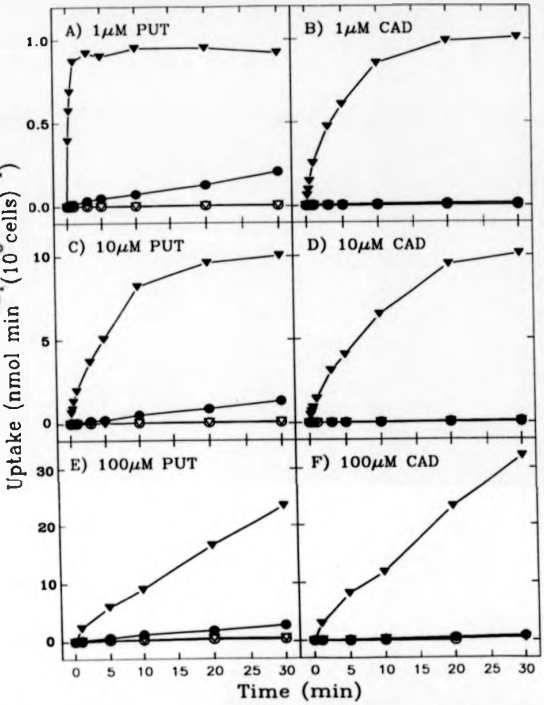

Figure 4.1 Diamine uptake in the trypanosomatids. *T.cruzi* epimastigotes (▼), *C.fasciculata* (●), *L.donovani* promastigotes (▽) and *T.b.brucei* procyclics (○). A background, in which cells were added to the appropriate radiolabel at 4°C and then pelleted immediately, was subtracted from each uptake measurement. Full details in section 2.2.3 of the methods.

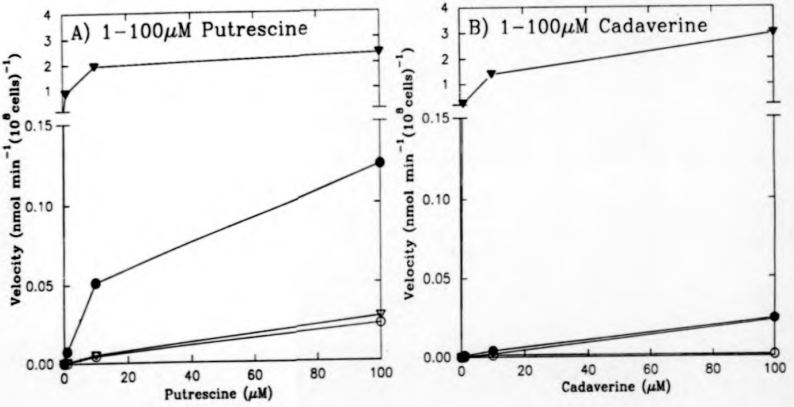

Figure 4.2 Relative velocities of 1-100 μM diamine uptake in the trypanosomatids. *T.cruzi* epimastigotes (▾), *C.fasciculata* (●), *L.donovani* promastigotes (▿) and *T.b.brucei* procyclics (○). The velocities were calculated from the values obtained in Figure 4.1.

CHAPTER 4 : RESULTS

PART 2 : DIAMINE UPTAKE IN *T.CRUZI*

4.1 A comparison of diamine uptake in four trypanosomatids

The time course of uptake of 1, 10 and 100 μM [^{3}H]putrescine and [^{3}H]cadaverine was measured at 28°C in exponentially growing *T.cruzi* epimastigotes, *C.fasciculata*, *L.donovani* promastigotes and *T.b.brucei* procyclics (Figure 4.1A-F). Over the 30 min time course putrescine uptake was extremely fast in *T.cruzi*. At a concentration of 1 μM over 85% of the putrescine had been accumulated by the *T.cruzi* cells after just 1 min (Figure 4.1A). The uptake of putrescine was substantially slower in all the other trypanosomatids. Even after 30 min incubation of the cells at each putrescine concentration, 4- to 8-fold less putrescine was accumulated by *C.fasciculata* and in the case of *T.b.brucei* and *L.donovani* putrescine uptake was at least 40-fold lower than that seen in *T.cruzi* (Figure 4.1A,C and E).

Cadaverine was also rapidly taken up by *T.cruzi*. At low (1 μM) concentrations the rate of cadaverine uptake was slower than with putrescine, requiring 20 min for over 95% of the cadaverine to be accumulated by the cells (Figure 4.1B). However at 10 and 100 μM concentrations uptake rates in *T.cruzi* for both putrescine and cadaverine were similar (Figure 4.1C-F), suggesting that cadaverine is accumulated at the same maximum rate by the cells only with a lower affinity than putrescine. The other trypanosomatids have an even lower ability to take up cadaverine than putrescine. After 30 min at least 40-fold more cadaverine has been incorporated into *T.cruzi* than in any of the other three trypanosomatids.

In *T.cruzi* saturable putrescine and cadaverine uptake was observed in the 1-100 μM range with a maximum velocity of 2-3 nmol min^{-1} (10^8cells)$^{-1}$ (Figure 4.2A-

B). Putrescine uptake in *C.fasciculata* also appears to be saturable in this concentration range with a maximum velocity of about 0.15 nmol min^{-1} $(10^8 cells)^{-1}$. In contrast, over this concentration range, cadaverine uptake in *C.fasciculata*, and both putrescine and cadaverine uptake in *L.donovani* and *T.b.brucei* appear to be non-saturable and of low velocity, <0.03 nmol min^{-1} $(10^8 cells)^{-1}$.

Putrescine and cadaverine uptake was also measured in freshly isolated bloodstream *T.b.brucei* and found to be of a similar low level to that seen in the procyclics (symbols not added to Figure 4.1, since they overlap the procyclic ones). *L.donovani* promastigotes (Figure 4.1) were grown at 28°C in Grace's insect medium prior to measurements of their diamine uptake in CBSS. It is worth noting that 1 μM putrescine uptake in *L.donovani* grown at 28°C in RTH+FCS medium (34 pmol 5 min^{-1}) or at 22°C in Grace's insect medium (29 pmol 5min^{-1}), is similar to the 1 μM putrescine uptake values observed for cells grown at 28°C in the Grace's insect medium (22 pmol 5 min^{-1}, Figure 4.1). However if the cells are grown at 22°C in RTH+FCS medium, prior to assay, then the uptake rates over 5 min for both 1 μM putrescine and 1 μM cadaverine become comparable to those shown in Figure 4.1 for *T.cruzi* (putrescine, 1005 versus 899 pmol 5 min^{-1}; cadaverine, 213 versus 605 pmol 5 min^{-1}; for *L.donovani* and *T.cruzi* respectively). *L.donovani* promastigotes grow better in both the media when incubated at 22 rather than 28°C. This suggests that *L.donovani* diamine uptake is either activated by something in the RTH+FCS medium or conversely that there is a molecule in the Grace's insect medium which inhibits diamine uptake. As this was not followed up, it is not possible to say here which is the more likely scenario.

Table 4.1 Intracellular fate of [^{3}H]Putrescine and [^{3}H]Cadaverine after a 90 s exposure of *T.cruzi* epimastigotes to each label

Diamine / Polyamine	[^{3}H]Putrescine equivalents[1] (%)	[^{3}H]Cadaverine equivalents[2] (%)
Putrescine	98	-
Spermidine	2	-
Spermine	nd[3]	-
Cadaverine	-	100
Aminopropylcadaverine	-	nd
Bis(aminopropyl)cadaverine	-	nd

T.cruzi cells were incubated with the appropriate radiolabel for 90 s and then centrifuged through silicone oil into acid. The acid extracts were then prepared for separation of the radiolabelled products by HPLC using method 2. Full details in section 2.2.4 of the methods. *T.cruzi* cells took up 80% of the 1μM [^{3}H]putrescine and 10% of the 1μM [^{3}H]cadaverine over the 90 s. Recovery of the labels from the column is 93% for [^{3}H]putrescine and 104% for [^{3}H]cadaverine. [1] % of the label coeluting with putrescine, spermidine and spermine; [2] % of the label coeluting with cadaverine, aminopropylcadaverine and *bis*(aminopropyl)cadaverine; [3] nd = not detectable (<0.5%).

4.2 Kinetics of putrescine and cadaverine transport in *T.cruzi*

4.2.1 Basic transport parameters

All the kinetic studies which are detailed below have been carried out on the epimastigote form of *T.cruzi*. This is an insect stage of the parasite which can be readily grown in axenic culture to quantities amenable for the biochemical characterisation of putrescine and cadaverine uptake. Before a more detailed kinetic analysis could be attempted the following basic parameters were established:

1) In order to obtain linear initial rates, putrescine and cadaverine uptake was determined by linear regression analysis on up to 5 time points spaced at 5 s intervals. All rates have a regression coefficient of $r > 0.95$. Over this time period < 10% of the radiolabelled diamine was taken up, except with exponential (day 3) *T.cruzi* cells incubated in 0.25 to 8 μM [^{3}H]putrescine concentrations where as much as 13% (8 μM) to 45% (0.25 μM) of the label was taken up by the cells.

2) A high cell concentration of approximately 1×10^8 cells ml^{-1} was maintained so that the cells would spin properly through the oil layer (84% have spun through the oil after 10 s and 98.5% after 1 min).

3) A temperature of 28°C was chosen to measure diamine uptake (unless otherwise stated) as the *T.cruzi* epimastigotes are cultured at this temperature.

4) Essentially there was no change in putrescine uptake rates in CBSS buffer in the pH 6.6 to 8.0 range. All the following diamine uptake studies were carried out in CBSS at pH 7.4.

5) Of the two diamine radiolabels which were taken up by the cells over the first 90 s there was <1.5% conversion of the putrescine label to spermidine and no metabolism of the cadaverine label (Table 4.1). As diamine uptake was only measured

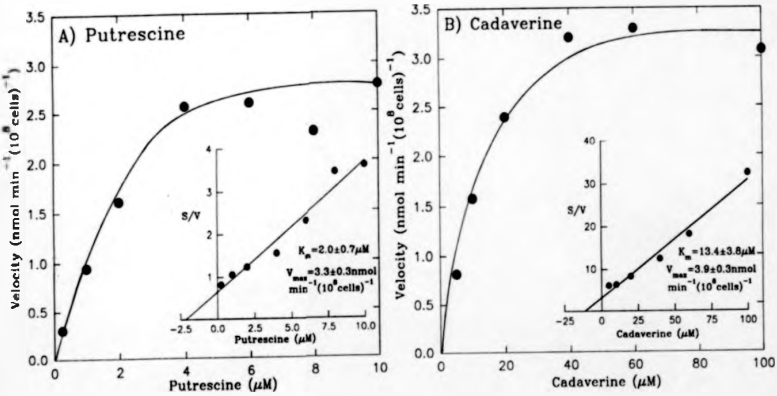

Figure 4.3 The kinetics of putrescine and cadaverine transport in *T.cruzi* epimastigotes. Main plots: The curve is fitted and the K_m and V_{max} values calculated from it using the Enzfitter software package. Insert: Hanes-Woolf plot, a linear S/V versus [S] transformation of the data. K_m = -[S] when S/V = 0; V_{max} = 1/slope.

over the first 20 to 25 s, it is concluded that we are solely measuring 'transport' and not 'uptake' comprising a mixture of transport and further metabolic conversion into other polyamines. However on a cautionary note, it is possible that in its present context the term 'transport' could encompass not only the entry of these diamines into the cell but also their subsequent sequestration within the cell by anionic compounds.

4.2.2 Determination of the kinetics of putrescine and cadaverine transport

The initial velocity of putrescine and cadaverine transport was measured in exponentially growing *T.cruzi* cells (day 3) over a range of putrescine (0.25 to 10 μM) and cadaverine (5 to 100 μM) concentrations (Figure 4.3).

By use of the Michaelis-Menten equation:

$$v = \frac{[S]}{K_m + [S]} . V_{max}$$

(where v is the initial velocity of the reaction (transport); V_{max} is the maximum velocity; [S] is the substrate (putrescine or cadaverine) concentration and K_m is equivalent to the substrate concentration which yields half-maximal velocity)

which describes the curve obtained for a simple unireactant system when the initial velocity is plotted against substrate concentration, rectangular hyperbolic curves were obtained for putrescine and cadaverine from which we can obtain V_{max} and K_m. (Figure 4.3). Hence the transport of both putrescine and cadaverine in exponentially growing *T.cruzi* cells exhibited saturable Michaelis-Menten type kinetics. Putrescine was transported by a rapid high-affinity saturable carrier with a $K_m = 2.0 \pm 0.7$ μM and a $V_{max} = 3.3 \pm 0.3$ nmol min^{-1} $(10^8$ cells$)^{-1}$ (which is equivalent to 14.1 nmol min^{-1} (mg protein$)^{-1}$, Figure 4.3A). Cadaverine was transported by a rapid 6-to 7-fold lower affinity

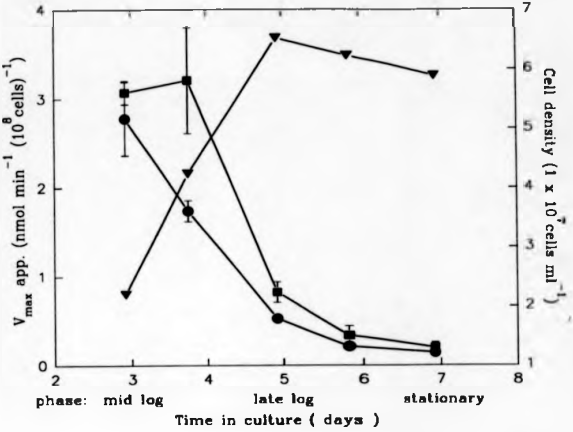

Figure 4.4 Changes in the maximum rate of putrescine and cadaverine transport with growth cycle phase. *T. cruzi* cells were diluted into fresh RTH media at 1 x 10^6 cells ml^{-1} and assayed over days 3 to 7 of their growth cycle for maximal diamine transport rates, V_{max} app., at 10μM putrescine (●) and 100μM cadaverine (■). For putrescine and cadaverine each point represents the mean value ± the standard deviation of 3 determinations. The cell density (▼) represents the mean of 2 determinations. Full details in section 2.2.2 of the methods.

Table 4.2 Alterations in K_m and V_{max} values for putrescine and cadaverine transport with cell growth phase

Time in culture (days)	Cell Density[1] (1×10^7 cells ml^{-1})	Putrescine Transport[2] K_m (μM)	Putrescine Transport[2] V_{max} (nmol min^{-1} $(10^8 cells)^{-1}$)	Cadaverine Transport[2] K_m (μM)	Cadaverine Transport[2] V_{max} (nmol min^{-1} $(10^8 cells)^{-1}$)
3	2.32	1.99 ± 0.67	3.29 ± 0.34	13.4 ± 3.9	3.86 ± 0.32
5	6.53	2.06 ± 0.65	0.61 ± 0.06*	45.0 ±19.3	1.01 ± 0.21*
7	5.89	2.69 ± 0.63	0.17 ± 0.01*	nd[3]	nd

[1] Cell density is the mean value of 2 determinations; [2] K_m and V_{max} values were determined using the Enzfitter software package (± SEM); [3] nd = not determinable; *Values differ significantly ($p<0.001$) as compared with 3 day cells, n=7 (the number of points used in Enzfit for the calculation of K_m and V_{max} values).

saturable carrier with a K_m =13.4 ± 3.8 μM and a V_{max} = 3.9 ± 0.3 nmol min^{-1} (10^8 cells)$^{-1}$ or 16.6 nmol min^{-1} (mg protein)$^{-1}$ (Figure 4.3B).

4.3 Alterations in the kinetics of putrescine and cadaverine transport during the growth cycle

Whilst measuring the kinetics of diamine transport in *T.cruzi* it was observed that cells assayed in late log or stationary phase (days 5-7) appeared to exhibit a similar K_m but lower V_{max} for both diamines than exponentially growing cells (day 3). When the kinetic parameters (K_m and V_{max}) were measured on the same batch of exponential (day 3), late log (day 5) and stationary (day 7) phase cells, it was found that the V_{max} decreased 20 fold as the cells went from exponential to stationary phase (Figure 4.4). However the K_m for putrescine remains essentially unchanged (Table 4.2), suggesting a loss of active transporters.

It should be noted here that most of the following work has been carried out solely on the tritiated diamine putrescine as it is a better substrate (lower K_m) and more readily available for characterisation of this transporter than cadaverine. Furthermore for Figure 4.4 and much of the subsequent work 10 μM putrescine (or 100 μM cadaverine for Figure 4.4) has been taken as a good estimate of the actual V_{max} for putrescine (cadaverine) transport, as can be seen from a comparison of Figures 4.4 and Table 4.2. Putrescine or cadaverine transport measured at 10 or 100 μM respectively has thus been termed the apparent maximum velocity (V_{max}app.).

4.4. Up- and down-regulation of diamine transport

In order to determine whether this up- and down-regulation of diamine

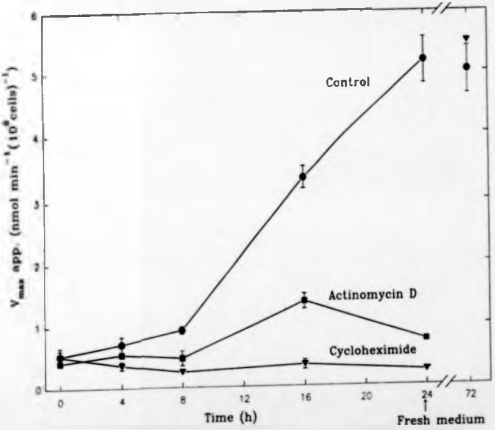

Figure 4.5 Recovery of putrescine transport. The *T.cruzi* cells were grown for 7 days and then diluted to 1 x 10^7 cells ml^{-1} into fresh medium supplemented with either 10 (or 100) µM cycloheximide (superposable values) or 2 µM actinomycin D and the velocity of 10 µM putrescine transport, V_{max} app., measured at 4 to 8 h intervals over the next 24 h. At 24 h control and 10 µM cycloheximide treated cells were washed once and resuspended at 1 x 10^{7}cells ml^{-1} in fresh medium and incubated for a further 48 h. Control cells were diluted into fresh medium alone. Each point represents the mean values ± the standard deviation of 3 determinations.

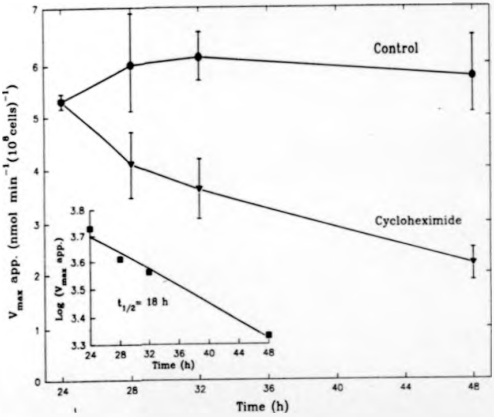

Figure 4.6 Effect of cycloheximide on maximal putrescine transport in *T.cruzi*. After 24 h the remaining control cells from Figure 4.5 were divided into two and incubated for a further 24 h ± 100 µM cycloheximide. The velocity of putrescine transport, V_{max} app., was measured at 24, 28, 32 and 48 hours, each point representing the mean ± the standard deviation of 3 determinations. **Insert:** From a log(V_{max} app.) versus time transformation of the data a half life of 18 hours was obtained for the transporter.

transport during the growth phase required new protein and RNA synthesis, stationary phase cells were diluted, into fresh RTH+FCS medium supplemented with the protein synthesis inhibitor cycloheximide, or the RNA synthesis inhibitor actinomycin D and their maximum rate (V_{max}app.) of putrescine transport measured over the next 24 h (Figure 4.5). In control cells, after an initial lag period lasting approximately 8 h, there was a progressive increase in the V_{max}app. of putrescine transport to a maximum after 24 h. No such corresponding increase in the V_{max}app. of putrescine transport was seen in the cells treated with cycloheximide or actinomycin D, suggesting that both protein and RNA synthesis are required for the up-regulation of putrescine transport upon dilution into fresh RTH+FCS medium.

At 24 h some of the control and cycloheximide treated cells were washed once in CBSS, resuspended at 1 x 10^7 cells ml^{-1}, in fresh RTH+FCS medium and incubated for a further 48 h (Figure 4.5). The recovery of putrescine transport activity in the cycloheximide treated cells to control levels indicates that the cycloheximide is having a specific and reversible effect on the synthesis of the transporter.

When, after 24 h in fresh RTH+FCS medium, cycloheximide was added to half the remaining control cells (Figure 4.5), then only in the cycloheximide treated cells was there a steady reduction in the V_{max} of putrescine transport over the next 24 h (Figure 4.6). From these data, it can be calculated that the putrescine transporter in *T.cruzi* turns over with a half-life of approximately 18 h (insert in Figure 4.6).

When *T.cruzi* cells exhibiting maximal putrescine transport velocities (24 h point on Figure 4.5) are incubated with 10 μM putrescine, 100 μM cycloheximide or 2 μM actinomycin D for 24 h then assayed for remaining [^{3}H]putrescine transport activity only 82, 38 or 42% respectively of the control cells activity (48 h point on

Table 4.3 Effect on maximal putrescine transport velocities of pre-incubation of *T.cruzi* cells with cycloheximide, actinomycin D or putrescine

Additions	V_{max} app.[1] (nmol min^{-1} $(10^8 cells)^{-1}$)	V_{max} app.[1] (% of control)
Control (no additions)	5.70 ± 0.69	100
10 μM Putrescine	4.65 ± 0.52	82
100 μM Cycloheximide	2.18 ± 0.32	38
2 μM Actinomycin D	2.46 ± 0.08	43

Day 7 (stationary phase) cells were diluted at 1 x 10^7 cells ml^{-1} into fresh RTH+FCS medium, grown for 24 hours in order to achieve maximal putrescine transport velocity, then washed once in CBSS and grown for a further 24 hours in fresh RTH+FCS medium containing the additions indicated above. [1] Measured at 10 μM putrescine ± the standard deviation of 3 determinations.

Table 4.4 Effect on maximal putrescine transport velocities of pre-incubation of *T.cruzi* cells in RTH+FCS supplemented with 100 μM exogenous putrescine or pre-conditioned for 4-7 days with *T.cruzi* cells (day 4 or 7 supernatant)

Addition	V_{max} app.[1] (nmol min^{-1} $(10^8 cells)^{-1}$)	V_{max} app.[1] (% of fresh medium control)
Day 7 cells	0.26 ± 0.03	6
24 hours after dilution into:		
Fresh media		
No additions	4.24 ± 0.35	100
+100μM Putrescine	0.49 ± 0.05	12
Day 4 supernatant		
No additions	1.48 ± 0.09	35
(Day 4 cells[2]	2.10 ± 0.27	49)
Day 7 supernatant		
No additions	0.99 ± 0.05	23
+100μM Putrescine	0.05 ± 0.03	1

Day 7 (stationary phase) *T.cruzi* cells were washed in CBSS then diluted at 1 x 10^7 ml^{-1} into either fresh RTH+FCS medium or day 4 or 7 supernatant (RTH+FCS medium which had been incubated with *T.cruzi* cells, seeded at 1 x 10^6 ml^{-1}, for 4 or 7 days respectively and then had the cells removed by centrifugation prior to use). 100μM putrescine was added at the start to half the cells incubated in fresh medium or day 7 supernatant. The V_{max} app. of putrescine transport was then measured 24 h later. [1] Measured at 10 μM putrescine ± standard deviation of 3 determinations; [2]*T.cruzi* cells seeded at 1 x 10^6 cells ml^{-1} into fresh RTH+FCS medium and grown for 4 days prior to measurement of putrescine transport.

Figure 4.6) was left (Table 4.3). Taken together these findings suggests both a loss of transporter protein molecules (cycloheximide) and the ribosomal message itself (actinomycin D) for this transporter. Over 24 h 10 μM exogenous unlabelled putrescine exerts a small negative feedback response on its own transport of about 20%, indicating only a weak regulatory response at this concentration.

Conversely addition of 100 μM putrescine for 24 h when stationary phase cells (day 7) were diluted into fresh RTH+FCS medium, prevented the normal increase in the velocity of putrescine transport which is observed in the control cells. Likewise when stationary phase cells were diluted into day 7 supernatant supplemented with or without 100 μM putrescine, the velocity of putrescine transport was lowered in the putrescine treated cells with respect to the untreated controls (Table 4.4). This demonstrates that addition of 100 μM exogenous putrescine causes negative feedback inhibition of the putrescine transporter, by preventing its up-regulation on dilution into fresh medium. Likewise incubation of stationary phase cells in day 4 supernatant caused the cells to attain a putrescine transport velocity which was intermediary to cells incubated in fresh RTH+FCS medium or day 7 supernatant. This suggests a number of possibilities including:

1) a component of the fresh RTH+FCS medium, which is used up as the cells proceed through the growth cycle, may be required to attain maximum putrescine transport velocity.

2) the cells release an inhibitor molecule into the medium as they proceed through the growth cycle, which then shuts off the putrescine transport system.

3) since polyamines are required for cell growth, then it is possible, if the putrescine supply is limiting, that the transporter is one of a number of cellular

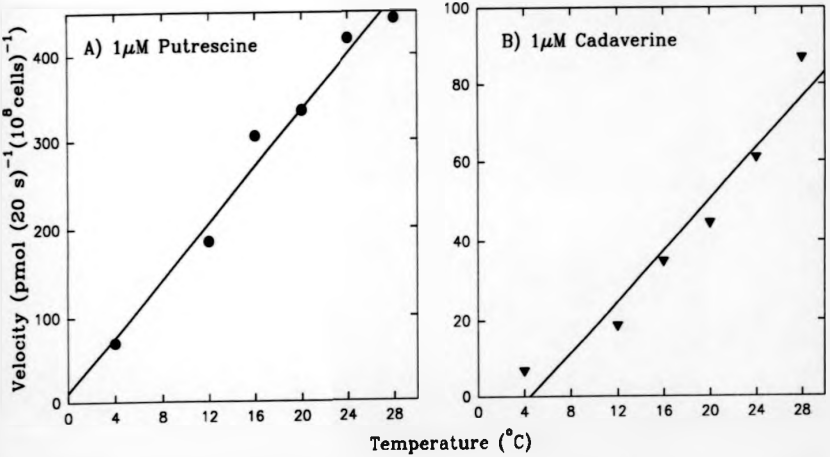

Figure 4.7 Changes in the rate of 1 μM putrescine and cadaverine transport with temperature. *T.cruzi* cells were taken on day 5 of the growth cycle and the transport of 1 μM putrescine and cadaverine was measured over 20 s at the temperatures indicated. Single observations were made and a background containing radiolabel but no cells was subtracted from each point.

systems which is switched on (up-regulated) when cell growth is initiated and conversely shut off (down-regulated) when the cells reach quiescence. However if the putrescine supply is not limiting, then there may be no need for the cells to up-regulate their diamine transport during cell growth.

4.5 Temperature dependence of transport

Unless otherwise stated further characterisation of the diamine transport system was carried out on exponentially growing (day 3) *T.cruzi* cells.

Initial results on late log phase cells seemed to indicate that both putrescine and cadaverine transport was highly temperature-dependent (Figure 4.7).

A more detailed analysis of the temperature dependence of putrescine transport was carried out over the temperature range of 12-28°C, using 10 μM putrescine to obtain the $Vmax_{app.}$ at 12, 16, 20, 24 and 28°C. Using this information the energy of activation for putrescine transport can be obtained from the Arrhenius equation:

$$k = Ae^{-Ea/RT}$$

where k is the rate constant for the reaction which is V_{max}app. in this case; A is the constant this reaction; E_a is the activation energy; R is the ideal gas constant (8.31 $J.K^{-1}mol^{-1}$) and T is the temperature in Kelvin.

The activation energy is most conveniently calculated from the Arrhenius equation by conversion into a logarithmic form:

$$\log (V_{max}\text{app.}) = \frac{-E_a}{2.3R} \cdot \frac{1}{T} + \log A$$

Plotting Log V_{max}app. against the reciprocal of temperature, measured in Kelvin gives

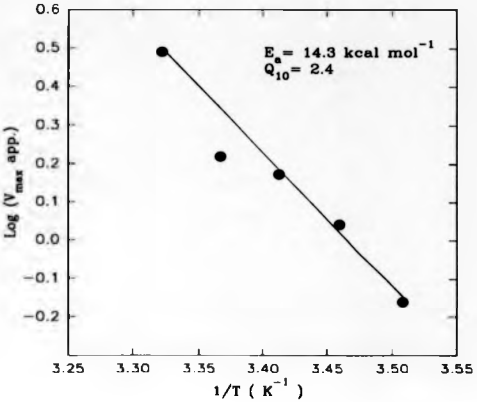

Figure 4.8 Arrhenius plot for putrescine transport. From the slope of the graph ($-E_a$ / 2.3R) an activation energy, E_a, of 14.3 kcal/mol was obtained for putrescine transport. The Q_{10} value which represents the increase in V_{max} app. observed when the temperature is raised by 10 degrees was found to be 2.4. The points shown here are mean values from 2 observations. They fit a straight line if the 24°C point is excluded. Therefore the 24°C point was omitted in the determination of the E_a and Q_{10} values.

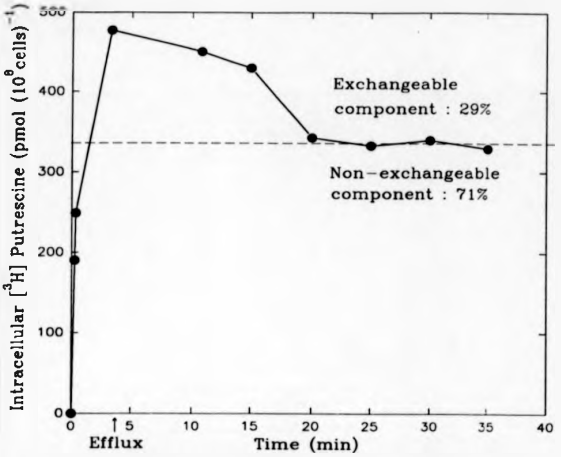

Figure 4.9 Pattern of putrescine efflux. *T.cruzi* cells taken on day 5 of their growth cycle, were preloaded with 1 μM [^{3}H]putrescine, at a concentration of 1 x 10^8 cells ml^{-1}, for 3 minutes 20 s, then the cells were pelleted, the supernatant removed and an equal volume of 1 μM unlabelled putrescine added. Cells were assayed at about 5 min intervals over the next 35 min, to estimate how much [^{3}H]putrescine was left in the cells at each time point. A background, in which cells were added to the appropriate radiolabel at 4°C and then spun down immediately, was subtracted from each uptake measurement.

an activation energy, E_a, of 14.3kcal mol^{-1} and an increase in the V_{max}app. over a 10^0 elevation of the temperature, Q_{10}, of 2.4 for the diamine transporter in the 12-28°C range (Figure 4.8). These values strongly suggest that diamine transport in *T.cruzi* epimastigotes is an active energy-dependent process.

4.6 Efflux of putrescine

In a preliminary experiment, late log phase *T.cruzi* cells were preloaded with 1 μM [^{3}H]putrescine, transferred to 1 μM unlabelled putrescine and assayed over the next 35 min to estimate how much [^{3}H]putrescine remained inside the cells (Figure 4.9). This suggested that about a quarter of the [^{3}H]putrescine taken up by the cells is free to exchange with the exogenous unlabelled putrescine, whilst the rest is presumably sequestered by the cells. As this work was not taken any further even this conclusion must be viewed with some caution. Furthermore, as the efflux of [^{3}H]putrescine was measured over minutes as opposed to seconds, a substantial amount of metabolism of the putrescine label is likely to have occured, since in section 3.1.2.1 it was found that 99% of the putrescine label was metabolised over a 2 h time period. These metabolites could then be affecting the observed pattern of 'putrescine' efflux from the cell.

4.7 Effect of ionophores, metabolic inhibitors and thiol reagents

The effect of these reagents on *T.cruzi* putrescine transport is summarized in Table 4.5. Of the ionophores and metabolic inhibitors only the mitochondrial proton gradient uncoupler, carbonylcyanide *m*-chlorophenyl hydrazone (CCCP) (Heytler, 1963) and 2,4 dinitrophenol, which uncouples electron transport from ATP formation

Table 4.5 Effect of ionophores, metabolic inhibitors and thiol reagents on putrescine transport

Inhibitor	Transport[1] (% Inhibition)
Ionophores:[2]	
20nM Gramicidin[4] (Na^+/K^+)	13
2μM Valinomycin (K^+)	28
10μM Carbonylcyanide *m*-chlorophenyl hydrazone (H^+ uncoupler)	90
2μM Calcimycin - A23187 (Ca^{2+})	29
Metabolic inhibitors:[3]	
1μg/ml Oligomycin (Mitochondrial ATPases)	-3
1mM Ouabain (Na^+/K^+ pump)	7
1mM 2,4 Dinitrophenol (uncoupler of electron transport)	67
1mM Iodoacetate (glycolysis)	37
Thiol reagents:[3]	
1mM *N*-Ethylmaleimide	>99
200μM *p*-Hydroxymercuribenzoate	>99
200μM *p*-Chloromercuriphenyl sulphonate	99

[1] Control 2 μM Putrescine Velocity = 3.0 nmol $min^{-1}(10^8 cells)^{-1}$ and represents zero percent inhibition. [2] 20 minute pre-incubation with the cells. [3] 10 minute pre-incubation with the cells. The results expressed here are the mean values of 2 observations. [4]The gramicidin is used at such a low concentration because it is a channel former and so approximately 1000 fold more efficient than the other ionophores which are all mobile ion carriers (Pressman 1976).

(Slater, 1967), were able to bring about greater than 30% inhibition of putrescine transport, causing 90 and 67% inhibition respectively. This suggests that putrescine transport in *T.cruzi* could be linked to a proton gradient like many other processes in micro-organisms (Henderson, 1990). Furthermore, the slight inhibitory effect (<30%) of the ionophores Gramicidin D, Valinomycin (in the presence of KCl) and A23187 and the strong inhibitory effect of CCCP (90%) on putrescine transport could all be linked, at least in part, to the fact that as well as dissipating the various ion gradients indicated (Table 4.5) they also disrupt the cells membrane potential (Kakinuma *et al.* 1988).

Three different thiol (sulphydryl) reagents of contrasting membrane permeabilities were used in order to probe the possible requirement for sulphydryl groups in the putrescine transport process. A 10 minute pre-incubation with any of the three reagents, at the concentrations indicated caused a 99% or greater inhibition of putrescine transport. The order of membrane permeability is *N*-ethylmaleimide (readily permeates membranes) > *p*-hydroxymercuribenzoate > *p*-chloromercuribenzene sulphonate (very membrane impermeable). As *N*-ethylmaleimide not only permeates the cells rapidly but is also highly toxic to the cells metabolism (Rothstein, 1970), causing reduced motility in *T.cruzi* cells after just 10 min incubation, it is difficult to separate its cytotoxic effects from that on external sulphydryl groups. However the fact that the relatively membrane impermeable reagents *p*-hydroxymercuribenzoate and *p*-chloromercuribenzene sulphonate (which have no affect on the motility of *T.cruzi*) also inhibit putrescine transport to the same extent as *N*-ethylmaleimide suggests that sulphydryl groups on the transporter itself or on some regulating external protein are important for the putrescine transport process.

Table 4.6 Effect of some amino acids and their derivatives on putrescine transport

AA System (side chain)	Amino acid (200μM)	Diamine precursor[1]	Transport (% inhibition[2])
A (neutral)	Asparagine	-	-12
	Serine	-	-13
L (neutral)	Leucine	-	-16
Ly^+ (basic)	Lysine	Cadaverine	0
	Arginine	Putrescine (via ornithine or agmatine)	-1
β^* (acidic)	Aspartic acid	-	7
-	Ornithine	Putrescine	-8
-	Agmatine	Putrescine	24

[1] Decarboxylation of these amino acids leads to the formation of putrescine or cadaverine, as indicated. Agmatine, the decarboxylated amino acid derivative of arginine, requires the removal of urea for putrescine formation. [2] Control 2 μM Putrescine Velocity = 2.5 nmol $min^{-1}(10^8 cells)^{-1}$ and represents zero percent inhibition. The results expressed here are the mean values of 2 observations.

4.8 Specificity of diamine transport process

4.8.1 Effect of some amino acids

The effect of selected amino acids on putrescine transport from each of the main amino acid transport systems found in cells (Christensen, 1979) together with the theoretical putrescine precursors ornithine and agmatine has been investigated (Table 4.6). None of the amino acids from either the system A (neutral side chains), L (neutral side chains), Ly^+ (basic side chains) or β^+ (acidic side chains) transport systems show any marked inhibitory affect on putrescine transport at a 100 fold excess. If anything the converse is true of the system A and L amino acids which actually appear to be stimulating putrescine transport by about 10-15%. Stimulation of polyamine uptake by system A amino acids is also observed in many mammalian cells (reviewed in section 1.5.5). Ornithine, the basic amino acid precursor to putrescine which is a homologue of lysine containing one less methylene group in its side chain does not influence putrescine transport. However agmatine, a decarboxylated analogue of arginine, is able to lower putrescine transport by 24% at a 100 fold excess. Commercial sources of agmatine are contaminated with putrescine (19% putrescine by peak area on HPLC analysis of Aldrich agmatine, M.R.Ariyanayagam and A.H.Fairlamb- unpublished observation) but this was removed prior to use in the transport assays by purification through a cation exchange column by M.R.Ariyanayagam of our laboratory. Hence agmatine seems to be a weak inhibitor of the putrescine transport system. Although agmatine is certainly taken up by *T.cruzi* (M.R.Ariyanayagam and A.H.Fairlamb, unpublished observations), this work is not able to distinguish the type of inhibition or indeed whether the agmatine is actually being taken up into the cells on the putrescine transporter.

Table 4.7 Effect of methylglyoxal *bis*(guanylhydrazone) and paraquat on putrescine transport

Inhibitor	Transport (% Inhibition[1])
50μM MGBG	14
500μM MGBG	20
50μM Paraquat	6
500μM Paraquat	17

Uptake measurements were carried out on *T.cruzi* cells which had been grown in RTH+FCS medium for 5 days. The ability of a 10- or 100-fold excess of MGBG or paraquat to inhibit 5 μM [^{3}H]putrescine transport when added simultaneously was investigated. [1] Control 5 μM Putrescine Velocity = 1.6 nmol $min^{-1}(10^8 cells)^{-1}$ and represents zero percent inhibition. The results expressed here are the mean values of 2 observations.

These results indicate that putrescine is not taken up on any of the known amino acid uptake systems. It also suggests that it is the presence of a carboxyl group, attached to the α-carbon atom of an amino acid, which prevents recognition of the basic amino acids by the putrescine transporter. Thus steric factors or alteration of charge may be involved.

4.8.2 Effect of MGBG, paraquat

Both MGBG and paraquat, which share structural similarities with the polyamines, are known to be at least partial inhibitors of many mammalian polyamine uptake systems (Byers *et al.* 1987; Rannels *et al.* 1989; Hyvonen *et al.* 1994; Balana-Fouce *et al.* 1989; Seiler & Dezeure, 1990; Khan *et al.* 1991). At a 100-fold excess of either MGBG or paraquat there is only a slight approximately 20% inhibition of putrescine transport suggesting that they are both weakly recognised by the putrescine transporter (Table 4.7).

4.8.3 Effect of sodium ions

Many mammalian polyamine uptake systems appear to exhibit sodium dependency (reviewed in section 1.5.3). When the Na^+ in CBSS was iso-osmotically replaced with Ch^+ and 1 μM putrescine transport activity measured, it was found that putrescine transport activity was 2.9-fold higher in *T.cruzi* cells incubated in the Na^+ free CBSS than in control cells assayed in the ordinary Na^+ containing CBSS. This suggests that Na^+ is actually exerting an inhibitory affect on putrescine transport activity in *T.cruzi* like that observed in the filamentous fungus *N.crassa*, where both putrescine and spermidine transport are inhibited by monovalent cations (Na^+, NH_4^+

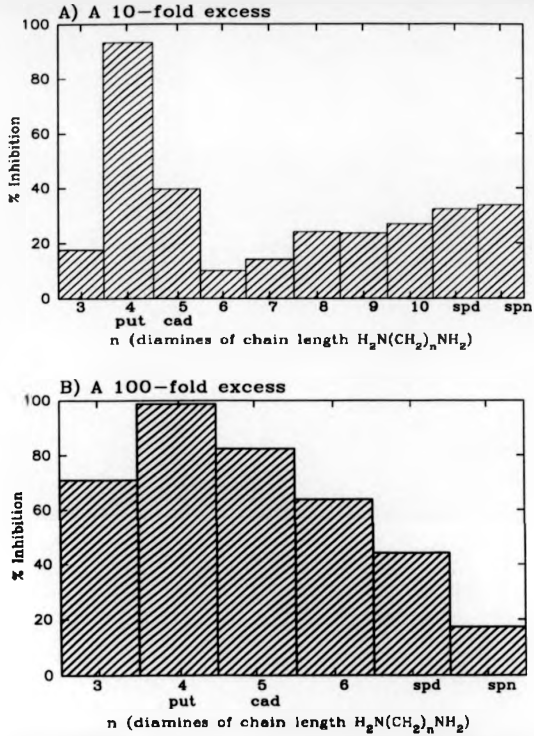

Figure 4.10 Inhibition of putrescine transport by diamines and polyamines. Cells were put into fresh RTH+FCS medium at 1 x 10^6 ml^{-1} and grown for 5 days. The ability of a 10- or 100-fold excess of the appropriate unlabelled diamine or polyamine to inhibit 5 μM [^{3}H]putrescine transport when added simultaneously was investigated. The values given here are the means of two observations. A) A 10-fold excess; control 5 μM putrescine velocity = 1.9 nmol min^{-1} 10^6cells^{-1}. B) A 100-fold excess; control 5 μM putrescine velocity = 1.3 nmol $min^{-1}10^6cells^{-1}$.

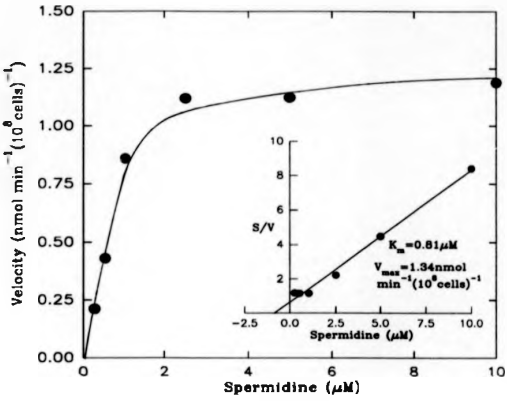

Figure 4.11 Kinetics of spermidine transport in *T.cruzi* epimastigotes. The curve is fitted and the K_m and V_{max} values calculated using the Enfitter software package. Insert: Hanes-Woolf plot, a linear S/V versus [S] transformation of the data.

and K^+) (Davis & Ristow, 1988). In addition as the Na^+/K^+ ionophore Gramicidin has only a small inhibitory affect this further suggests that putrescine transport is not Na^+-dependent (Table 4.5) especially when it is taken into account that Gramicidin D, along with the other ionophores tested in Table 4.5, also disrupt the membrane potential of the cells (Kakinuma *et al.* 1988).

4.8.4 Effect of other diamines and polyamines

Inhibition of 5 μM putrescine transport was carried out by using a 10- or 100-fold excess of diamines (of the general formula $NH_2(CH_2)_nNH_2$, where n=3-10) or the polyamines spermidine and spermine (Figure 4.10A and B). This indicates that putrescine has the highest affinity for the transporter followed by cadaverine, with the other diamines and polyamines showing a lower specificity for the transporter.

4.8.5 Evidence for the existence of multiple diamine and polyamine transporters

In order to try and establish whether there is either a single common or separate carrier(s) for the transport of diamines and polyamines, it was first necessary to establish the kinetics of polyamine transport. This was carried out by measuring [^{3}H]spermidine transport into *T.cruzi*. (Spermine kinetics were not established as no tritiated label was available and the [^{14}C]spermine was of too low a specific activity to get reliable measurements). [^{3}H]spermidine was transported into exponentially growing cells (day 3) with saturable Michaelis-Menten-type kinetics (Figure 4.11). Like putrescine, spermidine was transported with a similar high affinity, K_m = 0.81 ± 0.22 μM but with a V_{max} = 1.34 nmol min^{-1} (10^8cells)$^{-1}$ which was 3-4 fold lower than that observed for putrescine.

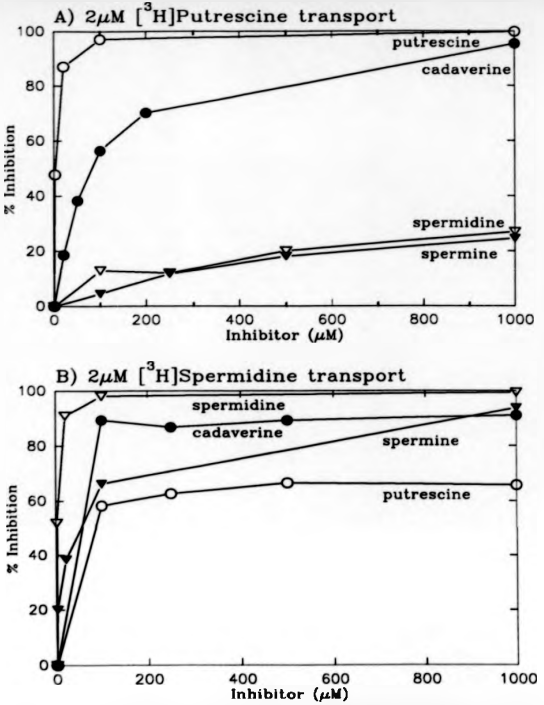

Figure 4.12 Inhibition of 2 μM [^{3}H]putrescine or [^{3}H]cadaverine transport into *T.cruzi* epimastigotes by unlabelled putrescine, cadaverine, spermidine or spermine. The pattern of inhibition of 2 μM [^{3}H]putrescine or [^{3}H]spermidine transport was examined on concurrent addition of 2-1000 μM concentrations unlabelled putrescine, cadaverine, spermidine and spermine. The points represent single observations. A) 2 μM [^{3}H]putrescine; control 2μM putrescine velocity = 3.1 nmol min^{-1} 10^{6}cells^{-1}. B) 2 μM [^{3}H]spermidine; control 2 μM spermidine velocity = 1.6 nmol min^{-1} 10^{6}cells^{-1}.

This was followed up by studies by using 2-1000 μM concentrations of the unlabelled diamines, putrescine and cadaverine and the polyamines, spermidine and spermine to inhibit the transport of 2 μM [^{3}H]putrescine or [^{3}H]spermidine (Figure 4.12A and B). Inhibition of [^{3}H]putrescine transport by unlabelled putrescine gives the expected pattern of inhibition with equimolar concentrations of unlabelled and labelled substrate reducing putrescine transport by 50% and a 10 fold excess of unlabelled putrescine reducing [^{3}H]putrescine transport by 90% (Figure 4.12A). At a 500 fold excess cadaverine caused >95% inhibition of [^{3}H]putrescine transport, whereas even at a 500 fold excess spermidine and spermine gave <30% inhibition (Figure 4.12A). Likewise inhibition of [^{3}H]spermidine by unlabelled spermidine (Figure 4.12B) gave the same pattern of inhibition as observed for [^{3}H]putrescine by unlabelled putrescine (Figure 4.12A). Inhibition of [^{3}H]spermidine transport by spermine (Figure 4.12B) follows a similar pattern to that observed with inhibition of [^{3}H]putrescine transport by cadaverine (Figure 4.12A). However the inhibition of [^{3}H]spermidine transport by the diamines putrescine and cadaverine (Figure 4.12B) is quite different from the inhibition pattern observed for [^{3}H]putrescine transport by the polyamines spermidine and spermine (Figure 4.10A), as at a 50 fold excess putrescine and cadaverine inhibit [^{3}H]spermidine transport by about 60 and 90% respectively whereas spermidine and spermine at a 50 fold excess only inhibit [^{3}H]putrescine transport by about 10%.

The K_m values of putrescine and spermidine transport are 1.34 ± 0.41 μM and 0.81 ± 0.22 μM respectively when measured at a similar time on exponentially growing cells. Therefore as the affinities for putrescine and spermidine transport are comparable this would suggest that in order for both compounds to be transported on a common transporter equimolar concentrations of each compound should inhibit the

others transport by about 50%. As this is not the case, the simplest interpretation of this data suggests the existence of at least two carrier systems for the transport of diamines and polyamines into *T.cruzi*. The first would be a diamine transporter with high specificity for the diamines, putrescine and cadaverine, and low specificity for the polyamines spermidine and spermine, which we have termed Pot1. The second would probably be a more general diamine and polyamine transporter, with the highest specificity being for spermidine and the lowest for putrescine, which we have called Pot2. In the light of this evidence it is possible that MGBG and paraquat (see section 4.8.2), which only inhibit putrescine transport by approximately 20% even at a 100 fold excess, may also go up predominantly on this putative general diamine and polyamine transporter.

4.9 Conclusions

T.cruzi contains specific high affinity saturable carriers for the transport of diamines and polyamines. A fairly selective diamine transporter has been characterised here, which exhibits a high affinity for putrescine, a moderate affinity for cadaverine and a low affinity for spermidine and spermine. This diamine transporter does not transport amino acids, is temperature dependent, appears to require sulphydryl groups and a proton gradient for maximal transport and its activity is altered as the cells proceed through the growth cycle. If the mammalian stages of *T.cruzi*, namely the bloodstream trypomastigotes and intracellular amastigotes possess similar rapid, high affinity transporters for diamines and polyamines to those found in the epimastigotes, then it will be important to take this into account when planning future chemotherapeutic strategies involving interference with *T.cruzi* polyamine metabolism.

CHAPTER 5 : DISCUSSION

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Previous to this study there have been very few reports on polyamine metabolism in *T.cruzi* (Schwarcz de Tarlovsky *et al.* 1993; Algranati *et al.* 1989), other than to indicate that the epimastigote form contains putrescine, cadaverine, spermidine and spermine. A minimum level of these diamines and polyamines are required by all cells for them to function normally (Tabor & Tabor, 1984; Pegg & McCann, 1988; Marton & Morris, 1987; Pegg, 1986; Tabor & Tabor, 1985). However, how *T.cruzi* acquires its putrescine (*de novo* synthesis versus uptake) is a subject open to much controversy.

Initial studies indicated that DFMO, which irreversibly inhibits ODC and so prevents the *de novo* synthesis of putrescine from ornithine, kills African trypanosomes (Bacchi *et al.* 1980b; Schechter & Sjoerdsma, 1986) but has no effect even at high concentrations against *T.cruzi* model infections (Hanson *et al.* 1982). DFMO treatment did not exert a marked inhibitory effect on the growth of *T.cruzi* epimastigotes (Tabor & Dobbs, 1970; Schwarcz de Tarlovsky *et al.* 1993) nor did it alter the ability of the trypomastigote stage to infect macrophages or myoblasts at concentrations up to 100 mM (Kierszenbaum *et al.* 1987b). As anticipated from these findings no more than trace amounts of ODC activity could be detected in these parasites (Algranati *et al.* 1989). Conversely DFMA, an irreversible inhibitor of ADC which prevents the *de novo* synthesis of putrescine from arginine, appeared at high concentrations to decrease the ability of *T.cruzi* to invade and multiply within mammalian cells (Kierszenbaum *et al.* 1987a). After much effort ADC activity was detected in *T.cruzi* trypomastigote / amastigote mixtures (Majumder *et al.* 1992) but at levels some 100,000 times lower than the ADC activity found in *E.coli* (Kallio *et*

al. 1981) and 1000-fold lower than the ODC activity found in *T.b.brucei* bloodstream trypomastigotes (Phillips *et al.* 1987). In the epimastigote form there was no detectable conversion of ornithine, arginine or lysine into polyamines (Hunter *et al.* 1994). In addition *T.cruzi* epimastigotes preferentially take up the polyamines putrescine and spermidine from the medium in comparison with the basic amino acids lysine, ornithine and arginine (Algranati *et al.* 1989). All this evidence suggests that the amino acid decarboxylases play a negligible role in the production of polyamines in these parasites. Instead, this work demonstrates that *T.cruzi* epimastigotes are able to scavenge trace amounts of diamines and polyamines from the medium on inducible, high affinity saturable transport systems supporting the notion that *T.cruzi* epimastigotes take up rather than synthesize *de novo* the putrescine and cadaverine found in their cells.

5.1 Transport studies

Firstly from the uptake studies in the trypanosomatids it can be seen that *T.b.brucei* (procyclics and bloodstream forms) which contain an active ODC, appear incapable of taking up any more than trace amounts of the diamines putrescine and cadaverine (Figure 4.1). Given their extracellular location (in tissues and later in the central nervous system) this may help to explain why DFMO is effective against these parasites. Conversely DFMO is ineffective against *T.cruzi* epimastigotes which do not appear to contain ODC but do rapidly accumulate putrescine and cadaverine from their surroundings (Figure 4.1). DFMO is not particularly effective against either *C.fasciculata* or *L.donovani* promastigotes, unless they are grown in a polyamine free medium (Hunter *et al.* 1991; Kaur *et al.* 1986, Gonzalez *et al.* 1991) as both of them

under the appropriate conditions can overcome the block by taking up diamines (in particular putrescine) from the medium.

The fact that K_m for putrescine and cadaverine in *T.cruzi* epimastigotes is in the micromolar range may be of physiological significance, since following a blood meal the excreta of one *T.cruzi* vector, *R.prolixus*, contains micromolar quantities of the diamines putrescine and cadaverine. Furthermore the *T.cruzi* diamine transporter appears to be induced in response to favourable growth conditions, for example on dilution into fresh growth medium containing FCS (section 4.4). A similar type of induction of both polyamine uptake (reviewed in section 1.5.6.3) and ODC activity (reviewed in (Bachrach, 1984)) can be observed in mammalian cells in response to various growth stimuli. Conversely, in *T.cruzi* epimastigotes the presence of large amounts of putrescine in the medium (100 μM) seems to inhibit this induction (section 4.4). The transport of diamines into *T.cruzi* seems to be an active temperature-dependent process requiring the presence of extracellular thiol groups. Transport can be strongly inhibited upon pre-incubation of the cells with the protonophore carbonylcyanide *m*-chlorophenyl hydrazone, suggesting that a membrane potential is involved. However, it is not possible to say whether diamine transport in *T.cruzi* is coupled to a proton gradient like those found in many bacterial metabolite-transport systems (Henderson, 1990). Both polyamine uptake systems in *E.coli* have nucleotide binding sites (Furuchi *et al.* 1991; Pistocchi *et al.* 1993). Furthermore it has been shown that the spermidine preferential uptake system in *E.coli* has an absolute requirement for ATP (Kashiwagi *et al.* 1993) and that a membrane potential is also involved (Kashiwagi *et al.* 1986; Kashiwagi *et al.* 1993). Hence it is possible that this could also be the case in *T.cruzi* although there is no direct evidence to support this

hypothesis at the moment. Both proton-motive force driven active transporters and facilitated diffusion systems have been described for the transport of nutrients in the trypanosomatids (reviewed in Zilbertsein, 1993). However the 'active' nature of some of these transport systems has since been questioned due to the difficulty of separating transport across the plasma membrane from either the subsequent rapid metabolism of molecules such as glucose and proline or their sequestration into intracellular organelles such as the glycosome for glucose (ter Kuile, 1993). Hence the apparent 'active' transport of diamines into *T.cruzi* observed here must be viewed with extreme caution.

The results indicate the presence of at least two diamine / polyamine transport systems in *T.cruzi* epimastigotes, one of which has high specificity for the diamines and low specificity for polyamines and the other is a more general diamine and polyamine transporter. Mammalian diamine / polyamine transporters usually seem to exhibit a higher affinity for polyamines than diamines (Table 1.3), with those diamines (1,7-diaminoheptane and 1,8-diaminooctane) of a similar chain length to spermidine being the best inhibitors of putrescine (Rajanayagam *et al.* 1992; Porter *et al.* 1984), spermidine (Porter & Bergeron, 1983; Porter *et al.* 1984) and spermine (Porter *et al.* 1984) uptake. This suggests that the diamine and polyamine transport systems of *T.cruzi* are more like those found in *E.coli*, which contains both putrescine specific and spermidine (spermine) preferential uptake systems (Furuchi *et al.* 1991; Piatocchi *et al.* 1993). Putrescine can then be excreted from *E.coli* cells through the potE protein (Kashiwagi *et al.* 1991) which is a putrescine-ornithine antiporter (Kashiwagi *et al.* 1992). It is not known whether a similar such system is present in *T.cruzi*. Either way, if *T.cruzi* epimastigotes truly are dependent on uptake for all their diamines with no

de novo synthesis, then it is not altogether surprising that they contain more than one uptake system for diamines and polyamines as a kind of insurance policy against one being lost. As polyamines are required for growth (Heby, 1981) and both dividing forms of *T.cruzi* (the epimastigote in the insect gut and the amastigote in host cells) are going to be bathed in micromolar levels of diamines and polyamines, it could be argued that these stages do not require a capacity for *de novo* synthesis when they can simply take them up from their surroundings. Perhaps then the activity of these transporters might play a central role in the overall control of *T.cruzi's* intracellular polyamine levels in a similar fashion to that observed with mammalian ODC, the dominant controlling factor of their entire polyamine pathway (McCann & Pegg, 1992). Alternatively a lack of tight regulation, similar to that observed with *T.b.brucei* ODC (Wang, 1991; Ghoda *et al.* 1992) potentially could be the downfall of *T.cruzi*.

However it first has to be established that diamine / polyamine transporter(s) are present in the mammalian forms of *T.cruzi*. If present, further characterization of this transporter(s) will then be required. Initially solubilization of the diamine transporter from the plasma membrane, followed by reconstitution into proteoliposomes could be attempted inorder to partially purify it and enable the properties of the transporter to be characterized in more detail. This has previously been carried out on the *T.b.brucei* D-glucose transporter (Seyfang & Duszenko 1993). Cloning, sequencing and expression of this transporter should then throw more light on both the nature and regulation of diamine transport in *T.cruzi*.

5.2 Metabolism

The labelling studies described here with putrescine and cadaverine show that

both compounds, in particular cadaverine, are much more avidly taken up and incorporated into other polyamines and thiols by *T.cruzi* epimastigotes than the non-pathogenic insect trypanosomatid *C.fasciculata* (section 3.1.1. and 3.1.2). The incorporation of putrescine into high levels of spermine (11.7 nmoles/10^8 cells) was unexpected as many trypanosomatids including *C.fasciculata* (section 3.1.1 and 3.1.2, (Fairlamb *et al.* 1986)) and *T.b.brucei* (Bacchi *et al.* 1977) do not appear to synthesize their own spermine. Although spermine has previously been detected in small amounts (<5 nmoles $(10^8 cells)^{-1}$) in *T.cruzi* epimastigotes (Algranati *et al.* 1989; Schwarcz de Tarlovsky *et al.* 1993) and trypomastigotes (Kierszenbaum *et al.* 1987a) this is the first report that the cells are able to synthesize it themselves under normal conditions as opposed to simply taking it up from the medium. The related trypanosomatid Leishmania appears to contain a small quantity of spermine (Table 1.2), which may be synthesized *de novo* (Bachrach *et al.* 1979). So what is the function of such high levels of spermine in *T.cruzi*? Radiolabelling studies with putrescine (Figure 3.2) indicate that the cells may be able to conjugate some of it to glutathione or other cellular components (U1 and U3), due to the increase in the spermine peak observed when the extract is acid hydrolysed. However spermine and the other polyamines do not appear to bind covalently to protein to any extent as there is very little radioactivity (<2%) found associated with the TCA precipitable material.

Labelling with spermidine and spermine shows these compounds are also readily taken up by *T.cruzi*. There is a trace amount of conversion of spermine to spermidine but this figure is <2% of the total label taken up by the cells. No putrescine is detected in either case, although it is possible that a small amount of conversion to *N*-acetylated polyamines occurs. This suggests that *T.cruzi* are unable

to oxidatively degrade (interconvert) spermine and spermidine to any great extent, unlike mammalian cells (Seiler, 1988).

While previous reports have detected cadaverine in *T.cruzi* epimastigotes (Algranati *et al.* 1989; Schwarcz de Tarlovsky *et al.* 1993), this work shows for the first time that these cells are able to convert cadaverine to aminopropylcadaverine and then through to homotrypanothione and *bis*(aminopropyl)cadaverine. In contrast, *C.fasciculata* can only convert cadaverine to aminopropylcadaverine. *T.b.brucei* bloodstream forms (at 2 x 10^8cells per injection onto the HPLC) do not under normal circumstances synthesize homotrypanothione (A.H.Fairlamb, unpublished observations), neither do *L.donovani* promastigotes even when grown on RTH+FCS supplemented with 5 μM cadaverine-confirm this. Furthermore, in various yeast and mammalian cells treated with DFMO, the addition of exogenous cadaverine in the absence of putrescine can lead to the production of aminopropylcadaverine and *bis*(aminopropyl)cadaverine (Hamana *et al.* 1989; Pohjanpelto *et al.* 1985b). However, this is the first report of a trypanosomatid being able to use cadaverine in place of putrescine for the synthesis of the trypanothione analogue homotrypanothione. As homotrypanothione is not formed in *C.fasciculata* and *L.donovani*, it is certainly not a process which is universal to trypanosomatids and so far appears to be unique to *T.cruzi*. Further confirmation of this is provided by the observation that the curative effect of DFMO in *T.b.brucei* treated mice can be antagonised by concurrent administration of putrescine, spermidine or spermine but not when treated similarly with cadaverine and 1,3-diaminopropane (Nathan *et al.* 1981).

These findings lead us to speculate whether *T.cruzi* has separate enzymes catalysing the synthesis of homotrypanothione and trypanothione from cadaverine and

putrescine respectively or whether there is just one set of enzymes present with a broader substrate specificity, enabling them to synthesize these analogues of the natural polyamines. The fact that conversion of cadaverine to aminopropylcadaverine appears to be slower than the equivalent putrescine to spermidine step (as judged by the 2 h labelling studies in PSG-BSA) perhaps suggests the latter hypothesis, especially as cadaverine only differs from putrescine by the presence of a single extra methylene group. However in order to answer these questions the polyamine synthase(s) from *T.cruzi* must first be purified then characterized. Only then can the specificity of the *T.cruzi* polyamine synthases be compared directly with those in mammalian cells.

An important question which arises from these studies is why do *T.cruzi* epimastigotes, which are normally resident in the mid- and hind-gut of the triatomine insect vector, take up and utilise cadaverine. In answer to this question analysis of the polyamine content of the excreta of a Chagas' disease vector, *R.prolixus*, immediately after a blood meal showed it to contain 4.5 μM putrescine, 1.1μM cadaverine, 0.9 μM spermidine and 0.5 μM spermine. It is likely that the cadaverine is produced by a genus of eubacteria, *Actinomyces*, which reside in the insect gut and are required for the successful development of the insect nymphs into the mature adult bugs (Brecher & Wigglesworth, 1944; Hamana & Matsuzaki, 1987). Hence from a physiological perspective, the epimastigote stage of the parasite could have adapted to metabolise cadaverine as well as putrescine for the production of polyamines and polyamine-glutathione conjugates. Whether a similar pattern of metabolism occurs in the mammalian host still needs to be determined although it has recently been established that the enzyme trypanothione reductase is present in the trypomastigote and

amastigote (mammalian stages) of the parasites life cycle (Moreno *et al.* 1994). However it is unclear whether there would be enough cadaverine present in mammalian cells to enable them to synthesize homotrypanothione under normal growth conditions. Mammalian cells do not contain a lysine decarboxylase, but their ODC can catalyse the decarboxylation of lysine to from cadaverine with a K_m that is about 100 times higher than for ornithine (McCann & Pegg, 1992). Thus this reaction may be of little physiological significance, occurring only when large amounts of ODC enzyme or a high lysine to ornithine ratio is present. This suggests that under normal physiological conditions there is probably relatively little cadaverine synthesized in mammalian cells. However it is worth noting that the intracellular amastigote stage of *T.cruzi* resides in the cytoplasm of the host's cells, and so will be bathed in micromolar levels of putrescine, spermidine and spermine (Morgan, 1990b) and therefore might not require the amino acid decarboxylases for the synthesis of putrescine (or cadaverine) at any stage of its life cycle involving growth and division.

Using homogenous recombinant *T.cruzi* trypanothione reductase it has been shown that the kinetic parameters for the reduction of homotrypanothione disulphide were similar to those of trypanothione disulphide giving rise to a k_{cat}/K_m ratios of 4.1 and 5.5 $\times$ $10^6 M^{-1} s^{-1}$ respectively (Hunter *et al.* 1994). Thus the kinetic properties of trypanothione reductase indicate that homotrypanothione is a physiological substrate of this enzyme, which is an important mediator in the cellular response to chemical oxidative stress by diamide and other agents (Fairlamb & Cerami, 1992; Kelly *et al.* 1993).

5.3 Prospects for chemotherapy

Provided the findings presented in this study are applicable to the amastigote

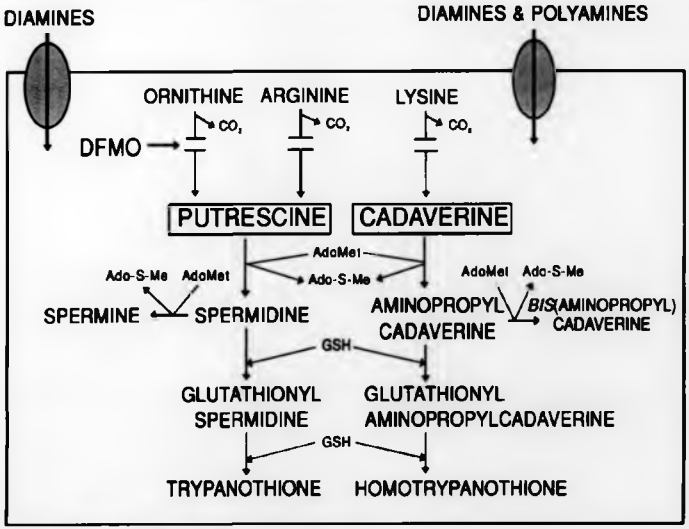

Figure 5.1 Uptake and metabolism of diamines in *T.cruzi* epimastigotes. There is no *de novo* synthesis of putrescine and cadaverine from the amino acids ornithine, arginine, and lysine.

stage then the results presented here are important with respect to the design of any prospective chemotherapeutic strategy which aims to interfere with polyamine biosynthesis. To help put all this in perspective a summary of diamine transport and metabolism in *T.cruzi* epimastigotes is provided (Figure 5.1). Firstly, inhibitors of the amino acid decarboxylases, for example DFMO and DFMA, have little effect on *T.cruzi*, due to the absence of these enzymes in the epimastigote, the detection of only trace levels of enzyme activity in the mammalian forms, and the ability of the parasite to overcome this block by taking up diamines from the environment. Secondly, it appears that *T.cruzi* epimastigotes can utilise a putrescine analogue, cadaverine, as a source of functional polyamines and polyamine-glutathione conjugates, unlike its human host. It is essential that this work be repeated in the mammalian forms of the parasite to establish whether they can utilise cadaverine in the same way as the epimastigotes. If they can, this apparent lack of specificity might be exploited in chemotherapy by producing compounds which could be metabolised by the less discerning enzymes of the parasite, but not recognised by the host. Thirdly, the presence of high affinity diamine and polyamine transport systems means that if they are also present in the mammalian stages, it may be possible to target toxic compounds, into the cell using these transporters. This of course presumes that they will not down-regulate the transporters in amastigotes stage, which is itself bathed in micromolar levels of the host cell's polyamines.

One of the main problems of drug design which has not been addressed thus far is the problem of getting the drug into the parasite. This is more complicated for *T.cruzi* than *T.b.brucei* as the former is an intracellular and the latter an extracellular parasite. Hence when considering drug design, ideally one would like them to be given orally as opposed to intravenously as this will cut both the cost of therapy and negate

the problems associated with having to be hospitalised during treatment. Therefore in order to exert its effect any drug designed would have to first be absorbed across the wall of the gut into the bloodstream, then be taken up by the mammalian cell and finally enter the parasite itself before exerting its activity. All this of course supposes that it doesn't get degraded before reaching the parasite itself! When coupled with the problem that once the disease enters its chronic phase autoimmunity may be involved (Petry & Eisen, 1989), this makes the process of drug design against *T.cruzi* an even greater challenge.

Therefore future strategies for the chemotherapy of Chagas' disease, instead of trying to inhibit the amino acid decarboxylases, might be more profitable if they concentrated on using the polyamine transport system of *T.cruzi* to target inhibitors of its aminopropyltransferases and the polyamine-glutathione synthetases into the cell.

CHAPTER 6 : REFERENCES

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