

LONDON  
SCHOOL of  
HYGIENE  
& TROPICAL  
MEDICINE



LSHTM Research Online

Le Quesne, SA; (1995) Transport and metabolism of polyamines in *Trypanosoma cruzi*. PhD thesis, London School of Hygiene & Tropical Medicine. DOI: <https://doi.org/10.17037/PUBS.04656691>

Downloaded from: <https://researchonline.lshtm.ac.uk/id/eprint/4656691/>

DOI: <https://doi.org/10.17037/PUBS.04656691>

**Usage Guidelines:**

Please refer to usage guidelines at <https://researchonline.lshtm.ac.uk/policies.html> or alternatively contact [researchonline@lshtm.ac.uk](mailto:researchonline@lshtm.ac.uk).

Available under license. To note, 3rd party material is not necessarily covered under this license: <http://creativecommons.org/licenses/by-nc-nd/3.0/>

<https://researchonline.lshtm.ac.uk>

**TRANSPORT AND METABOLISM  
OF POLYAMINES  
IN *TRYPANOSOMA CRUZI***

A thesis submitted to the Board of Studies in Biochemistry,  
University of London,  
for the degree of Doctor of Philosophy.

**1995**

**Sarah Ann Le Queene  
BSc.(Hons.)**

**Department of Medical Parasitology  
London School of Hygiene and Tropical Medicine**



### 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. [ $^3\text{H}$ ]Putrescine was rapidly taken up from the medium and incorporated into the polyamines spermidine, spermine and the glutathione-polyamine conjugate  $\text{N}^1, \text{N}^8$ -bis(glutathionyl)spermidine (trypanothione). Likewise [ $^3\text{H}$ ]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  $\text{N}^1, \text{N}^9$ -bis(glutathionyl)-aminopropylcadaverine (homotrypanothione). Detailed analysis has revealed that *T. cruzi* epimastigotes (clone X10/6) transport exogenous [ $^3\text{H}$ ]putrescine and [ $^3\text{H}$ ]cadaverine by a rapid, high affinity, temperature dependent, diamine transport system which exhibits saturable kinetics (putrescine  $K_m = 2.0 \mu\text{M}$ ,  $V_{max} = 3.3 \text{ nmol min}^{-1} (10^8 \text{ cells})^{-1}$ ; cadaverine  $K_m = 13.4 \mu\text{M}$ ,  $V_{max} = 3.9 \text{ nmol min}^{-1} (10^8 \text{ cells})^{-1}$ ). 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.

### ACKNOWLEDGEMENTS

I would like to thank Prof. Alan Fairlamb and Dr. Karl Hunter for all their help and advice in supervising this project and the rest of the group for their constant encouragement. In particular I would like to thank Dr. Karl Hunter for patiently explaining the intricacies of an HPLC system to me and Mr. Mark Ariyanayagam for all his advice on cell culture.

I would also like to thank Drs. P.P. McCann and A.J. Bitonti (Marion-Merrell-Dow Research institute, Cincinnati, USA) for the provision of aminopropylcadaverine, Dr. P.J. Rodrigues and Prof. M. Israel (Cancer Pharmacology Laboratories, University of Tennessee College of Medicine, USA) for the provision of *but*(aminopropyl)cadaverine, Dr. M.H. Park (National Institutes of Health, USA), for the provision of [1,5<sup>3</sup>H]cadaverine and Dr. C.J. Bacchi (Haskins Laboratories and Biology Department, Pace University, New York, USA) for communicating his initial findings on polyamine uptake in *T.cruzi*. I also wish to express thanks to the Wellcome Trust for their financial support of this work.

Finally I want to thank Paul and my family for their unfailing love and support and for keeping me going through the ups and downs of these past three years.

# TABLE OF CONTENTS

	PAGE
List of figures	9-10
List of tables	11-12
<b><u>CHAPTER 1: INTRODUCTION</u></b>	14-51
<b>1.1 Chagas' disease</b>	15
1.1.1 Disease burden	15
1.1.2 Life cycle of <i>Trypanosoma cruzi</i>	15
1.1.3 Clinical symptoms	16
1.1.4 Existing chemotherapy for Chagas' disease	17
1.1.5 An Integrated approach to the control of Chagas' disease	17
1.1.5.1 Transmission prevention	17
1.1.5.2 Future prospects for chemotherapy	19
1.1.5.3 Rational drug design	20
<b>1.2 Polyamines: Structure and function</b>	20
<b>1.3 Polyamine biosynthesis, retroconversion and regulation</b>	22
1.3.1 General characteristics	22
1.3.2 Polyamines in mammalian cells	23
1.3.2.1 An overview	23
1.3.2.2 Regulation	24
1.3.3 Polyamines in the Trypanosomatidae	27
1.3.3.1 Polyamine content	27
1.3.3.2 Trypanothione	28
1.3.3.3 Regulation	28
<b>1.4 Prospects for chemotherapy using agents which interfere with polyamine metabolism</b>	29
1.4.1 Inhibitors of ornithine and arginine decarboxylase	30
1.4.2 Inhibitors of S-Adenosylmethionine biosynthesis and metabolism	33
1.4.3 Interference with trypanothione metabolism	34
1.4.4 Interference with polyamine regulation	35
1.4.5 Potential problems with this approach	36

	6
	PAGE
1.5 Polyamine Uptake	36
1.5.1 General Characteristics	36
1.5.2 Energy dependency	37
1.5.3 Sodium dependency	39
1.5.4 Requirement for sulphhydryl groups	41
1.5.5 Interaction with amino acid transport systems	41
1.5.6 Other factors affecting polyamine uptake	42
1.5.6.1 Polyamine content of the cell's environment	42
1.5.6.2 Intracellular polyamine content	42
1.5.6.3 Growth conditions	43
1.5.7 Regulation of polyamine uptake	46
1.5.7.1 A role for antizyme	46
1.5.7.2 A possible role for the Na <sup>+</sup> -K <sup>+</sup> pump, protein kinase C and Ca <sup>2+</sup>	46
1.5.8 Specificity of uptake	47
1.5.9 Molecular Characterisation	49
1.5.10 Therapeutic approaches	50
<b>CHAPTER 2: MATERIALS AND METHODS</b>	52-65
2.1 Metabolic labelling studies	53
2.1.1 Cell culture	53
2.1.2 Purification of the radiolabels	53
2.1.2.1 [ <sup>3</sup> H]Cadaverine	53
2.1.2.2 [ <sup>14</sup> C]Spermidine and [ <sup>14</sup> C]Spermine	54
2.1.3 Radiolabelling experiments	55
2.1.3.1 Long term labelling in cell culture medium	55
2.1.3.2 Short term labelling in PSG-BSA	55
2.1.3.3 Preparation of cell extracts	56
2.1.4 Analysis of the polyamine content of the medium	56
2.1.5 Analysis of the polyamine content of the excreta of <i>Rhodnius prolixus</i> after a blood meal	56
2.1.6 High-Performance Liquid Chromatography (HPLC) analysis.	57
2.1.7 Polyamine oxidation.	58

	7
	PAGE
2.1.7.1 Assay for Polyamine Oxidase.	58
2.1.7.2 Culture of <i>T.cruzi</i> in Spermidine or Spermidine	59
2.1.8 Measurement of the cell volume of <i>T.cruzi</i> epimastigotes by the inulin exclusion method	59
2.2 Transport of polyamines	59
2.2.1 Cell culture	59
2.2.2. General uptake/transport method for diamines and polyamines involving centrifugation through silicone oil	60
2.2.3 A comparison of [ $^3\text{H}$ ]putrescine and [ $^3\text{H}$ ]cadaverine uptake in the trypanosomatids	61
2.2.4 Metabolism of the [ $^3\text{H}$ ]putrescine and [ $^3\text{H}$ ]cadaverine labels over 90 s	62
2.2.5 Determination of the maximum rate of 2 $\mu\text{M}$ Putrescine transport within the 6.6-8.0 pH range	63
2.2.6 Effect of cycloheximide, actinomycin D or putrescine on the rate of putrescine transport	63
2.2.7 The effect of potential inhibitors on diamine transport	63
2.3 Protein assay	64
2.4 Statistical analysis	64
2.4 Chemicals	65

### **CHAPTER 3 : RESULTS**

<b><u>PART 1 : DIAMINE AND POLYAMINE METABOLISM IN <i>T.CRUZI</i></u></b>	66-77
3.1 Uptake and metabolism of tritiated diamines	67
3.1.1 Long term labelling	67
3.1.1.1 [ $^3\text{H}$ ]Putrescine	67
3.1.1.2 [ $^3\text{H}$ ]Cadaverine	69
3.1.1.3 Identification of peak U5 as homotrypanothione	70
3.1.1.4 Possible identity of U6	70
3.1.1.5 Use of RTH + 10% chicken serum	71
3.1.1.6 Recoveries	71
3.1.2 Short term labelling	72
3.1.2.1 [ $^3\text{H}$ ]Putrescine	73



	8
	PAGE
3.1.2.2 [ <sup>3</sup> H]Cadaverine	73
3.2 Uptake and metabolism of [ <sup>14</sup> C]polyamines	73
3.3 Determination of the cell volume of <i>T.cruzi</i>	75
3.4 Polyamine content of the excreta from <i>Rhodnius prolixus</i> after a blood meal	76
3.5 Summary	77

## **CHAPTER 4 : RESULTS**

### **PART 2 : DIAMINE UPTAKE IN *T.CRUZI*** 78-93

4.1 A comparison of diamine uptake in four trypanosomatids	79
4.2 Kinetics of putrescine and cadaverine transport in <i>T.cruzi</i>	81
4.2.1 Basic transport parameters	81
4.2.2 Determination of the kinetics of putrescine and cadaverine transport	82
4.3 Alterations in the kinetics of putrescine and cadaverine transport during the growth cycle	83
4.4 Up- and down-regulation of diamine transport	83
4.5 Temperature dependence of transport	86
4.6 Efflux of putrescine	87
4.7 Effect of ionophores, metabolic inhibitors and thiol reagents	87
4.8 Specificity of diamine transport process	89
4.8.1 Effect of some amino acids	89
4.8.2 Effect of MGBG, paraquat	90
4.8.3 Effect of sodium ions	90
4.8.4 Effect of other diamines and polyamines	91
4.8.5 Evidence for the existence of multiple diamine and polyamine transporters	91
4.9 Conclusions	93

### **CHAPTER 5 : DISCUSSION** 94-105

5.1 Transport studies	96
5.2 Metabolism	99
5.3 Prospects for chemotherapy	103

### **CHAPTER 6 : REFERENCES** 106-140

**LIST OF FIGURES****FACING PAGE**

Figure 1.1 Classification of Kinetoplastida with special reference to <i>Trypanosoma cruzi</i> .	15
Figure 1.2 The life cycle of <i>Trypanosoma cruzi</i> .	15
Figure 1.3 Structure of some naturally occurring diamines and polyamines.	20
Figure 1.4 A generalised diagram showing the polyamine biosynthetic and retroconversion pathway.	22
Figure 1.5 Regulation of the polyamine content in a mammalian cell.	24
Figure 1.6 The biosynthesis of trypanothione.	28
Figure 1.7 The structure and some possible functions of trypanothione.	28
Figure 3.1 Representative HPLC traces depicting the elution times of polyamines, <i>N</i> -acetyl polyamines and polyamine-glutathione conjugates.	67
Figure 3.2 HPLC chromatograms of <i>T. cruzi</i> cells labelled with tritiated putrescine and cadaverine.	68
Figure 3.3 HPLC chromatograms of <i>C. fasciculata</i> cells labelled with tritiated putrescine and cadaverine.	68
Figure 3.4 A comparison of the elution characteristics of authentic homotrypanothione disulphide with peak U5.	70
Figure 3.5 The structure of homotrypanothione.	70
Figure 3.6 HPLC chromatograms of <i>T. cruzi</i> cells labelled with [ <sup>14</sup> C]spermidine and spermine.	74
Figure 3.7 HPLC chromatogram depicting the polyamine content of <i>Rhodnius prolixus</i> excreta.	76
Figure 4.1 Diamine uptake in the trypanosomatids.	79
Figure 4.2 Relative velocities of 1-100 $\mu$ M diamine uptake in the trypanosomatids.	79
Figure 4.3 The kinetics of putrescine and cadaverine transport in <i>T. cruzi</i> epimastigotes.	82

## FACING PAGE

Figure 4.4 Changes in the maximum rate of putrescine and cadaverine transport with growth cycle phase.	83
Figure 4.5 Recovery of putrescine transport.	84
Figure 4.6 Effect of cycloheximide on maximal putrescine transport in <i>T.cruzi</i> .	84
Figure 4.7 Changes in the rate of 1 $\mu$ M putrescine and cadaverine transport with temperature.	86
Figure 4.8 Arrhenius plot for putrescine transport.	87
Figure 4.9 Pattern of putrescine efflux.	87
Figure 4.10 Inhibition of putrescine transport by diamines and polyamines.	91
Figure 4.11 Kinetics of spermidine transport in <i>T.cruzi</i> epimastigotes.	91
Figure 4.12 Inhibition of 2 $\mu$ M [ $^3$ H]putrescine or [ $^3$ H]cadaverine transport into <i>T.cruzi</i> epimastigotes by unlabelled putrescine, cadaverine, spermidine or spermine.	92
Figure 5.1 Uptake and metabolism of diamines in <i>T.cruzi</i> epimastigotes.	104

## LIST OF TABLES

## FACING PAGE

Table 1.1 Prevalence of some human diseases caused by parasitic protozoa.	15
Table 1.2 The polyamine content of selected trypanosomatidae (Genus: <i>Leishmania</i> , <i>Trypanosoma</i> and <i>Crithidia</i> ).	27
Table 1.3 Characteristics of the different polyamine uptake systems found in mammalian cell lines.	36
Table 1.4 Characteristics of some polyamine uptake systems found in fungi, bacteria and kinetoplastids.	36
Table 3.1 Comparison of the pattern of uptake and incorporation of [ <sup>3</sup> H]putrescine and [ <sup>3</sup> H]cadaverine between <i>T.cruzi</i> grown in RTH medium which has been supplemented with either 10% foetal calf (FCS) or chicken serum (CS).	71
Table 3.2 Radiolabelling of <i>T.cruzi</i> and <i>C.fasciculata</i> with [ <sup>3</sup> H]putrescine and [ <sup>3</sup> H]cadaverine in PSG-BSA for 2 h.	73
Table 3.3 Radiolabelling of <i>T.cruzi</i> with [ <sup>14</sup> C]spermidine and [ <sup>14</sup> C]spermine in PSG-BSA for 2 h.	75
Table 3.4 Amounts of the radiolabelled diamines and polyamines taken up by <i>T.cruzi</i> and <i>C.fasciculata</i> over 2 h from the PSG-BSA and the intracellular concentration that this represents.	76
Table 3.5 Possible identity of the unknown peak found at 12 minutes on the HPLC trace of the excreta produced by <i>Rhodnius prolixus</i> immediately after a blood meal.	76
Table 4.1 Intracellular fate of [ <sup>3</sup> H]Putrescine and [ <sup>3</sup> H]Cadaverine after a 90 seconds exposure of <i>T.cruzi</i> epimastigotes to each label.	81
Table 4.2 Alterations in $K_m$ and $V_{max}$ values for putrescine and cadaverine transport with cell growth phase.	83
Table 4.3 Effect on maximal putrescine transport velocities of pre-incubation of <i>T.cruzi</i> cells with cycloheximide, actinomycin D or putrescine.	85
Table 4.4 Effect on maximal putrescine transport velocities of pre-incubation of <i>T.cruzi</i> cells in RTH+PCS supplemented with 100 $\mu$ M exogenous putrescine or pre-conditioned for 4-7 days with <i>T.cruzi</i> cells (day 4 or 7 supernatant).	85

## FACING PAGE

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

88

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

89

Table 4.7 Effect of methylglyoxal bis(guanyldrazone) and paraquat on putrescine transport.

90

# LIST OF ABBREVIATIONS

<u>Abbreviation</u>	<u>Name in full</u>
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 <sup>1</sup> -acetyltransferase
DFMA	DL- $\alpha$ -difluoromethylarginine
DFMO	DL- $\alpha$ -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+PCS	RPMI/Trypticase/haemin plus 10% foetal calf serum
RNA	ribonucleic acid
TCA	trichloroacetic acid
V <sub>max</sub> app.	the apparent maximum velocity

## **CHAPTER 1 : INTRODUCTION**

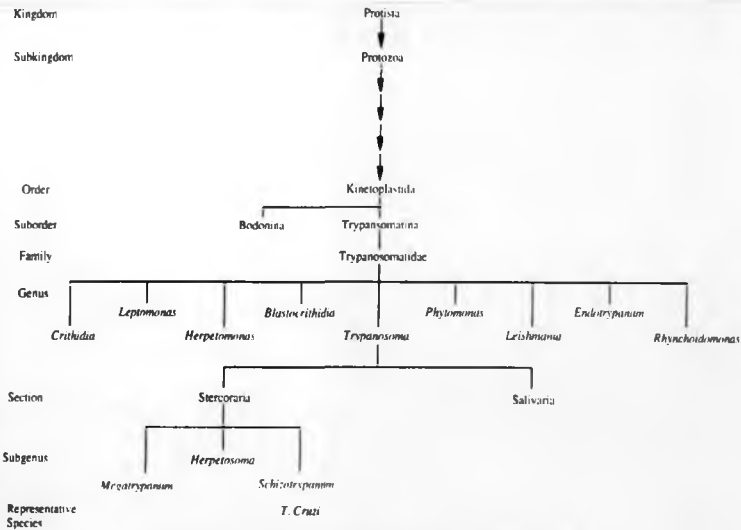


Figure 1.1 Classification of Kinetoplastida with special reference to *Trypanosoma Cruzi*

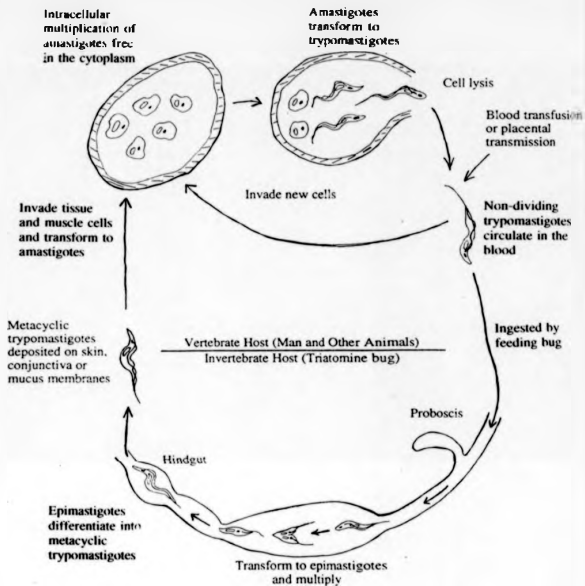


Table 1.1 Prevalence of some human diseases caused by parasitic protozoa

Disease	Distribution (continents)	Incidence of infection <sup>1</sup> (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 ( <i>T. cruzi</i> )	South and Central America	16-18	90	45	(Moncayo, 1992) (WHO, 1993)
African ( <i>T. brucei</i> group)	Africa	0.015-0.020 <sup>2</sup>	50	?	(WHO, 1993) (Kuzne, 1993)

<sup>1</sup> Number of people infected with the parasite.

<sup>2</sup> 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 Kinetoplastida (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'-nitro(furfurylideneamino)tetrahydro-4H-1,4-thiazine-1,1-dioxide) sold under the name Lampit (Bayer company) and benznidazole

(*N*-benzyl-2-nitroimidazole acetamide) sold as Rochagan or Radilol (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 (Petty & Elsen, 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 committed 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

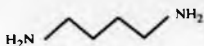
prevented by prior treatment of potentially infected blood with Gentian violet (Van-Voorhis, 1990; Docampo *et al.* 1991). This therapy is not ideal as a frequent blue discolouration of the skin is observed after transfusion making it difficult to spot early signs of anoxia and the gentian violet itself 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.

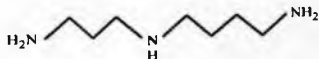


Putrescine  
(1,4-diaminobutane)

#### Diamines

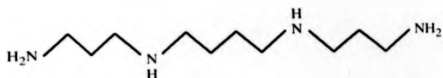


Cadaverine  
(1,5-diaminopentane)



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 metabolic, 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 & Sjoerdama, 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  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ . 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

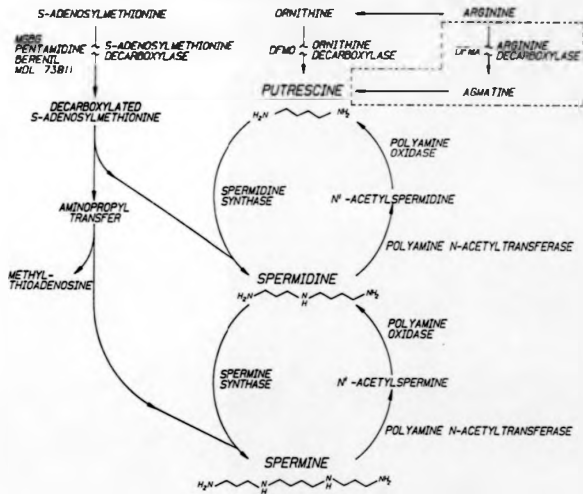


Figure 1.4 A generalised diagram showing the polyamine biosynthetic and retroconversion pathway. Adapted from Pegg and McCann 1988.

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:

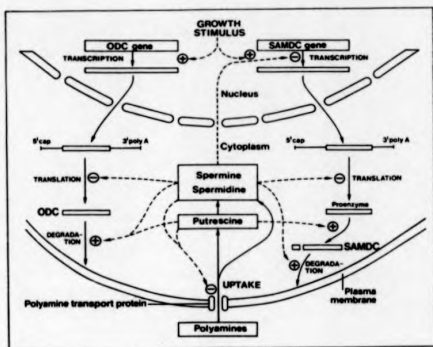
- 1) The reactions converting arginine to putrescine via agmatine (surrounded by a dashed line in Figure 1.4) only occur in plants, bacteria (Kallio *et al.* 1981) and possibly *T. cruzi* (Kierzenbaum *et al.* 1987a; Majumder *et al.* 1992; Yakubu *et al.* 1992).
- 2) Eukaryotic protozoans such as *Trypanosoma 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 & Yariett, 1993; Majumder & Kierzenbaum, 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*<sup>1</sup>-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 proteasome (Murakami *et al.* 1992) or the 20S proteasome (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*<sup>1</sup>-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(guanyldiazone) (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; Branton *et al.* 1991).

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

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

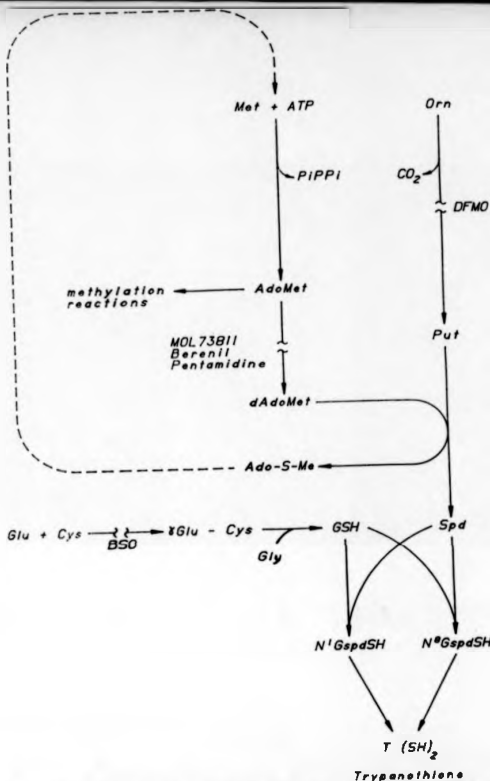
Most cells were assayed in mid to late exponential phase of growth. PUT = putrescine, SPD = spermidine, SPN = spermine, P=promastigote, A=amastigote, E=epimastigote, BT=bloodstream trypomastigote. <sup>2</sup>Polyamine content measured in nmol (mg protein)<sup>-1</sup>; ( ) cadaverine concentration in nmol (10<sup>6</sup> cells)<sup>-1</sup>; <sup>1</sup>free spermidine concentration (not bound to glutathione); <sup>2</sup>not detectable; <sup>3</sup>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.

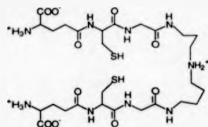
### 1.3.3 Polyamines in the Trypanosomatidae

#### 1.3.3.1 Polyamine content

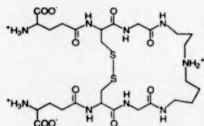
In most trypanosomatidae the major diamines and polyamines present are putrescine and spermidine respectively (Bacchi, 1981; Bacchi *et al.* 1977) (Table 1.2). Many of the *Leishmania* species studied also appear to contain trace amounts of spermine (Table 1.2). Some of this could be taken up from the 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 1999a. Met = methionine, ATP = adenosine triphosphate, Ornithine = L-ornithine, Orn = ornithine, Put = putrescine, Spd = spermidine, Glu = glutamic acid, cys = cysteine,  $\gamma$ Glu-Cys =  $\gamma$ -glutamyl cysteine, Gly = glycine, GSH = glutathione, N<sup>1</sup>GspdSH = N<sup>1</sup>-glutathionylspermidine, N<sup>6</sup>GspdSH = N<sup>6</sup>-glutathionylspermidine, BSO = trypanothione synthase, dAdoMet = 5-deoxyadenosine, Ado-S-Me = S-adenosylmethionine, DFMO = difluoromethylornithine.



Trypanothione (T[SH]<sub>2</sub>)



Trypanothione disulphide (T[S]<sub>2</sub>)

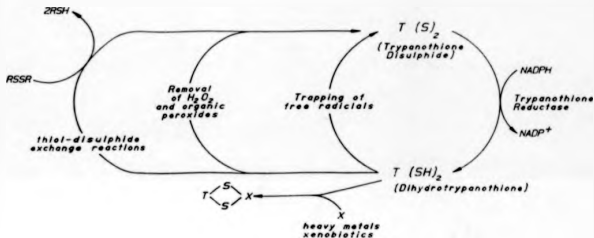


Figure 1.7 The structure and some possible functions of trypanothione.

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 Resynthesis

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 & Sjoerdama, 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- $\alpha$ -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 & Sjoerdama, 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<sup>and</sup> 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 <sup>may be effective against</sup> *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.rhodinense* (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* (Kierzenbaum *et al.* 1987b). However, inhibitors of arginine decarboxylase, for example DL- $\alpha$ -difluoromethylarginine (DFMA), at very high concentrations (12-50mM), appear to decrease the capacity of *T.cruzi* to infect and multiply within mammalian cells (Kierzenbaum *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 extracts containing up to  $2.4 \times 10^9$  trypanosomes (McCann *et al.* 1988) and radiolabelling epimastigotes with [ $^{14}\text{C}$ ]ornithine or [ $^{14}\text{C}$ ]arginine also failed to demonstrate any putrescine formation (Algranati *et al.* 1989). Recently trace amounts of ADC activity (11.5 pmol  $\text{CO}_2$  mg protein $^{-1}$  h $^{-1}$ ) and slight (<0.1%) conversion of radiolabelled arginine to agmatine, putrescine and spermidine was observed in mixed trypomastigote / amastigote preparations but this required high cell numbers, ( $0.3 \times 10^9$  ml $^{-1}$  for radiolabelling and  $3 \times 10^9$  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-10 $\mu\text{M}$ ) 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) (MOBG) 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 ( $K_i$  1.5 $\mu$ M), leading to a reduction in spermidine (50%) and increase in putrescine levels (Bitonti *et al.* 1990). MDL 73811 also causes a 20-fold increase in AdoMet levels in *T.b.brucel*, 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.brucel*, lead to an increase in the cell's methylation index (ratio of AdoMet:S-Adenosylhomocysteine) (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 aberrant 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 sulfoximine 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 dihydrottrypanothione as the dithioarsane adduct MeIT (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  $RNH(CH_2)_xNH(CH_2)_yNH(CH_2)_zNHR$  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 <sub>m</sub> (μM)			Transporter	Energy dependence	Other characteristics			Reference
	Put	Spd	Spn			<sup>1</sup> Na <sup>+</sup>	<sup>2</sup> SH	<sup>3</sup> AA	
<u>Normal</u>									
Fibroblast (human)	1.1	<sup>4</sup> nd	nd	Single	Yes	nd	nd	No	(Pohjanpelto, 1976)
(Swiss 3T3)	10-14	nd	nd	nd	nd	nd	nd	nd	(DiPasquale <i>et al.</i> 1978)
Hepatocyte (mouse)	8-11	nd	nd	nd	nd	nd	nd	nd	(Bethell & Pegg, 1981)
(rat)	nd	nd	20	nd	Yes	Yes	nd	nd	(Marin <i>et al.</i> 1990)
Mammary gland (mouse)	nd	47.6	nd	Single	Yes	nd	nd	No	(Auberger <i>et al.</i> 1983)
Enterocyte (rat)	12.3	2.51	nd	Multiple	Yes	No	nd	No	(Kano & Oka, 1976)
Adrenocortical (bovine)	10	nd	nd	Multiple	Yes	Yes	No	No	(Kumagai <i>et al.</i> 1989)
Embryonic palate	5.8	nd	nd	Single	Yes	Yes	Yes	No	(Kumagai & Johnson, 1988)
mesenchymal (mouse)									(Feige & Chambaz, 1985)
Endothelial, aortic (pig)	9	0.6	nd	Multiple	Yes	Yes	Yes	No	(Gewiel-Thompson & Greene, 1988)
Lymphocytes (bovine)	3.7	0.4	0.2	Single	Yes	No	Yes	No	(Bogle <i>et al.</i> 1994)
Endothelial, umbilical-vein (human)	3.0	0.8	0.5	Multiple	Yes	Yes	Yes	nd	(Kakinuma <i>et al.</i> 1988)
Macrophage, pulmonary alveolar (rabbit)	3.0	0.7	1.0	Multiple	Yes	Yes	Yes	nd	(Rajanyagam <i>et al.</i> 1992)
Pulmonary, epithelial Type II (rat)	2.1	0.2	nd	Single	Yes	nd	nd	nd	(Morgan, 1992)
Perfused lung (rat)	nd	0.5	nd	Single	Yes	Yes	nd	No	(Saunders <i>et al.</i> 1989)
	nd	0.6	nd	Multiple	nd	Yes	nd	nd	(Kameiji <i>et al.</i> 1989)
	14	nd	nd	nd	nd	nd	nd	nd	(Rannels <i>et al.</i> 1989)
									(Wyatt <i>et al.</i> 1988)

Ovary (Chinese hamster)	6.2	6.3	1.0
Erythrocyte (human)	37	1.6	nd
(human)	nd	nd	nd
<u>Transformed</u>			
ADI/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
O6 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

<sup>1</sup>Na<sup>+</sup>-dependent; <sup>2</sup>requires thiol groups for maximal activity.

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

<sup>3</sup>interaction with amino acid transport systems; <sup>4</sup>nd = not determined.



Table 1A Characteristics of some polyamine uptake systems found in fungi, bacteria and kinetoplastids

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

<sup>1</sup>Na<sup>+</sup>-dependent; <sup>2</sup>requires thiol groups for maximal activity; <sup>3</sup>interaction with amino acid transport systems; <sup>4</sup>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 & Varley, 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  $\mu$ M (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) whereas 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 radiolabel's 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-Pouze *et al.* 1989). For example, in fibroblasts the rate of 100  $\mu$ M 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 permeabilisation 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 & Risow, 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 ( $\text{Na}^+$ -dependent) polyamine uptake. In these systems it is common for the uptake of putrescine and spermidine to be  $\text{Na}^+$ -dependent whilst spermine transport is often  $\text{Na}^+$ -independent (Feige & Chambaz, 1985; Rannels *et al.* 1989; Nuttall *et al.* 1990; Morgan, 1992). Also many cellular systems have a non-saturable  $\text{Na}^+$ -independent component to their polyamine transport (Feige & Chambaz, 1985; De Smedt *et al.* 1989; Auburger *et al.* 1983). However the criteria for  $\text{Na}^+$ -dependent

transport used in Table 1.3, which is only based on either the iso-osmotic replacement of sodium,  $\text{Na}^+$ , with choline,  $\text{Ch}^+$ , (or other monovalent cations such as lithium and *N*-methyl-D-glucamine) and / or the use of ionophores (gramicidin ( $\text{Na}^+/\text{K}^+$ ), monensin ( $\text{Na}^+$ )) and the  $\text{Na}^+/\text{K}^+$  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; Nustall *et al.* 1990), does not give the full picture. Replacement of  $\text{Na}^+$  with  $\text{Ch}^+$  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  $\text{Na}^+$  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  $\text{Na}^+$  (Tabor & Tabor, 1966). This suggests that in *E.coli*, rat enterocytes and pancreatic acini polyamine uptake is not a  $\text{Na}^+$ -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  $\text{Na}^+$  in a 1:1 stoichiometric relationship, suggesting that the spermidine is directly coupled in a ternary complex,  $\text{Na}^+$ -spermidine-carrier (Khan *et al.* 1990).

In addition some ionophores such as gramicidin ( $\text{Na}^+/\text{K}^+$ ), carbonylcyanide *m*-chlorophenylhydrazone ( $\text{H}^+$ ) and calcimycin (A23187,  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ) 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 (Kalkinuma *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*-chloromercuribenzenesulphonate (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 *T. infantum* putrescine uptake can be partially inhibited by the amino acids lysine, arginine (system  $Ly^+$ ) and aspartic acid (system  $\beta^+$ ) (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  $<0.81\mu\text{M}$  putrescine,  $6-34\mu\text{M}$  spermidine and  $4-10\mu\text{M}$  spermine (Cooper *et al.* 1978; Claverie *et al.* 1987), whilst plasma and serum contain much lower levels of polyamines at  $0.03-0.5\mu\text{M}$  (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  $0.1-10\mu\text{M}$  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<sup>cells</sup> (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 dibutyl 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 in 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<sup>+</sup>-K<sup>+</sup> pump, protein kinase C and Ca<sup>2+</sup>

In L1210 leukaemia cells protein kinase C activators such as the phorbol ester, phorbol myristic acid (PMA), increase spermidine, Na<sup>+</sup> and Rb<sup>+</sup> (a measure of the Na<sup>+</sup>-K<sup>+</sup> pump activity) uptake whereas inhibitors such as H-7 lower Na<sup>+</sup> and Rb<sup>+</sup> uptake (Khan *et al.* 1992). Likewise PMA stimulates putrescine uptake in pancreatic acini (Stüber *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<sup>+</sup> and Na<sup>+</sup>-K<sup>+</sup> 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<sup>+</sup>-K<sup>+</sup> pump perhaps via activation of protein kinase C, thereby stimulating the efflux of sodium from the cell and hence polyamine-Na<sup>+</sup> coupled entry into the cell (Khan *et al.* 1989b;

Khan *et al.* 1994). In addition an increase in free intracellular  $\text{Ca}^{2+}$ , may cause the translocation of cytosolic protein kinase C to the plasma membrane where it activates the  $\text{Na}^+/\text{K}^+$  pump and the activation of calmodulin (by translocation to the plasma membrane). It is possible that this calmodulin may then influence the efflux of  $\text{Ca}^{2+}$  from the cell (via the  $\text{Ca}^{2+}$  ATPase), which in turn might stimulate both the uptake of extracellular  $\text{Ca}^{2+}$  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  $\text{Na}^+$ -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 whose 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 positively 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; O'Sullivan *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; O'Sullivan *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; O'Sullivan *et al.* 1991). Conversely addition of an aziridine moiety to putrescine to form *N*-(4-aminobutyl)aziridine (O'Sullivan *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(guanyldrazone) (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  $\alpha,\omega$ -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).

### 1.5.2 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 putrescine-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 & Flinoff, 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*<sup>4</sup>-spermidine



derivatives are good potential candidates as they are good substrates for the polyamine uptake system (Porter *et al.* 1982). Chloroambucil has been conjugated to the N<sup>4</sup>-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**

## **CHAPTER 2 : MATERIALS AND METHODS**

### **2.1 Metabolic labelling studies**

#### **2.1.1 Cell culture**

*Crithidia fasciculata* cells (clone HS6), seeded at  $1 \times 10^6$  cells  $\text{ml}^{-1}$ , were grown at  $28^\circ\text{C}$  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  $1 \times 10^6$  cells  $\text{ml}^{-1}$  and maintained at  $28^\circ\text{C}$  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 Trypsinase (Becton Dickinson Microbiology Systems) at  $0.175 \text{ g ml}^{-1}$ , 60 ml heat-inactivated (30 min at  $56^\circ\text{C}$ ) foetal calf serum (FCS) (Life Technologies Ltd), 6 ml penicillin/streptomycin ( $5000 \text{ units ml}^{-1} / 5 \text{ mg ml}^{-1}$ ) (Life Technologies Ltd) and 4ml haemin at  $2.5 \text{ mg ml}^{-1}$ . 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 [ $^3\text{H}$ ]Cadaverine**

[ $^3\text{H}$ ]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  $500 \mu\text{l}$  6 M HCl and  $25 \mu\text{l}$  (0.5mCi) of the crude [ $^3\text{H}$ ]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 (Heio 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  $\text{NH}_4\text{OH}$ . 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 [ $^3\text{H}$ ]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 [ $^3\text{H}$ ] cadaverine were pooled, freeze-dried and resuspended in 1 ml 10 mM HCl yielding 263  $\mu\text{Ci}$  [ $^3\text{H}$ ]cadaverine with a purity of >98% (26% recovery).

#### 2.1.2.2 [ $^{14}\text{C}$ ]Spermidine and [ $^{14}\text{C}$ ]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  $\text{C}_{18}$  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  $1 \times 10^6$  cells  $\text{ml}^{-1}$  and incubated to late log phase in RTH+PCS (6 days) or yeast/tryptone broth (2 days) respectively in the presence of  $1 \mu\text{Ci ml}^{-1}$  [ $1,4\text{-}^3\text{H}$ ]putrescine.2HCl (11 Ci  $\text{mmol}^{-1}$ , Amersham International plc) or [ $1,5\text{-}^3\text{H}$ ]cadaverine.2HCl (18 Ci  $\text{mmol}^{-1}$ ). *T.cruzi* cells were also cultured under identical conditions at  $1 \mu\text{Ci ml}^{-1}$  [tetramethylene 1,4- $^{14}\text{C}$ ]spermidine.3HCl (10.3 mCi  $\text{mmol}^{-1}$ , New England Nuclear) or [tetramethylene 1,4- $^{14}\text{C}$ ]spermine.4HCl (13.4 mCi  $\text{mmol}^{-1}$ , New England Nuclear) except that RTH+CS was used in place of RTH+PCS. At the end of this time cells were harvested by centrifugation ( $1500 \times g$ , 10 min,  $4^\circ\text{C}$ ) 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 Fraction V (BSA) and 2% penicillin/streptomycin solution).

#### **2.1.3.2 Short term labelling in PSG-BSA**

Late log phase cells ( $1.5 \times 10^8$ ) were pelleted by centrifugation ( $1500 \times g$ , 10 min,  $4^\circ\text{C}$ ), the supernatant discarded and then resuspended in 2 ml of PSG-BSA containing 10  $\mu\text{Ci}$  of the [ $^3\text{H}$ ]-diamines or 5  $\mu\text{Ci}$  of the [ $^{14}\text{C}$ ]-polyamines. The cells were incubated at  $28^\circ\text{C}$  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 norxamine 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  $\mu\text{m}$  guard column (Anachem) and onto a Beckman Ultrasphere Ion Pairing 5  $\mu\text{m}$  C<sub>18</sub> (ODS 2, 250 mm x 4.6 mm) column (method 1) or a Beckman C<sub>18</sub> 5  $\mu\text{m}$  (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 fluorescamine (Fluoram, Roche) (Weigle *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<sup>-1</sup>. 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 (Cammerra Packard) and counting on a Beckman LS-6000LL series scintillation counter (Beckman

Instruments Ltd).

Method 2. 50  $\mu$ l 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<sup>1</sup>-glutathionylpermidine and N<sup>1</sup>-glutathionylpermidine 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<sub>2</sub>O<sub>2</sub> as a reaction product. The assay mixture contained 0.1 M potassium phosphate, pH 8, 0.04 mg horseradish peroxidase (175 U mg<sup>-1</sup>), 150  $\mu$ l of serum under test, 0.25 mg homovanillic acid, 0-100 nmol polyamine substrate and  $\pm$ 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  $4 \times 10^6$  *T. cruzi* cells and 0-500  $\mu$ M spermidine or spermine in 2 ml of RTH+FCS or RTH+CS medium. Aminoguanidine (1mM) was added to half the RTH+PCS 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 ( $11.9 \text{ mCi mmol}^{-1}$ , Amersham International plc) which had previously been dialysed against 0.9% (w/v) NaCl. This gave a cell volume for *T. cruzi* epimastigotes of  $5.5 \pm 0.4 \mu\text{l}$  ( $10^8 \text{ cells}^{-1}$ ) ( $n = 4$ ).

## 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  $1 \times 10^6 \text{ cells ml}^{-1}$  into Grace's 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  $1 \times 10^5 \text{ cells ml}^{-1}$  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  $1 \times 10^6 \text{ ml}^{-1}$  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  $\text{ml}^{-1}$  and  $5 \text{mg ml}^{-1}$  respectively) (CBSS+PS), 28 °C, 0.1 ml) containing [ $^3\text{H}$ ]putrescine, [ $^3\text{H}$ ]cadaverine or [terminal methylenes $^3\text{H}(\text{N})$ -] spermidine.3HCl (15.6Ci  $\text{mmol}^{-1}$ , 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  $\text{g ml}^{-1}$ ; 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 (1500 $\times$ g, 10 min, 4 °C) and then resuspended to  $2 \times 10^8$  cells  $\text{ml}^{-1}$  in CBSS+PS and prewarmed to 28 °C. At intervals, aliquots (0.1 ml) were added to the radiolabel-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 ( $16,000 \times g$ , 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 [ $^3H$ ]estradiol and [ $^3H$ ]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  $\mu M$  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  $^{\circ}C$  and then centrifuging them immediately through the silicone oil.

### 2.2.4 Metabolism of the $^3\text{H}$ putrescine and $^3\text{H}$ 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+PFS were prepared as described in section 2.2.2 and then resuspended at  $2 \times 10^8$  cells  $\text{ml}^{-1}$  in CBSS. The cells and label (both at  $2 \times$  final concentration) were prewarmed to  $28^\circ\text{C}$  for 10 min. At time zero an equal volume of cells were added to the label, mixed, and immediately 100  $\mu\text{l}$  aliquots were withdrawn with an Eppendorf automatic pipette and layered on top of 0.1 ml of silicone oil which was overlaid on 100  $\mu\text{l}$  of 10% TCA containing  $3.5 \mu\text{M}$  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 ( $12,500 \times g$ ) 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  $4^\circ\text{C}$  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  $^3\text{H}$ putrescine and  $^3\text{H}$ 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  $\frac{\text{total}}{\text{label}}$   $^3\text{H}$ putrescine or  $^3\text{H}$ cadaverine label in putrescine or cadaverine equivalents respectively.

### 2.2.5 Determination of the maximum rate of 2 $\mu$ M 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  $\mu$ M 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  $\mu$ m 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  $\mu$ M cycloheximide, 2  $\mu$ M actinomycin D and 10 or 100  $\mu$ M putrescine. Putrescine transport was measured under saturating conditions (10  $\mu$ M) 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 ( $8 \times 10^8$  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  $\mu$ M 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  $233 \pm 19 \mu\text{g}$  ( $10^8$  cells) $^{-1}$  ( $n = 4$ ) was determined for *T.cruzi*.

### 2.4 Statistical analysis

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

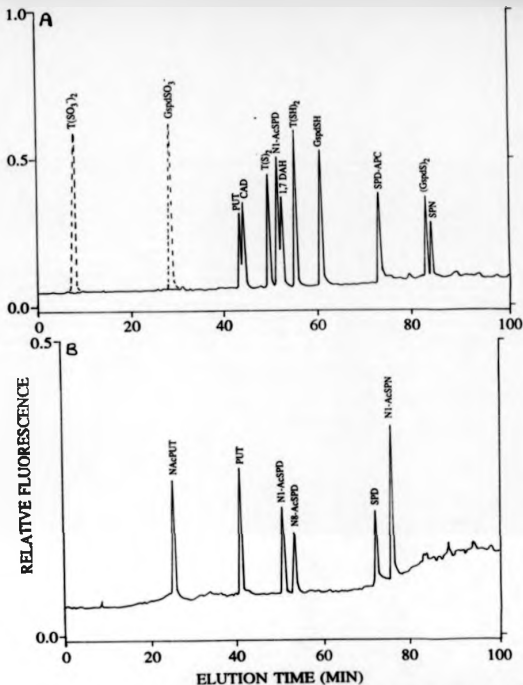
a matrix inversion method using the enzfitter program (Elsevier Biosoft, Cambridge, UK). Where appropriate, all the other data points shown are the arithmetical means  $\pm$  the standard deviation (SD). In Table 4.2 the data was analysed by student's paired t-test. Confidence levels were set at 99.9% ( $p > 0.001$  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<sup>1</sup>-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 purchased 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-acetypolyamines and polyamine-glutathione conjugates.** A) Polyamines and polyamine-glutathione conjugates; B) N-Acetypolyamines. 1,7-Diaminooheptane (1,7 DAH) is the internal standard. PUT = putrescine, CAD = cadaverine, SPD = spermidine, APC = aminopropylcadaverine, SPN = spermine, NAcPUT = N-Acetylputrescine, N1AcSPD = N<sup>1</sup>-acetylspermidine, N8AcSPD = N<sup>8</sup>-Acetylspermidine, N1AcSPN = N<sup>1</sup>-Acetylspermine, GcpdSH = glutathionylspermidine, (GcpdS)<sub>2</sub> = glutathionylspermidine disulphide, T(SH)<sub>2</sub> = dihydrotryanthonine, T(S)<sub>2</sub> = trypanthione disulphide. Glutathionylspermidine sulphonate (Gcpd(SO<sub>3</sub>)<sub>2</sub>) and trypanthione bis(sulphonate) (T(SO<sub>3</sub>)<sub>2</sub>) are the performic acid oxidation products of glutathionylspermidine and trypanthione respectively. *Bis*(aminopropylcadaverine) co-elutes with spermine under this system.

## CHAPTER 3 : RESULTS

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

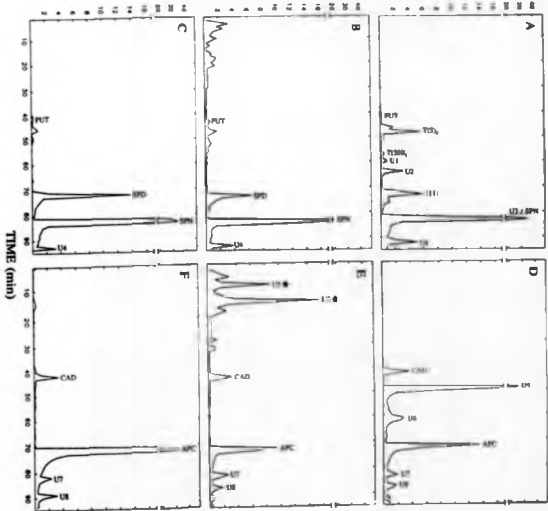
#### 3.1 Uptake and metabolism of tritiated diamines

##### 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 [<sup>3</sup>H]putrescine and [<sup>3</sup>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 [<sup>3</sup>H]Putrescine

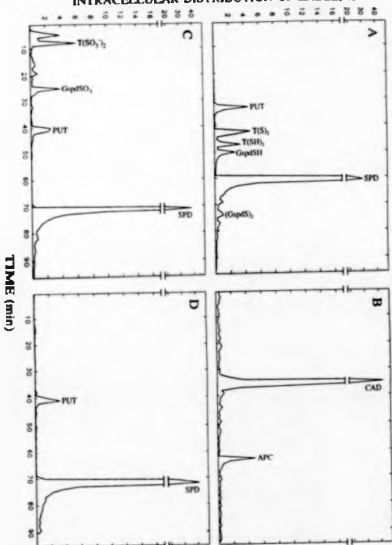
These experiments showed that 75% of the total (label plus medium) exogenous putrescine (0.77 nmol ml<sup>-1</sup>) was taken up by *T.cruzi*. Following HPLC separation, only 7% of the radioactivity taken up was recovered as putrescine. The majority was incorporated into spermidine (14%), spermine (37%),



**Figure 3.** 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. Mass recovery of the label was 66%. The polyamine content of the RTM+PES medium was: putrescine, 1.09 mM; spermidine, 1.10 mM; spermidine and 0.54 mM spermine. A) [Putrescine-<sup>3</sup>H]-labelled *T. cruzi* cells. B) After metabolic conversion, [1,10-dimethyl-<sup>3</sup>H]-cadaverine-labelled *T. cruzi* cells. C) After perfomate acid oxidation, acid oxidized, D) after acid hydrolysis, POT = putrescine, CAD = cadaverine, SPD = spermidine, SPN = spermine, TSSD = dihydroxyputrescine, TOS = tyrosinovaldehyde disulphide, U5 = histonepyrophosphate disulphide, U7 = histonepyrophosphate disulphide, U8 are formed following pyroglutamic acid oxidation of authentic disulphides. The chemical identities of U1, U3, U4, U6 and U9 are not known. See text for further details.

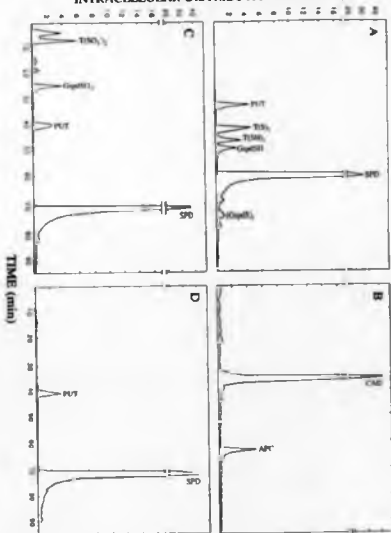
**Figure 3.3 HPLC chromatograms of *C. fasciculata* cells labelled with tritiated putrescine and cadaverine.** Extracts were prepared as detailed in the Materials and Methods. Percentages represent the amount of the label in each fraction compared to the total recovered from the column. Mean recovery 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) [<sup>3</sup>H]Putrescine-labelled *C. fasciculata* cells, B) [<sup>3</sup>H]cadaverine-labelled *C. fasciculata* cells, C) [<sup>3</sup>H]Putrescine-labelled cells after performic acid oxidation, D) after acid hydrolysis. PUT = putrescine, CAD = cadaverine, SPD = spermidine, APC = aminopropylcadaverine, Gaps5H = glutathionylspermidine, (Gaps5)<sub>2</sub> = glutathionylspermidine disulphide, T(SH)<sub>2</sub> = dihydrotrypanothione, T(S)<sub>2</sub> = 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.

# INTRACELLULAR DISTRIBUTION OF LABEL. %



**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 recoveries 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) [<sup>3</sup>H]Putrescine-labelled *C. fasciculata* cells, B) [<sup>3</sup>H]cadaverine-labelled *C. fasciculata* cells, C) [<sup>3</sup>H]Putrescine labelled cells after performic acid oxidation, D) after acid hydrolysis. PUT = putrescine, CAD = cadaverine, SPD = spermidine, APC = aminopropylcadaverine, GpdsH = glutathionylspermidine, (Gpds)<sub>2</sub> = glutathionylspermidine disulphide, T(SH)<sub>2</sub> = dithyocryptosporidione, T(S)<sub>2</sub> = 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.

# INTRACELLULAR DISTRIBUTION OF LABEL, %



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.

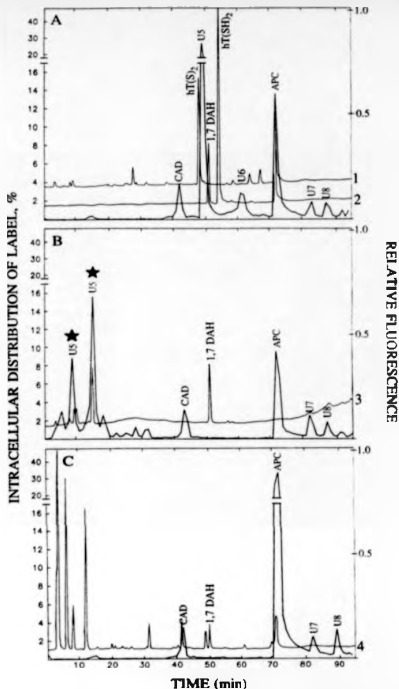
In comparison, 41% of the [ $^3\text{H}$ ]putrescine in the medium that was taken up by *C. fasciculata*. Most of this radioactivity was recovered as putrescine (7%) spermidine (63%) and glutathionyl-spermidine conjugates (18%). No spermine or unidentified peaks were formed (Figure 3.3A). Performic acid 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 [<sup>3</sup>H]Cadaverine

With [<sup>3</sup>H]cadaverine, 79% of the total (label plus medium) exogenous diamine (1.06 mmol.ml<sup>-1</sup>) was incorporated into *T. cruzi*. 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<sup>a</sup>, Figure 3.2E). The sum of U5<sup>a</sup> (fractions 2-20) represents 51% of the radioactivity recovered, suggesting that U5 and U6 were converted to U5<sup>a</sup> 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 [<sup>3</sup>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 US.** A) [ $^3\text{H}$ ]Cadaverine cell extracts and homotrypanothione standards, B) performic acid oxidation of [ $^3\text{H}$ ]cadaverine cell extracts and homotrypanothione disulphide and C) acid hydrolysis of [ $^3\text{H}$ ]cadaverine cell extracts and homotrypanothione disulphide. Base line traces in A, B and C correspond to the equivalent chromatograms in Figure 3.2 and give the percent intracellular distribution of the label from 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 Q(TSH) $_2$ , 4 nmol spermidine (coelutes with homotrypanothione under this system, see Figure 3.1). Trace 2: 2 nmol of homotrypanothione maximum (hTSH) $_2$  and 4 nmol of spermidine. Trace 3: 0.5 nmol 1,7 DAH and 2 nmol of performic acid oxidized homotrypanothione maximum. Trace 4: 0.5 nmol 1,7 DAH and 4 nmol acid hydrolyzed 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  $N^1,N^6$ -bis(glutathionyl)-aminopropylcadaverine ("homotrypanothione") disulphide. Unsuccessful attempts were made to synthesize enough  $N^1,N^6$ -bis(glutathionyl)aminopropylcadaverine for formal identification, either enzymatically using glutathionylperimidase 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  $N^1,N^6$ -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\* 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 [<sup>3</sup>H]putrescine and [<sup>3</sup>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, %			
	[ <sup>3</sup> H]putrescine		[ <sup>3</sup> H]cadaverine	
	RTH+FCS	RTH+CS	RTH+FCS	RTH+CS
putrescine	0.7	1.5	-	-
spermidine	13.9	14.0	-	-
spermine / U3 <sup>1</sup>	49.9	61.5	-	-
N <sup>1</sup> -acetylspermine	nd <sup>2</sup>	2.9	-	-
glutathionylspermidine <sup>3</sup>	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 7% <sup>4</sup> 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 <sup>5</sup>	89	58 <sup>5</sup>	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  $\mu$ M putrescine, 1.0  $\mu$ M cadaverine, 1.1  $\mu$ M spermidine and 0.5  $\mu$ M spermine; ii) RTH+CS was 2.3  $\mu$ M putrescine, 0.8  $\mu$ M spermidine and 0.5  $\mu$ M spermine, prior to the addition of the radiolabel. Tentative assignments for some of these unidentified compounds (U1-8) are suggested in the accompanying text. <sup>1</sup>In *T. cruzi* some of the 'spermine' could be U3 which coelutes with it; <sup>2</sup>not detectable; <sup>3</sup>both the free form and the mixed disulphide of glutathionylspermidine and glutathione (U2 in Figure 3.2) are included in the percentages depicted here; <sup>4</sup>amount of spermine / U3 peak present as U3 not determined; <sup>5</sup>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 disulphide 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  $2 \text{ nmol} \cdot 10^8 \text{ cells}^{-1}$ ) (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 [ $^3\text{H}$ ]putrescine and [ $^3\text{H}$ ]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 2.3 $\mu$ M putrescine and 0.2 $\mu$ M 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 [ $^3\text{H}$ ]putrescine and [ $^3\text{H}$ ]cadaverine in PSG-BSA for 2 h.

component detected	Radioactivity in acid extracts, %			
	[ $^3\text{H}$ ]putrescine		[ $^3\text{H}$ ]cadaverine	
	<i>T.cruzi</i>	<i>C.fasciculata</i>	<i>T.cruzi</i>	<i>C.fasciculata</i>
putrescine	1.3	92.3	-	-
spermidine	34.5	4.1	-	-
<i>N</i> <sup>6</sup> -acetylspermidine	2.37 <sup>1</sup>	nd	-	-
spermine / U3 <sup>2</sup>	16.8	nd <sup>3</sup>	-	-
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  $\mu\text{M}$  putrescine and 0.2  $\mu\text{M}$  spermidine. <sup>1</sup>tentative assignment only; <sup>2</sup>In *T.cruzi* some of the 'spermine' could be U3 which coelutes with it; <sup>3</sup>not detectable.

### 3.1.2.1 [ $^3\text{H}$ ]Putrescine

In *T.cruzi* 69% of the total exogenous [ $^3\text{H}$ ]putrescine ( $2.8\mu\text{M}$ ) 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 [ $^3\text{H}$ ]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 [ $^3\text{H}$ ]putrescine taken up by the cells into polyamines and then conjugate these polyamines to glutathione.

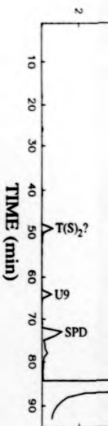
### 3.1.2.2 [ $^3\text{H}$ ]Cadaverine

With [ $^3\text{H}$ ]cadaverine ( $0.3\mu\text{M}$ ), *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 ( $0.3\mu\text{M}$ ), 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 [ $^{14}\text{C}$ ]polyamines

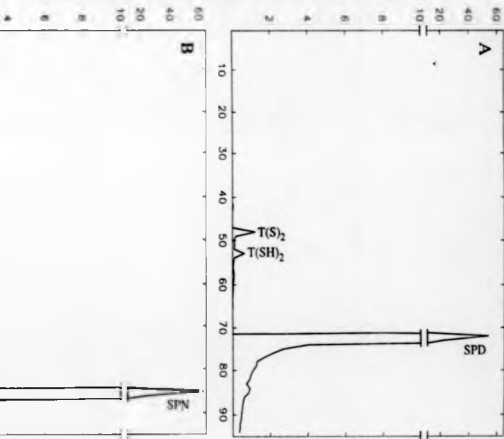
Initial attempts to label *T.cruzi* cells with  $1\mu\text{Ci.ml}^{-1}$  [ $^{14}\text{C}$ ]spermidine and [ $^{14}\text{C}$ ]spermine to late log phase (6 days) in RTH+FCS medium were unsuccessful as all the cells rounded up and in the case of spermine nearly half of them died. Others have reported 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 >> putrescine = 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  $\geq 100 \mu\text{M}$  spermidine or spermine alone died. If 1mM aminoguanidine was also added to the wells then the cells incubated in the presence of 100  $\mu\text{M}$  spermidine grew normally whereas those in 100 $\mu\text{M}$  spermine still grew to a lower density than the control cells. *T.cruzi* incubated for 6 days in RTH+CS  $\pm$  1mM aminoguanidine grew normally up to spermidine and spermine concentrations of 500  $\mu\text{M}$  (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 ( $98 \text{ nmol ml}^{-1}$ ) and 10% of the total spermine ( $75 \text{ nmol ml}^{-1}$ ) 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 [ $^{14}\text{C}$ ]spermidine and [ $^{14}\text{C}$ ]spermine in PSG-BSA for 2 h.

component detected	Radioactivity in acid extracts, %	
	[ $^{14}\text{C}$ ]spermidine	[ $^{14}\text{C}$ ]spermine
putrescine	nd. <sup>1</sup>	nd
spermidine	85.7	0.7
spermine	4.5	92.4
<i>N</i> <sup>1</sup> -acetylspermine	0.97 <sup>2</sup>	1.47
glutathionylspermidine	nd	nd
trypanothione	3.7	nd
Unassigned peaks	0	U9 <sup>3</sup> 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  $\mu\text{M}$  putrescine and 0.2  $\mu\text{M}$  spermidine. <sup>1</sup>not detectable; <sup>2</sup>tentative assignment only; <sup>3</sup>for the position of U9 refer to Figure 3.6.

PSG-BSA, 12% of the total exogenous spermidine ( $243 \text{ nmol ml}^{-1}$ ) and 17% of the spermine ( $187 \text{ nmol ml}^{-1}$ ) was taken up by the cells (Table 3.3). However, the [ $^{14}\text{C}$ ]spermidine and [ $^{14}\text{C}$ ]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  $39 \text{ nmol. spermidine } (10^8 \text{ cells})^{-1}$  and  $42 \text{ nmol. spermine } (10^8 \text{ cells})^{-1}$ . 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 [ $^{14}\text{C}$ ]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.

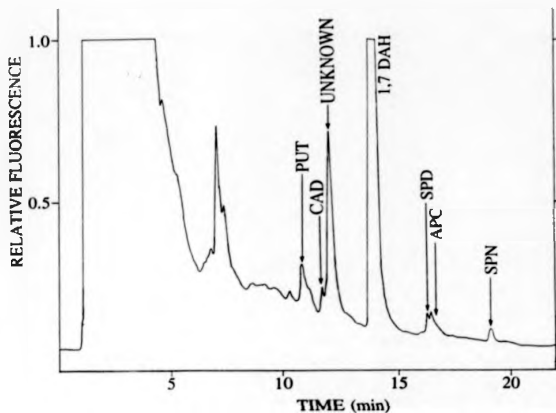
### 3.3 Determination of the cell volume of *T. cruzi*

Using the inulin exclusion method (Damper & Patton, 1976) the cell volume of *T. cruzi* epimastigotes was determined to be  $5.5 \pm 0.4 \mu\text{l } (10^8 \text{ cells})^{-1}$  ( $n = 4$ ), while the previously published value of  $10.5 \pm 0.6 \mu\text{l } (10^8 \text{ cells})^{-1}$  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 ( $\mu$ l ( $10^8$ cells) $^{-1}$ )	Internal concentration of radiolabel and its metabolites (mM)
<i>T.cruzi</i>	putrescine	2.57	5.5 $\pm$ 0.4	0.47
	cadaverine	0.32		0.06
	spermidine	38.91		7.08
	spermine	42.39		7.71
<i>C.fasciculata</i>	putrescine	2.24	10.5 $\pm$ 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  $1.5 \times 10^8$  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 co-elution 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 'unknowns' 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
<u>Nitrogenous excretion</u>		
Uric acid	No	Main route for nitrogenous excretion in <i>R. prolixus</i>
Allantoin/Allantoic acid	No	Breakdown products of uric acid
Creatine	No	
Xanthine	No	Precursors to uric acid - possible excretory products
Hypoxanthine	No	
Urea	No	From the blood meal
<u>Pigments</u>		
Biliverdin	No	Derived from the breakdown of haemoglobin in the blood meal
3-hydroxykynurenine	No	<i>R. prolixus</i> 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  $^{14}\text{C}$ -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  $\mu\text{M}$  putrescine, 1.1  $\mu\text{M}$  cadaverine, 0.9  $\mu\text{M}$  spermidine and 0.5  $\mu\text{M}$  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***

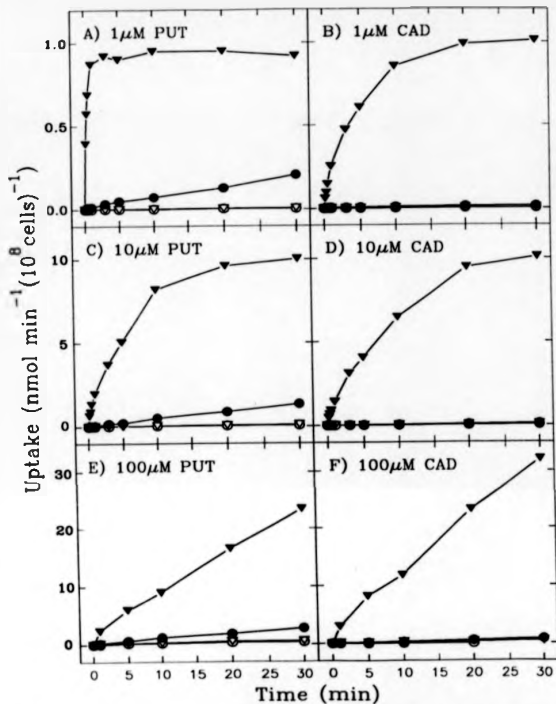


Figure 4.1 Diamine uptake in the trypanosomatids. *T. cruzi* epimastigotes (▼), *C. fasciculata* (●), *L. donovani* promastigotes (▽) and *T. b. brucei* procyclics (○). A background, in which cells were added to the appropriate radiolabel at 4°C and then pelleted immediately, was subtracted from each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

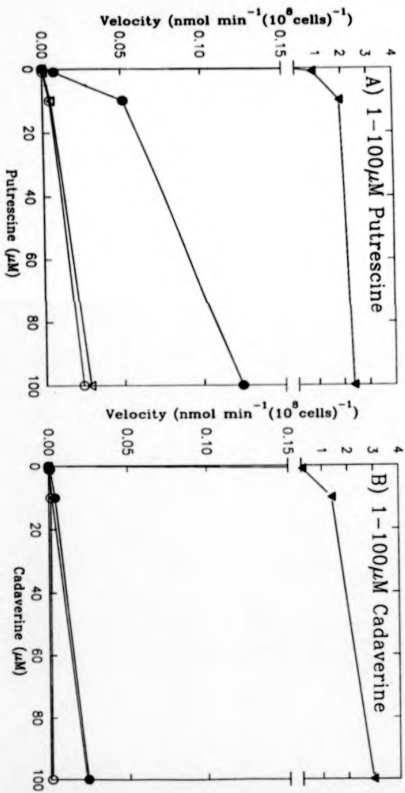


Figure 4.2 Relative velocities of 1-100  $\mu$ M diamine uptake in the trypanosomatids, *T. brucei* epimastigotes (○), *C. fascicularis* (△), *L. donovani* promastigotes (●) and *T. brucei* procyclics (○). The velocities were calculated from the values obtained in Figure 4.1.

## 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  $\mu\text{M}$  [ $^3\text{H}$ ]putrescine and [ $^3\text{H}$ ]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  $\mu\text{M}$  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  $\mu\text{M}$ ) 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  $\mu\text{M}$  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  $\mu\text{M}$  range with a maximum velocity of 2-3  $\text{nmol min}^{-1}$  ( $10^8\text{cells}$ ) $^{-1}$  (Figure 4.2A-

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

Putrescine and cadaverine uptake was also measured in freshly isolated bloodstream *T.b.brucel* 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  $28^\circ\text{C}$  in Grace's insect medium prior to measurements of their diamine uptake in CBSS. It is worth noting that  $1 \mu\text{M}$  putrescine uptake in *L.donovani* grown at  $28^\circ\text{C}$  in RTH+FCS medium ( $34 \text{ pmol } 5 \text{ min}^{-1}$ ) or at  $22^\circ\text{C}$  in Grace's insect medium ( $29 \text{ pmol } 5 \text{ min}^{-1}$ ), is similar to the  $1 \mu\text{M}$  putrescine uptake values observed for cells grown at  $28^\circ\text{C}$  in the Grace's insect medium ( $22 \text{ pmol } 5 \text{ min}^{-1}$ , Figure 4.1). However if the cells are grown at  $22^\circ\text{C}$  in RTH+FCS medium, prior to assay, then the uptake rates over 5 min for both  $1 \mu\text{M}$  putrescine and  $1 \mu\text{M}$  cadaverine become comparable to those shown in Figure 4.1 for *T.cruzi* (putrescine, 1005 versus 899  $\text{pmol } 5 \text{ min}^{-1}$ ; cadaverine, 213 versus 605  $\text{pmol } 5 \text{ min}^{-1}$ ; for *L.donovani* and *T.cruzi* respectively). *L.donovani* promastigotes grow better in both the media when incubated at 22 rather than  $28^\circ\text{C}$ . 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 [ $^3\text{H}$ ]Putrescine and [ $^3\text{H}$ ]Cadaverine after a 90 s exposure of *T.cruzi* epimastigotes to each label

Diamine / Polyamine	[ $^3\text{H}$ ]Putrescine equivalents <sup>1</sup> (%)	[ $^3\text{H}$ ]Cadaverine equivalents <sup>2</sup> (%)
Putrescine	98	-
Spermidine	2	-
Spermine	nd <sup>3</sup>	-
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 1  $\mu\text{M}$  [ $^3\text{H}$ ]putrescine and 10% of the 1  $\mu\text{M}$  [ $^3\text{H}$ ]cadaverine over the 90 s. Recovery of the labels from the column is 93% for [ $^3\text{H}$ ]putrescine and 104% for [ $^3\text{H}$ ]cadaverine. <sup>1</sup> % of the label coeluting with putrescine, spermidine and spermine; <sup>2</sup> % of the label coeluting with cadaverine, aminopropylcadaverine and bis(aminopropyl)cadaverine; <sup>3</sup> nd = not detectable (<0.5%).

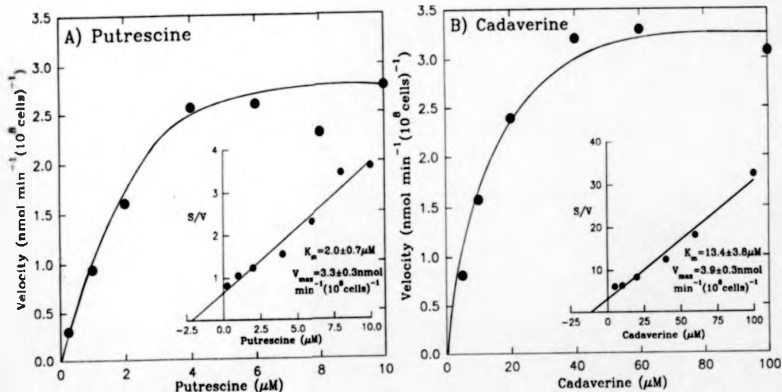


#### 4.2 Kinetics of putrescine and cadaverine transport in *T.cruzi*

##### 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 asexual 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  $\mu\text{M}$  [ $^3\text{H}$ ]putrescine concentrations where as much as 13% (8  $\mu\text{M}$ ) to 45% (0.25  $\mu\text{M}$ ) of the label was taken up by the cells.
- 2) A high cell concentration of approximately  $1 \times 10^8$  cells  $\text{ml}^{-1}$  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  $S/V = 0$ ;  $V_{\max} = 1/\text{slope}$ .

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  $\mu\text{M}$ ) and cadaverine (5 to 100  $\mu\text{M}$ ) concentrations (Figure 4.3).

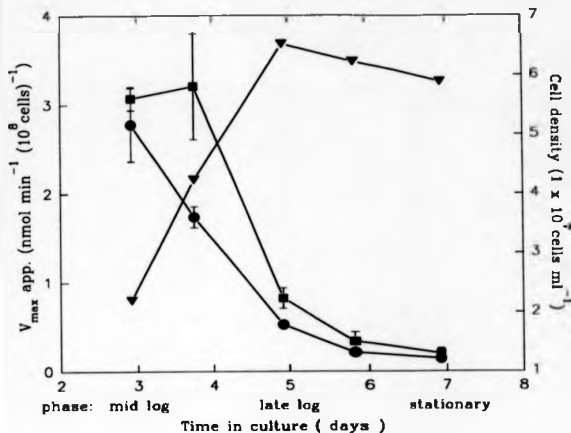
By use of the Michaelis-Menten equation:

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

(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 unreactant system when the initial velocity is plotted against substrate concentration, rectangular hyperbolic curves were obtained for putrescine and cadaverine from which you 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  $K_m = 2.0 \pm 0.7 \mu\text{M}$  and a  $V_{\max} = 3.3 \pm 0.3 \text{ nmol min}^{-1} (10^8 \text{ cells})^{-1}$  (which is equivalent to  $14.1 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$ , 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  $1 \times 10^5$  cells ml<sup>-1</sup> and assayed over days 3 to 7 of their growth cycle for maximal diamine transport rates,  $V_{max\ app.}$ , at 10  $\mu$ M putrescine (□) and 100  $\mu$ M cadaverine (●). For putrescine and cadaverine each point represents the mean value  $\pm$  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 <sup>1</sup> ( $1 \times 10^7$ cells $ml^{-1}$ )	Putrescine Transport <sup>2</sup>		Cadaverine Transport <sup>2</sup>	
		$K_m$ ( $\mu M$ )	$V_{max}$ ( $nmol\ min^{-1}$ ( $10^8$ cells) $^{-1}$ )	$K_m$ ( $\mu M$ )	$V_{max}$ ( $nmol\ min^{-1}$ ( $10^8$ cells) $^{-1}$ )
3	2.32	$1.99 \pm 0.67$	$3.29 \pm 0.34$	$13.4 \pm 3.9$	$3.86 \pm 0.32$
5	6.53	$2.06 \pm 0.65$	$0.61 \pm 0.06^*$	$45.0 \pm 19.3$	$1.01 \pm 0.21^*$
7	5.89	$2.69 \pm 0.63$	$0.17 \pm 0.01^*$	nd <sup>3</sup>	nd

<sup>1</sup> Cell density is the mean value of 2 determinations; <sup>2</sup>  $K_m$  and  $V_{max}$  values were determined using the Eazfitter software package ( $\pm$  SEM);

<sup>3</sup> nd = not determinable; \*Values differ significantly ( $p < 0.001$ ) as compared with 3 day cells,  $n=7$  (the number of points used in Eazfit for the calculation of  $K_m$  and  $V_{max}$  values).

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

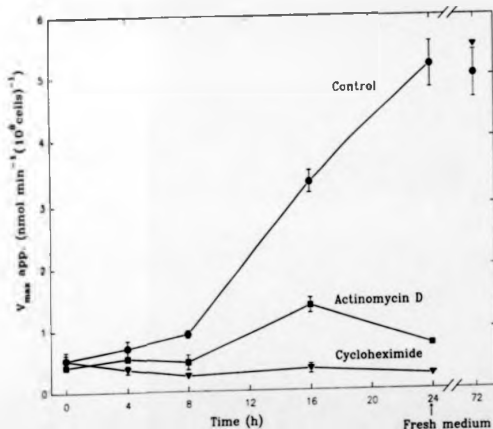
#### 4.3 Alterations in the kinetics of putrescine and cadaverine transport during the growth cycle

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.

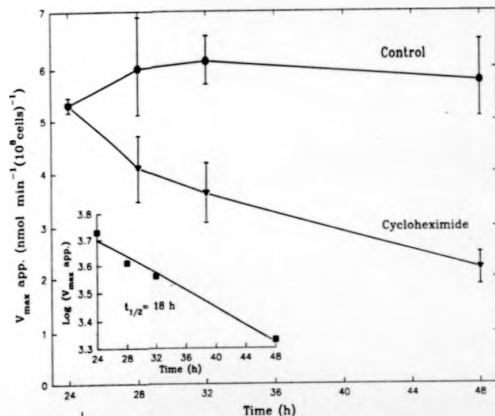
It should be noted here that most of the following work has been carried out solely on the tritiated diamine putrescine as it is a better substrate (lower  $K_m$ ) and more readily available for characterisation of this transporter than cadaverine. Furthermore for Figure 4.4 and much of the subsequent work  $10 \mu\text{M}$  putrescine (or  $100 \mu\text{M}$  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  $100 \mu\text{M}$  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 postreincubation transport.** The *Y. crassus* cells were grown for 7 days and then diluted to  $1 \times 10^7$  cells ml<sup>-1</sup> into fresh medium supplemented with either 10 (or 100)  $\mu$ M cycloheximide (superposable values) or 2  $\mu$ M Actinomycin D and the velocity of 10  $\mu$ M postreincubation transport,  $V_{max\ app}$ , measured at 4 to 8 h intervals over the next 24 h. At 24 h control and 10  $\mu$ M cycloheximide treated cells were washed once and reseeded at  $1 \times 10^7$  cells ml<sup>-1</sup> in fresh medium and incubated for a further 48 h. Control cells were diluted into fresh medium alone. Each point represents the mean values  $\pm$  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  $\pm$  100  $\mu$ M cycloheximide. The velocity of putrescine transport,  $V_{max} \text{ app.}$ , was measured at 24, 28, 32 and 48 hours, each point representing the mean  $\pm$  the standard deviation of 3 determinations. Insert: From a  $\log(V_{max} \text{ app.})$  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 ( $V_{\max, \text{app.}}$ ) 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  $V_{\max, \text{app.}}$  of putrescine transport to a maximum after 24 h. No such corresponding increase in the  $V_{\max, \text{app.}}$  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  $1 \times 10^7$  cells  $\text{ml}^{-1}$ , 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  $\mu\text{M}$  putrescine, 100  $\mu\text{M}$  cycloheximide or 2  $\mu\text{M}$  actinomycin D for 24 h then assayed for remaining [ $^3\text{H}$ ]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	$V_{\max \text{ app.}}^1$ (nmol min <sup>-1</sup> (10 <sup>8</sup> cells) <sup>-1</sup> )	$V_{\max \text{ app.}}^1$ (% 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  $1 \times 10^7$  cells ml<sup>-1</sup> 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. <sup>1</sup> 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+PFS supplemented with 100  $\mu$ M exogenous putrescine or pre-conditioned for 4-7 days with *T.cruzi* cells (day 4 or 7 supernatant)

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

Day 7 (stationary phase) *T.cruzi* cells were washed in CBSS then diluted at  $1 \times 10^7$  ml<sup>-1</sup> into either fresh RTH+PFS medium or day 4 or 7 supernatant (RTH+PFS medium which had been incubated with *T.cruzi* cells, seeded at  $1 \times 10^6$  ml<sup>-1</sup>, for 4 or 7 days respectively and then had the cells removed by centrifugation prior to use). 100 $\mu$ M 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. <sup>1</sup> Measured at 10  $\mu$ M putrescine  $\pm$  standard deviation of 3 determinations; <sup>2</sup> *T.cruzi* cells seeded at  $1 \times 10^6$  cells ml<sup>-1</sup> into fresh RTH+PFS 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  $\mu\text{M}$  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  $\mu\text{M}$  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  $\mu\text{M}$  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  $\mu\text{M}$  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+PCS medium or day 7 supernatant. This suggests a number of possibilities including:

- 1) a component of the fresh RTH+PCS 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

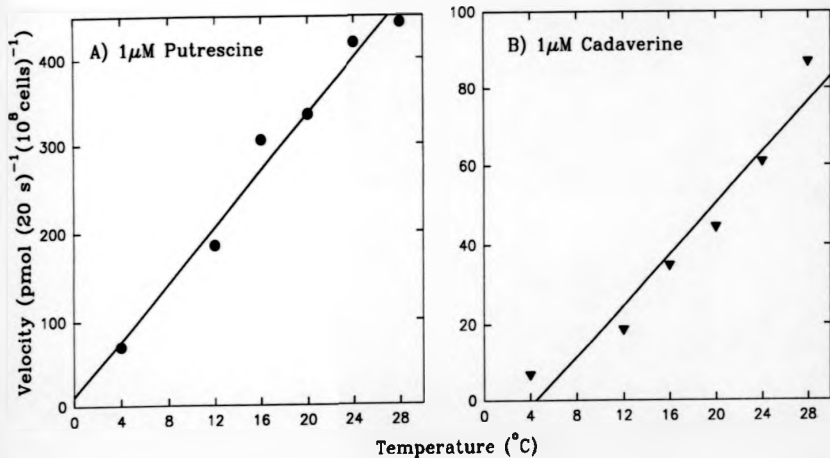


Figure 4.7 Changes in the rate of 1  $\mu$ M putrescine and cadaverine transport with temperature. *T. cruzi* cells were taken on day 5 of the growth cycle and the transport of 1  $\mu$ M putrescine and cadaverine was measured over 20 s at the temperatures indicated. Single observations were made and 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  $\mu$ M putrescine to obtain the  $V_{max,app}$  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:

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

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<sup>-1</sup>mol<sup>-1</sup>) and  $T$  is the temperature in Kelvin.

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

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

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

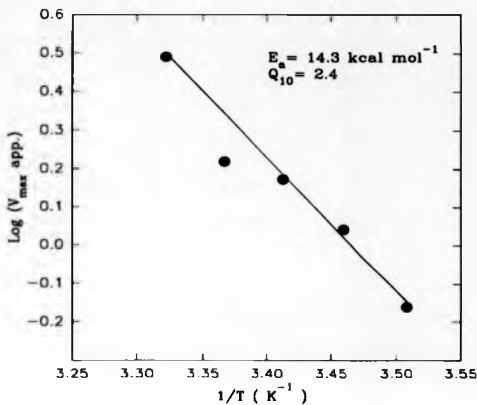
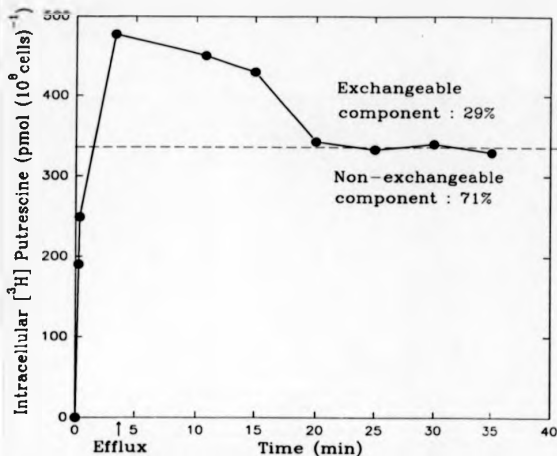


Figure 4.8 Arrhenius plot for putrescine transport. From the slope of the graph ( $-E_a / 2.3R$ ) an activation energy,  $E_a$ , of 14.3 kcal/mol was obtained for putrescine transport. The  $Q_{10}$  value which represents the increase in  $V_{\text{max app.}}$  observed when the temperature is raised 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. crust* cells taken on day 5 of their growth cycle, were preloaded with 1  $\mu$ M [<sup>3</sup>H]putrescine, at a concentration of  $1 \times 10^6$  cells  $\text{ml}^{-1}$ , for 3 minutes 20 s, then the cells were pelleted, the supernatant removed and an equal volume of 1  $\mu$ M unlabelled putrescine added. Cells were assayed at about 5 min intervals over the next 35 min, to estimate how much [<sup>3</sup>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 14.3 kcal mol<sup>-1</sup> and an increase in the  $V_{max,app}$  over a 10° 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  $\mu$ M [<sup>3</sup>H]putrescine, transferred to 1  $\mu$ M unlabelled putrescine and assayed over the next 35 min to estimate how much [<sup>3</sup>H]putrescine remained inside the cells (Figure 4.9). This suggested that about a quarter of the [<sup>3</sup>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 [<sup>3</sup>H]putrescine was measured over minutes as opposed to seconds, a substantial amount of metabolism of the putrescine label is likely to have occurred, 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 <sup>1</sup> (% Inhibition)
<u><b>Ionophores:</b></u> <sup>2</sup>	
20nM Gramicidin <sup>4</sup> (Na <sup>+</sup> /K <sup>+</sup> )	13
2μM Valinomycin (K <sup>+</sup> )	28
10μM Carbonylcyanide <i>m</i> -chlorophenyl hydrazone (H <sup>+</sup> uncoupler)	90
2μM Calcimycin - A23187 (Ca <sup>2+</sup> )	29
<u><b>Metabolic inhibitors:</b></u> <sup>3</sup>	
1μg/ml Oligomycin (Mitochondrial ATPases)	-3
1mM Ouabain ( Na <sup>+</sup> /K <sup>+</sup> pump)	7
1mM 2,4 Dinitrophenol (uncoupler of electron transport)	67
1mM Iodoacetate (glycolysis)	37
<u><b>Thiol reagents:</b></u> <sup>3</sup>	
1mM <i>N</i> -Ethylmaleimide	>99
200μM <i>p</i> -Hydroxymercuribenzoate	>99
200μM <i>p</i> -Chloromercuriphenyl sulphonate	99

<sup>1</sup> Control 2 μM Putrescine Velocity = 3.0 nmol min<sup>-1</sup>(10<sup>8</sup>cells)<sup>-1</sup> and represents zero percent inhibition. <sup>2</sup> 20 minute pre-incubation with the cells. <sup>3</sup> 10 minute pre-incubation with the cells. The results expressed here are the mean values of 2 observations. <sup>4</sup>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*-chloromercuribenzenesulphonate (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*-chloromercuribenzenesulphonate (which have no effect 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 (200 $\mu$ M)	Diamine precursor <sup>1</sup>	Transport (% inhibition <sup>2</sup> )
A ( neutral )	Asparagine	-	-12
	Serine	-	-13
L ( neutral )	Leucine	-	-16
Ly <sup>+</sup> ( basic )	Lysine	Cadaverine	0
	Arginine	Putrescine (via ornithine or agmatine)	-1
$\beta^+$ ( acidic )	Aspartic acid	-	7
-	Ornithine	Putrescine	-8
-	Agmatine	Putrescine	24

<sup>1</sup> 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. <sup>2</sup> Control 2  $\mu$ M Putrescine Velocity = 2.5 nmol min<sup>-1</sup>(10<sup>8</sup>cells)<sup>-1</sup> 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  $\beta^+$  (acidic side chains) transport systems show any marked inhibitory effect 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(guanyldrazone) and paraquat on putrescine transport

Inhibitor	Transport (% Inhibition <sup>1</sup> )
50 $\mu$ M MGBG	14
500 $\mu$ M MGBG	20
50 $\mu$ M Paraquat	6
500 $\mu$ M 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  $\mu$ M [<sup>3</sup>H]putrescine transport when added simultaneously was investigated. <sup>1</sup> Control 5  $\mu$ M Putrescine Velocity = 1.6 nmol min<sup>-1</sup>(10<sup>8</sup>cells)<sup>-1</sup> and represents zero percent inhibition. The results expressed here are the mean values of 2 observations.

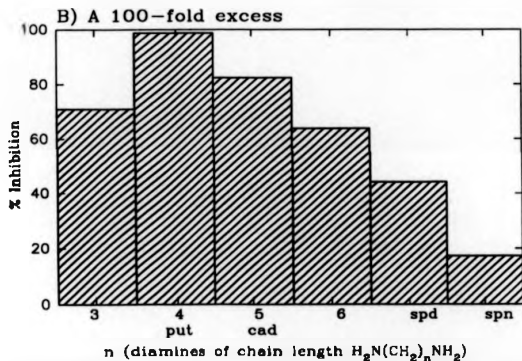
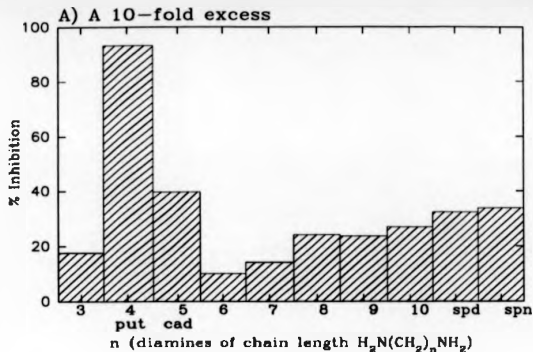
These results indicate that putrescine is not taken up on any of the known amino acid uptake systems. It also suggests that it is the presence of a carboxyl group, attached to the  $\alpha$ -carbon atom of an amino acid, which prevents recognition of the basic amino acids by the putrescine transporter. 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).

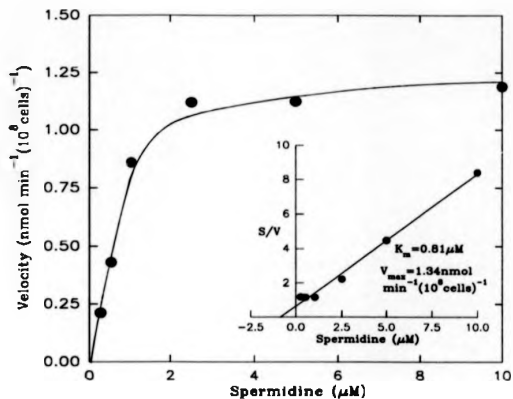
#### 4.8.3 Effect of sodium ions

Many mammalian polyamine uptake systems appear to exhibit sodium dependency (reviewed in section 1.5.3). When the  $\text{Na}^+$  in CBSS was iso-osmotically replaced with  $\text{Ch}^+$  and 1  $\mu\text{M}$  putrescine transport activity measured, it was found that putrescine transport activity was 2.9-fold higher in *T. cruzi* cells incubated in the  $\text{Na}^+$  free CBSS than in control cells assayed in the ordinary  $\text{Na}^+$  containing CBSS. This suggests that  $\text{Na}^+$  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 ( $\text{Na}^+$ ,  $\text{NH}_4^+$



**Figure 4.10 Inhibition of putrescine transport by diamines and polyamines.** Cells were put into fresh RTH+PCS medium at  $1 \times 10^6$  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  $\mu$ M [ $^3$ 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  $\mu$ M putrescine velocity = 1.9 nmol min $^{-1}$   $10^6$  cells $^{-1}$ . B) A 100-fold excess; control 5  $\mu$ M putrescine velocity = 1.3 nmol min $^{-1}$   $10^6$  cells $^{-1}$ .





**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 Enzfitter software package. Inset: 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 effect 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  $\mu M$  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 [ $^3H$ ]spermidine transport into *T. crust.* (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). [ $^3H$ ]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,  $K_m = 0.81 \pm 0.22 \mu M$  but with a  $V_{max} = 1.34 \text{ nmol min}^{-1} (10^8 \text{ cells})^{-1}$  which was 3-4 fold lower than that observed for putrescine.

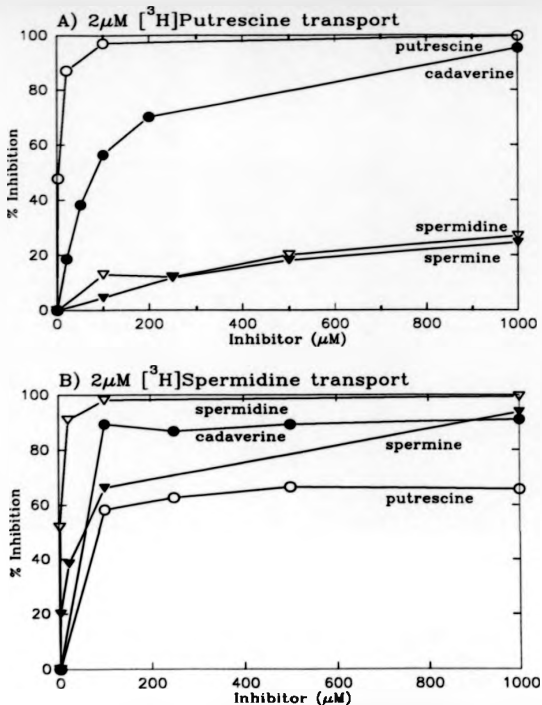


Figure 4.12 Inhibition of 2  $\mu$ M [ $^3$ H]putrescine or [ $^3$ H]cadaverine transport into *T. cruzi* epimastigotes by unlabelled putrescine, cadaverine, spermidine or spermine. The pattern of inhibition of 2  $\mu$ M [ $^3$ H]putrescine or [ $^3$ H]spermidine transport was examined on concurrent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

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

The  $K_m$  values of putrescine and spermidine transport are  $1.34 \pm 0.41 \mu\text{M}$  and  $0.81 \pm 0.22 \mu\text{M}$  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 sulphhydryl 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**

## CHAPTER 5 : DISCUSSION

Previous to this study there have been very few reports on polyamine metabolism in *T.cruzi* (Schwartz 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 & Sjoerdama, 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; Schwartz 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 (Philippe *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, González *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 PCS (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  $\mu$ M) 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 Zilberstein, 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; Pistocchi *et al.* 1993). Putrescine can then be excreted from *E.coli* cells through the *potE* protein (Kaashiwagi *et al.* 1991) which is a putrescine-ornithine antiporter (Kaashiwagi *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.brucel* 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 in order 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.brucel* D-glucose transporter (Seyfang & Duszzenko 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.brucel* bloodstream forms (at  $2 \times 10^8$  cells 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  $\mu$ M 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.brucel* 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  $\mu\text{M}$  putrescine, 1.1  $\mu\text{M}$  cadaverine, 0.9  $\mu\text{M}$  spermidine and 0.5  $\mu\text{M}$  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 form cadaverine with a  $K_m$  that is about 100 times higher than for ornithine (McCaan & 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

## DIAMINES

## DIAMINES &amp; POLYAMINES

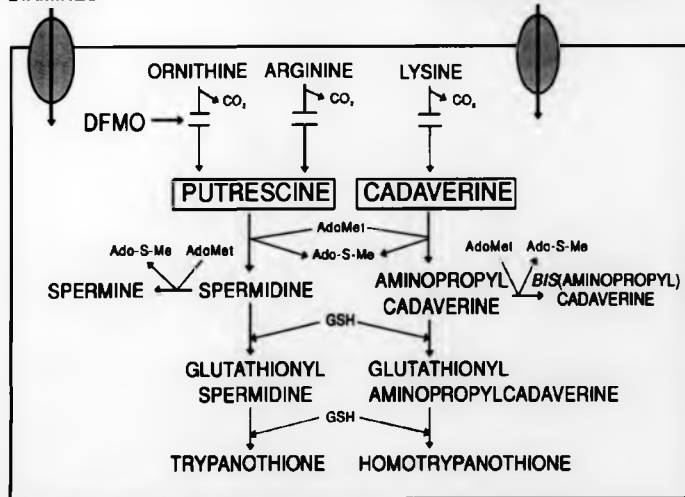


Figure 3.1 Uptake and metabolism of diamines in *T. cruzi* epimastigotes. There is no *de novo* synthesis of putrescine and cadaverine from the amino acids ornithine, arginine, and lysine.



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**

## CHAPTER 6 : REFERENCES

- Algranati, I.D., Sanchez, C. & Gonzales, N.S. (1989). Polyamines in *Trypanosoma cruzi* and *Leishmania mexicana*. In: *The Biology and Chemistry of Polyamines*, Goldenberg, S.H. & Algranati, I.D., (editors). Oxford: Oxford University Press, pp. 137-146.
- Alves, F., Ståhl, E., Höcker, M. & Fölach, U.R. (1992). Putrescine uptake and metabolism in isolated rat pancreatic acini. *Digestion*, 53, 8-16.
- Aronow, B., Allen, K., Patrick, J. & Ullman, B. (1985). Altered nucleoside transporters in mammalian cells selected for resistance to the physiological effects of inhibitors of nucleoside transport. *Journal of Biological Chemistry*, 260, 6226-6233.
- Arrick, B.A., Griffith, O.W. & Cerami, A. (1981). Inhibition of glutathione synthesis as a chemotherapeutic strategy for trypanosomiasis. *Journal of Experimental Medicine*, 153, 720-725.
- Auberger, P., Samson, M. & Le Cam, A. (1983). Inhibition of hormonal induction of tyrosine aminotransferase by polyamines in freshly isolated rat hepatocytes. *Biochemical Journal*, 214, 679-685.
- Auelli, R., Stjernborg, L., Khomutov, A.R., Knomutov, R.M. & Persson, L. (1991). Regulation of S-adenosylmethionine decarboxylase in L1210 leukemia cells: studies using an irreversible inhibitor of the enzyme. *European Journal of Biochemistry*, 196, 551-556.
- Avila, J.L. & Polegre, M.A. (1993). Uptake and metabolism of S-adenosyl-L-methionine by *Leishmania mexicana* and *Leishmania braziliensis* promastigotes. *Molecular and Biochemical Parasitology*, 58, 123-134.

Bacchi, C.J. (1981). Content, synthesis and function of polyamines in trypanosomatids: relationship to chemotherapy. *Journal of Protozoology*, 2867, 20-27.

Bacchi, C.J., Goldberg, S., Yarett, N. & Garofalo, J. (1992). Increase in methylation of proteins during exposure of *Trypanosoma brucei* to a antipolyamine agents. *American Journal of Tropical Medicine and Hygiene*, 47, 277. (Abstract)

Bacchi, C.J., Lipschik, G.P. & Nathan, H.C. (1977). Polyamines in trypanosomatids. *Journal of Bacteriology*, 131, 657-661.

Bacchi, C.J. & McCann, P.P. (1987). Parasitic protozoan and polyamines. In: *Inhibition of Polyamine Metabolism: Biological Significance and Basis for New Therapies*, McCann, P.P., Pegg, A.E. & Sjoerdama, A., (editors). Orlando, Florida: Academic Press, pp. 317-344.

Bacchi, C.J., Nathan, H.C., Hutner, S.H., Duch, D.S. & Nichol, C.A. (1980a). Negation of trypanocidal drug cures by polyamines. In: *Current Chemotherapy and Infectious Disease*, Nelson, J.D. & Grassi, C., (editors). Washington, DC: The American Society for Microbiology, pp. 1119-1121.

Bacchi, C.J., Nathan, H.C., Hutner, S.H., McCann, P.P. & Sjoerdama, A. (1980b). Polyamine metabolism: a potential therapeutic target in trypanosomes. *Science*, 210, 332-334.

Bacchi, C.J., Nathan, H.C., Yarett, N., Goldberg, B., McCann, P.P., Bitonti, A.J. & Sjoerdama, A. (1992). Cure of murine *Trypanosoma brucei rhodesiense* infections with an S-adenosylmethionine decarboxylase inhibitor. *Antimicrobial Agents and Chemotherapy*, 36, 2736-2740.

Bacchi, C.J., Vergara, C., Garofalo, J., Lipschik, G.Y. & Hutner, S.H. (1979). Synthesis and content of polyamines in bloodstream *Trypanosoma brucei*. *Journal of Protozoology*, 26, 484-488.

- Bacchi, C.J. & Yarlett, N. (1993). Effects of antagonists of polyamine metabolism on African trypanosomes. *Acta Tropica*, **54**, 225-236.
- Bachrach, U. (1984). Physiological aspects of ornithine decarboxylase. *Cell Biochemistry and Function*, **2**, 6-10.
- Bachrach, U., Brem, S., Wertman, S.B., Schnur, L.F. & Greenblatt, C.L. (1979). *Leishmania* spp.: cellular levels and synthesis of polyamines during growth cycles. *Experimental Parasitology*, **48**, 457-463.
- Balana-Fouce, R., Escribano, M.I. & Alunda, J.M. (1991). *Leishmania infantum* - polyamine biosynthesis and levels during the growth of promastigotes. *International Journal of Biochemistry*, **23**, 1213-1217.
- Balana-Fouce, R., Ordóñez, D. & Alunda, J.M. (1989). Putrescine transport system in *Leishmania infantum* promastigotes. *Molecular and Biochemical Parasitology*, **35**, 43-50.
- Ballas, S.K., Mohandas, N., Maron, L.J. & Shohet, S.B. (1983). Stabilization of erythrocyte membranes by polyamines. *Proceedings of the National Academy of Sciences of the United States of America*, **80**, 1942-1946.
- Baumann, R.J., Hanson, W.L., McCann, P.P., Sjoerdama, A. & Bitonti, A.J. (1990). Suppression of both antimony-susceptible and antimony-resistant *Leishmania donovani* by a bis(benzyl)polyamine analog. *Antimicrobial Agents and Chemotherapy*, **34**, 722-727.
- Berger, B.J., Carter, N.S. & Fairlamb, A.H. (1993). Polyamine and pentamidine metabolism in African trypanosomes. *Acta Tropica*, **54**, 215-224.
- Bethell, D.R. & Pegg, A.E. (1981). Uptake of putrescine by 3T3 and SV3T3 cells and

its effect on ornithine decarboxylase activity. *Journal of Cellular Physiology*, **109**, 461-468.

Bey, P., Danzin, C. & Jung, M. (1987). Inhibition of basic amino acid decarboxylases involved in polyamine biosynthesis. In: *Inhibition of Polyamine Metabolism: Biological Significance and Basis for New Therapies*, McCann, P.P., Pegg, A.E. & Sjoerdama, A., (editors). Orlando: Academic Press, pp. 1-31.

Bitonti, A.J., Byers, T.L., Bush, T.L., Casara, P.J., Bacchi, C.J., Clarkson, A.B., Jr., McCann, P.P. & Sjoerdama, A. (1990). Cure of *Trypanosoma brucei brucei* and *Trypanosoma brucei rhodesiense* infections in mice with an irreversible inhibitor of S-adenosylmethionine decarboxylase. *Antimicrobial Agents and Chemotherapy*, **34**, 1485-1490.

Bitonti, A.J., Cross-Doersen, E. & McCann, P.P. (1988). Effects of  $\alpha$ -difluoromethylornithine on protein synthesis and synthesis of the variant-specific glycoprotein (VSG) in *Trypanosoma brucei brucei*. *Biochemical Journal*, **250**, 295-298.

Bitonti, A.J., Dumont, J.A. & McCann, P.P. (1986). Characterization of *Trypanosoma brucei brucei* S-adenosyl-L-methionine decarboxylase and its inhibition by Berenil, pentamidine and methylglyoxal bis(guanyldrazone). *Biochemical Journal*, **237**, 685-689.

Bogle, R.G., Mann, G.E., Pearson, J.D. & Morgan, D.M.L. (1994). Endothelial polyamine uptake: Selective stimulation by L-arginine deprivation or polyamine depletion. *American Journal of Physiology*, **266**, C776-C783.

Bolkenius, F.N. & Seiler, N. (1989). Polyamine oxidase inhibitors. In: *Design of Enzyme Inhibitors as Drugs*, Sandler, M. & Smith, H.J., (editors). New York: Oxford university press, pp. 245-255.

- Braunlin, W.H., Strick, T.J. & Record, M.T., Jr. (1982). Equilibrium dialysis studies of polyamine binding to DNA. *Biopolymers*, **21**, 1301-1314.
- Brecher, G. & Wigglesworth, V.B. (1944). The transmission of *Actinomyces rhodni* Erikson in *Rhodnius prolixus* stål (Hemiptera) and its influence on the growth of the host. *Parasitology*, **35**, 220-224.
- Brun, R. & Schonenberger, M. (1979). Cultivation and in vitro cloning of procyclic culture forms of *Trypanosoma brucei* in a semi-defined medium. *Acta Tropica*, **36**, 289-292.
- Brunton, V.G., Grant, M.H. & Wallace, H.M. (1991). Mechanisms of spermine toxicity in baby-hamster kidney (BHK) cells. The role of amine oxidases and oxidative stress. *Biochemical Journal*, **280**, 193-198.
- Bueb, J.L., De-Silva, A., Mouali, M. & Landry, Y. (1992). Natural polyamines stimulate G-proteins. *Biochemical Journal*, **282**, 545-550.
- Byers, T.L., Bitonti, A.J. & McCann, P.P. (1990). Bis(benzyl)polyamine analogues are substrates for a mammalian cell-transport system which is distinct from the polyamine-transport system. *Biochemical Journal*, **269**, 35-40.
- Byers, T.L., Bush, T.L., McCann, P.P. & Bitonti, A.J. (1991). Antitrypanosomal effects of polyamine biosynthesis inhibitors correlate with increases in *Trypanosoma brucei brucei* S-adenosyl-L-methionine. *Biochemical Journal*, **274**, 527-533.
- Byers, T.L., Kameji, R., Rannels, D.E. & Pegg, A.E. (1987). Multiple pathways for uptake of paraquat, methylglyoxal bis(guanyldiazide), and polyamines. *American Journal of Physiology*, **252**, C663-C669.
- Byers, T.L. & Pegg, A.E. (1989). Properties and physiological function of the polyamine transport system. *American Journal of Physiology - Cell Physiology*, **257**, C545-C553.



- Byers, T.L. & Pegg, A.E. (1990). Regulation of polyamine transport in Chinese hamster ovary cells. *Journal of Cellular Physiology*, 143, 460-467.
- Byers, T.L., Wechter, R., Nuttall, M.E. & Pegg, A.E. (1989). Expression of a human gene for polyamine transport in Chinese hamster ovary cells. *Biochemical Journal*, 263, 745-752.
- Carrera, L., Balana-Fouce, R. & Alunda, J.M. (1994). Polyamine content and drug sensitivities of different clonal lines of *Leishmania infantum* promastigotes. *Parasitology Research*, 80, 203-207.
- Carter, N.S. & Fairlamb, A.H. (1993). Arsenical-resistant trypanosomes lack an unusual adenosine transporter. *Nature*, 361, 173-176.
- Casara, P., Marchal, P., Wagner, J. & Danzin, C. (1989). 5'-((Z)-4-amino-2-butenyl)methylamino)-5'-deoxy-adenosine: a potent enzyme-activated irreversible inhibitor of S-adenosyl-L-methionine decarboxylase from *Escherichia coli*. *Journal of the American Chemical Society*, 111, 9111-9113.
- Chen, K.Y. & Rinehart, C.A. (1981). Difference in putrescine transport in undifferentiated versus differentiated mouse NB-15 neuroblastoma cells. *Biochemical and Biophysical Research Communications*, 101, 243-249.
- Christensen, H.N. (1979). Exploiting amino acid structure to learn about membrane transport. *Advances in Enzymology*, 49, 41-101.
- Chun, P.W., Saffen, E.E., DiToro, R.J., Rennert, O.M. & Weinstein, N.H. (1977). Polyamines-sickling red blood cell interaction. *Biophysical Chemistry*, 6, 321-335.
- Claverie, N., Wagner, J., Knodgen, B. & Seiler, N. (1987). Inhibition of polyamine oxidase improves the antitumoral effect of ornithine decarboxylase inhibitors. *Anticancer Research*, 7, 765-772.

- Coombs, G.H. & Sanderson, B.E. (1985). Amine production by *Leishmania mexicana*. *Annals of Tropical Medicine and Parasitology*, **79**, 409-415.
- Coons, T., Hanson, S., Bitonti, A.J., McCann, P.P. & Ullman, B. (1990). Alpha-difluoromethylornithine resistance in *Leishmania donovani* is associated with increased ornithine decarboxylase activity. *Molecular and Biochemical Parasitology*, **39**, 77-90.
- Cooper, K.D., Shukla, J.B. & Rennert, O.M. (1978). Polyamine compartmentalization in various human disease states. *Clinica Chimica Acta*, **82**, 1-7.
- Damper, D. & Patton, C.L. (1976). Pentamidine transport and sensitivity in brucei-group trypanosomes. *Journal of Protozoology*, **23**, 349-356.
- Davis, R.H., Morris, D.R. & Coffino, P. (1992). Sequestered end products and enzyme regulation: The case of ornithine decarboxylase. *Microbiological Reviews*, **56**, 280-290.
- Davis, R.H. & Ristow, J.L. (1988). Polyamine transport in *Neurospora crassa*. *Archives of Biochemistry and Biophysics*, **267**, 479-489.
- Davis, R.H., Ristow, J.L., Howard, A.D. & Barnett, G.R. (1991). Calcium modulation of polyamine transport is lost in a putrescine-sensitive mutant of *Neurospora crassa*. *Archives of Biochemistry and Biophysics*, **285**, 297-305.
- de Castro, S.L. (1993). The challenge of Chagas' disease chemotherapy: an update of drugs assayed against *Trypanosoma cruzi*. *Acta Tropica*, **53**, 83-98.
- De Smedt, H., Van Den Bosch, L., Geuns, J. & Borghgraef, R. (1989). Polyamine transport systems in the LLC-PK<sub>1</sub> renal epithelial established cell line. *Biochimica et Biophysica Acta*, **1012**, 171-177.

- DeBenedene, M., Olson, J.W. & Snow, E.C. (1993). Expression of polyamine transporter activity during B lymphocyte cell cycle progression. *Journal of Immunology*, **150**, 4218-4224.
- Desjeux, P. (1992). Human Leishmaniasis: epidemiology and public health aspects. *World Health Organization - World Health Statistics Quarterly*, **45**, 267-275.
- Dezeure, F., Sarhan, S. & Seiler, N. (1988). Chain-fluorinated polyamines as tumor-markers-IV. comparison of 2-fluoroputrescine and 2,2-difluoroputrescine as substrates of spermidine synthase *in vitro* and *in vivo*. *International Journal of Biochemistry*, **20**, 1299-1312.
- DiPaquale, A., White, D. & McGuire, J. (1978). Epidermal growth factor stimulates putrescine transport and ornithine decarboxylase activity in cultivated human fibroblasts. *Experimental Cell Research*, **116**, 317-323.
- Docampo, R., Gadelha, F.R. & Moreno, S.N. (1991). Metabolism and mode of anti-*Trypanosoma cruzi* action of Gentian violet. In: *Molecular and Immunological Aspects of Parasitism*, Wang, C.C., (editor). Washington: American Association for the Advancement of Science, pp. 95-105.
- Dusanic, D.G. (1991). *Trypanosoma (Schizotrypanum) cruzi*. In: *Parasitic protozoa*, Kreier, J.P. & Baker, J.R., (editors). San Diego: Academic Press, Inc., pp. 137-194.
- Expanded Program on Immunisation, (1992). New initiative will halt Chagas disease in six years, says Argentine health official. *Tropical Disease Research news*, **40**, 3-5.
- Fairlamb, A.H. (1988). The role of glutathionylspermidine and trypanothione in regulation of intracellular spermidine levels during growth of *Crithidia fasciculata*. In: *Progress in Polyamine Research: Novel Biochemical, Physiological and Clinical Aspects*, Zappia, V. & Pegg, A.E., (editors). New York: Plenum Press, pp. 667-674.

- Fairlamb, A.H. (1989a). Novel biochemical pathways in parasitic protozoa. *Parasitology*, **99S**, 93-112.
- Fairlamb, A.H. (1991). Trypanothione metabolism in the chemotherapy of Leishmaniasis and Trypanosomiasis. In: *Molecular & Immunological Aspects of Parasitism*, Wang, C.C., (editor). Washington, D.C.: American Association for the Advancement of Science, pp. 107-121.
- Fairlamb, A.H., Blackburn, P., Ulrich, P., Chait, B.T. & Cerami, A. (1985). Trypanothione: a novel bis(glutathionyl)spermidine cofactor for glutathione reductase in trypanosomatids. *Science*, **227**, 1485-1487.
- Fairlamb, A.H., Carter, N.S., Cunningham, M. & Smith, K. (1992). Characterisation of melarsen-resistant *Trypanosoma brucei brucei* with respect to cross-resistance to other drugs and trypanothione metabolism. *Molecular and Biochemical Parasitology*, **53**, 213-222.
- Fairlamb, A.H. & Cerami, A. (1992). Metabolism and functions of trypanothione in the Kinetoplastida. *Annual Review of Microbiology*, **46**, 695-729.
- Fairlamb, A.H., Henderson, G.B. & Cerami, A. (1986). The biosynthesis of trypanothione and N<sup>1</sup>-glutathionylspermidine in *Crithidia fasciculata*. *Molecular and Biochemical Parasitology*, **21**, 247-257.
- Fairlamb, A.H., Henderson, G.B., Bacchi, C.J. & Cerami, A. (1987). *In vivo* effects of difluoromethylornithine on trypanothione and polyamine levels in bloodstream forms of *Trypanosoma brucei*. *Molecular and Biochemical Parasitology*, **24**, 185-191.
- Fairlamb, A.H., Henderson, G.B. & Cerami, A. (1989b). Trypanothione is the primary target for arsenical drugs against African trypanosomes. *Proceedings of the National Academy of Sciences of the United States of America*, **86**, 2607-2611.

- Pecker, L.F., Beier, H. & Berlin, J. (1986). Cloning and characterization of a lysine decarboxylase gene from *Hafnia alvei*. *Molecular and General Genetics*, 203, 177-184.
- Feige, J.J. & Chambaz, E.M. (1985). Polyamine uptake by bovine adrenocortical cells. *Biochimica et Biophysica Acta*, 846, 93-100.
- Ferrante, A., Allison, A.C. & Hirumi, H. (1982). Polyamine oxidase-mediated killing of African trypanosomes. *Parasite Immunology*, 4, 349-354.
- Ferrante, A., Rzepczyk, C.M. & Saul, A.J. (1984). Polyamine oxidase-mediated trypanosome killing: the role of hydrogen peroxide and aldehydes. *Journal of Immunology*, 133, 2157-2162.
- Folk, J.E. (1980). Transglutaminases. *Annual Review of Biochemistry*, 49, 517-531.
- Fong, W.F., Heller, J.S. & Canellakis, E.S. (1976). The appearance of an ornithine decarboxylase inhibitory protein upon the addition of putrescine to cell cultures. *Biochimica et Biophysica Acta*, 428, 456-465.
- Frydman, L., Rossomando, P.C., Frydman, V., Fernandez, C.O., Frydman, B. & Samejima, K. (1992). Interactions between natural polyamines and tRNA: an  $^{15}\text{N}$  NMR analysis. *Proceedings of the National Academy of Sciences of the United States of America*, 89, 9186-9190.
- Fujiwara, S. & Harada, Y. (1989). Fast-growing root nodule bacteria produce a novel polyamine, aminobutylhomospermidine. *Biochemical and Biophysical Research Communications*, 165, 659-666.
- Fukuchi, J., Kashiwagi, K., Kusama-Eguchi, K., Terao, K., Shirahata, A. & Igarashi, K. (1992). Mechanism of the inhibition of cell growth by  $\text{N}^1, \text{N}^{12}$ -bis(ethyl)spermine. *European Journal of Biochemistry*, 209, 689-696.

- Furuchi, T., Kashiwagi, K., Kobayashi, H. & Igarashi, K. (1991). Characteristics of the gene for a spermidine and putrescine transport system that maps at 15 min on the *Escherichia coli* chromosome. *Journal of Biological Chemistry*, **266**, No. 31, 20928-20933.
- Gawel-Thompson, K. & Greene, R.M. (1988). Characterization of a polyamine transport system in murine embryonic palate mesenchymal cells. *Journal of Cellular Physiology*, **136**, 237-246.
- Gawel-Thompson, K.J. & Greene, R.M. (1989). Epidermal growth factor: Modulator of murine embryonic palate mesenchymal cell proliferation, polyamine biosynthesis, and polyamine transport. *Journal of Cellular Physiology*, **140**, 359-370.
- Ghoda, L., Basu, H.S., Porter, C.W., Marion, L.J. & Coffino, P. (1992). Role of ornithine decarboxylase suppression and polyamine depletion in the antiproliferative activity of polyamine analogs. *Molecular Pharmacology*, **42**, 302-306.
- Ghoda, L., Phillips, M.A., Bass, K.E., Wang, C.C. & Coffino, P. (1990). Trypanosome ornithine decarboxylase is stable because it lacks sequences found in the carboxyl terminus of the mouse enzyme which target the laser for intracellular degradation. *Journal of Biological Chemistry*, **265**, 11823-11826.
- Ghoda, L., Sidney, D., Macrae, M. & Coffino, P. (1992). Structural elements of ornithine decarboxylase required for intracellular degradation and polyamine-dependent regulation. *Molecular and Cellular Biology*, **12**, 2178-2185.
- Ghoda, L., van Daalen Wetters, T., Macrae, M., Ascherman, D. & Coffino, P. (1989). Prevention of rapid intracellular degradation of ODC by a carboxyl-terminal truncation. *Science*, **243**, 1493-1495.
- Gibson, W.C. & Miles, M.A. (1986). The karyotype and ploidy of *Trypanosoma cruzi*. *EMBO Journal*, **5**, 1299-1305.

- Giffin, B.F. & McCann, P.P. (1989). Physiological activation of the mitochondrion and the transformation capacity of DFMO induced intermediate and short stumpy bloodstream form trypanosomes. *American Journal of Tropical Medicine and Hygiene*, **40**, 487-493.
- González, N.S., Sánchez, C.P., Sterco, L. & Algranati, I.D. (1991). Control of *Leishmania mexicana* proliferation by modulation of polyamine intracellular levels. *Biochemical and Biophysical Research Communications*, **180**, 797-804.
- González, N.S., Coriani, C. & Algranati, I.D. (1992). Differential regulation of putrescine uptake in *Trypanosoma cruzi* and other trypanosomatids. *Biochemical and Biophysical Research Communications*, **188**, 120-128.
- González, N.S., Coriani, C. & Algranati, I.D. (1993). Transporte de poliaminas en trypanosomatidos. *Anales de la Asociacion Química Argentina*, **81**, 117-125.
- Greenberg, C.S., Birkbichler, P.J. & Rice, R.H. (1991). Transglutaminases: Multifunctional cross-linking enzymes that stabilize tissues. *FASEB Journal*, **5**, 3071-3077.
- Gretlin, D.P., Bartos, D., Bartos, F., Smith, R.G. & Campbell, R.A. (1972). Purification of radiolabelled polyamines and isolation of polyamines from serum by silica gel chromatography. *Advances in Polyamine Research*, **7**, 13-21.
- Gutteridge, W.E. (1987). New anti-protozoal agents. *International Journal for Parasitology*, **17**, 121-129.
- Hamana, K. & Matsuzaki, S. (1987). Distribution of polyamines in actinomycetes. *FEMS Microbiology Letters*, **41**, 211-215.
- Hamana, K., Matsuzaki, S., Hosaka, K. & Yamashita, S. (1989). Interconversion of polyamines in wild-type strains and mutants of yeasts and the effects of polyamines on their growth. *FEMS Microbiology Letters*, **52**, 231-236.

- Hanson, W.L., Bradford, M.M., Chapman, W.L., Waits, V.B., McCann, P.P. & Sjoerdama, A. (1982).  $\alpha$ -Difluoromethylornithine: A promising lead for preventive chemotherapy for coccidiosis. *American Journal of Veterinary Research*, 43, 1651-1653.
- He, Y., Suzuki, T., Kashiwagi, K., Kusama-Eguchi, K., Shirahata, A. & Igarashi, K. (1994). Correlation between the inhibition of cell growth by bis(ethyl)polyamine analogues and the decrease in the function of mitochondria. *European Journal of Biochemistry*, 221, 391-398.
- Heston, M.A. & Flinnoff, W.F. (1988). Methylglyoxal-bis(guanyldiazotone)-resistant Chinese hamster ovary cells: genetic evidence that more than a single locus controls uptake. *Journal of Cellular Physiology*, 136, 133-139.
- Heby, O. (1981). Role of polyamines in the control of cell proliferation and differentiation. *Differentiation*, 19, 1-20.
- Heby, O. & Persson, L. (1990). Molecular genetics of polyamine synthesis in eukaryotic cells. *Trends in Biochemical Sciences*, 15, 153-158.
- Heller, J.S., Fong, W.F. & Canellakis, E.S. (1976). Induction of a protein inhibitor to ornithine decarboxylase by the end products of its reaction. *Proceedings of the National Academy of Sciences of the United States of America*, 73, 1858-1862.
- Henderson, G.B., Ulrich, P., Fairlamb, A.H., Rosenberg, I., Pereira, M., Sela, M. & Cerami, A. (1988). "Subversive" substrates for the enzyme trypanothione disulfide reductase: alternative approach to chemotherapy of Chagas' disease. *Proceedings of the National Academy of Sciences of the United States of America*, 85, 5374-5378.
- Henderson, P.J.F. (1990). Proton-linked sugar transport systems in bacteria. *Journal of Bioenergetics and Biomembranes*, 22, 525-569.



- Heston, W.D.W., Kadmon, D., Covey, D.F. & Fair, W.R. (1984). Differential effect of  $\alpha$ -difluoromethylornithine on the *in vivo* uptake of  $^{14}\text{C}$ -labeled polyamines and methylglyoxal bis(guanyldrazone) by a rat prostate-derived tumor. *Cancer Research*, **44**, 1034-1040.
- Heston, W.D.W., Watanabe, K.A., Pankiewicz, K.W. & Covey, D.F. (1987). Cytotoxic and non-cytotoxic *N*-alkyl derivatives of putrescine: Effect on polyamine uptake and growth of prostatic cancer cells *in vitro*. *Biochemical Pharmacology*, **36**, 1849-1852.
- Heytler, P.G. (1963). Uncoupling of oxidative phosphorylation by carbonyl cyanide phenylhydrazones. I. Some characteristics of *m*-Cl-CCP action on mitochondria and chloroplasts. *Biochemistry*, **2**, 357-361.
- Hirs, C.H.W. (1967). Performic acid oxidation. *Methods in Enzymology*, **11**, 197-199.
- Holley, J.L., Mather, A., Wheelhouse, R.T., Cullis, P.M., Hartley, J.A., Bingham, J.P. & Cohen, G.M. (1992). Targeting of tumor cells and DNA by a chlorambucil-spermidine conjugate. *Cancer Research*, **52**, 4190-4195.
- Hunter, K.J., Le Quecne, S.A. & Fairlamb, A.H. (1994). Identification and biosynthesis of  $N^1, N^9$ -bis(glutathionyl)aminopropylcadaverine (homotrypanothione) in *Trypanosoma cruzi*. *European Journal of Biochemistry*, **226**, 1019 - 1027.
- Hunter, K.J., Strobos, C.A.M. & Fairlamb, A.H. (1991). Inhibition of polyamine biosynthesis in *Crithidia fasciculata* by D,L- $\alpha$ -difluoromethylornithine and D,L- $\alpha$ -difluoromethylarginine. *Molecular and Biochemical Parasitology*, **46**, 35-44.
- Hyvonen, T., Seiler, N. & Persson, L. (1994). Characterization of a COS cell line deficient in polyamine transport. *Biochimica et Biophysica Acta*, **1221**, 279-285.

- Igarashi, K., Sakamoto, I., Goto, N., Kashiwagi, K., Honma, R. & Hirose, S. (1982). Interaction between polyamines and nucleic acids or phospholipids. *Archives of Biochemistry and Biophysics*, **219**, 438-443.
- Janne, J., Alhonen, L. & Leinonen, P. (1991). Polyamines: from molecular biology to clinical applications. *Annals of Medicine*, **23**, 241-259.
- Jennings, F.W. (1990). Future prospects for the chemotherapy of human trypanosomiasis 2. Combination chemotherapy and African trypanosomiasis. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **84**, 618-621.
- Kabra, P.M., Lee, H.K., Lubich, W.P. & Marion, L.J. (1986). Solid-phase extraction and determination of dansyl derivatives of unconjugated and conjugated polyamines by reversed-phase liquid chromatography: improved separation systems for polyamines in cerebrospinal fluid, urine and tissue. *Journal of Chromatography*, **388**, 19-32.
- Kakinuma, Y., Hoshino, K. & Igarashi, K. (1988). Characterization of the inducible polyamine transporter in bovine lymphocytes. *European Journal of Biochemistry*, **176**, 409-414.
- Kakinuma, Y., Masuda, N. & Igarashi, K. (1992). Proton potential-dependent polyamine transport by vacuolar membrane vesicles of *Saccharomyces cerevisiae*. *Biochimica et Biophysica Acta*, **1107**, 126-130.
- Kallio, A., McCann, P.P. & Bey, P. (1981). DL- $\alpha$ -(difluoromethyl)arginine: a potent inhibitor of bacterial arginine decarboxylase. *Biochemistry*, **20**, 3163-3166.
- Kameji, R., Rannels, S.R., Pegg, A.E. & Rannels, D.E. (1989). Spermidine uptake by type II pulmonary epithelial cells in primary culture. *American Journal of Physiology*, **256**, C160-C167.
- Kameji, T. & Pegg, A.E. (1987). Effect of putrescine on the synthesis of S-adenosylmethionine decarboxylase. *Biochemical Journal*, **243**, 285-288.

- Kano, K. & Oka, T. (1976). Polyamine transport and metabolism in mouse mammary gland: general properties and hormonal regulation. *Journal of Biological Chemistry*, **251**, 2795-2800.
- Kashiwagi, K., Kobayashi, H. & Igarashi, K. (1986). Apparently unidirectional polyamine transport by proton motive force in polyamine-deficient *Escherichia coli*. *Journal of Bacteriology*, **165**, 972-977.
- Kashiwagi, K., Miyamoto, S., Suzuki, F., Kobayashi, H. & Igarashi, K. (1992). Excretion of putrescine by the putrescine-ornithine antiporter encoded by the *potE* gene of *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America*, **89**, 4529-4533.
- Kashiwagi, K., Miyamoto, S., Nukui, E., Kobayashi, H. & Igarashi, K. (1993). Functions of *potA* and *potD* proteins in spermidine-preferential uptake system in *Escherichia coli*. *Journal of Biological Chemistry*, **268**, 19358-19363.
- Kashiwagi, K., Suzuki, T., Suzuki, F., Furuchi, T., Kobayashi, H. & Igarashi, K. (1991). Coexistence of the genes for putrescine transport protein and ornithine decarboxylase at 16 min on *Escherichia coli* chromosome. *Journal of Biological Chemistry*, **266** No.31, 20922-20927.
- Kaur, K., Emmett, K., McCann, P.P., Sjoerdama, A. & Ullman, B. (1986). Effects of DL- $\alpha$ -difluoromethylornithine on *Leishmania donovani*. *Journal of Protozoology*, **33**, 518-521.
- Keithly, J.S. & Fairlamb, A.H. (1989). Inhibition of *Leishmania* species by  $\alpha$ -difluoromethylornithine. In: *Leishmaniasis: The Current Status and New Strategies for Control*, Hart, D.T., (editor). New York: Plenum Press/NATO ASI Series Vol. 163, pp. 749-756.
- Kelly, J.M., Taylor, M.C., Smith, K., Hunter, K.J. & Fairlamb, A.H. (1993).

- Phenotype of recombinant *Leishmania donovani* and *Trypanosoma cruzi* which overexpress trypanothione reductase: sensitivity towards agents that are thought to induce oxidative stress. *European Journal of Biochemistry*, 218, 29-37.
- Kelly, J.M., Ward, H.M., Miles, M.A. & Kendall, G. (1992). A shuttle vector which facilitates the expression of transfected genes in *Trypanosoma cruzi* and *Leishmania*. *Nucleic Acids Research*, 20, 3963-3969.
- Khan, N.A., Fardel, O., Havouis, R., Fauchet, R. & Moulinoux, J.-P. (1994). Transport and metabolism of polyamines in wild and multidrug resistant human leukemia (K 562) cells. *Leukemia Research*, 18, 283-291.
- Khan, N.A., Masson, I., Quemener, V. & Moulinoux, J.-P. (1990). Coupling of Na<sup>+</sup> with the spermidine transporter protein in NIH Balb/c 3T3 cells. *Cellular and Molecular Biology*, 36, 345-348.
- Khan, N.A., Quemener, V. & Moulinoux, J.-P. (1989a). Role of protein kinase C activators and inhibitors, calmodulin antagonists and membrane sialic acids in polyamine transport in murine leukemia cells. *Cellular and Molecular Biology*, 35, 215-224.
- Khan, N.A., Quemener, V. & Moulinoux, J.-P. (1989b). Characterization of Na<sup>+</sup>-dependent and system A-independent polyamine transport in normal human erythrocytes. *Biochemical Archives*, 5, 161-169.
- Khan, N.A., Quemener, V., Seiler, N. & Moulinoux, J.-P. (1990). Mechanism of spermidine uptake in cultured mammalian cells and its inhibition by some polyamine analogues. *Pathobiology*, 58, 172-178.
- Khan, N.A., Quemener, V. & Moulinoux, J.-P. (1991). Polyamine membrane transport regulation. *Cell Biology International Reports*, 15, 9-24.
- Khan, N.A., Quemener, V. & Moulinoux, J.-P. (1992). Phorbol esters augment

polyamine transport by influencing  $\text{Na}^+\text{-K}^+$  pump in murine leukemia cells. *Experimental Cell Research*, 199, 378-382.

Khan, N.A., Quemener, V. & Moulinoux, J.-P. (1994). Characterization of polyamine transport pathways. In: *Neuropharmacology of polyamines*, Carter, C., (editor). London: Academic Press, Ltd., pp. 37-60.

Khan, N.A., Sezan, A., Quemener, V. & Moulinoux, J.-P. (1993). Polyamine transport regulation by calcium and calmodulin: Role of  $\text{Ca}^{2+}$ -ATPase. *Journal of Cellular Physiology*, 157, 493-501.

Kierszenbaum, F., Wirth, J.J., McCann, P.P. & Sjoerdsma, A. (1987a). Arginine decarboxylase inhibitors reduce the capacity of *Trypanosoma cruzi* to infect and multiply in mammalian host cells. *Proceedings of the National Academy of Sciences of the United States of America*, 84, 4278-4282.

Kierszenbaum, F., Wirth, J.J., McCann, P.P. & Sjoerdsma, A. (1987b). Impairment of macrophage function by inhibitors of ornithine decarboxylase activity. *Infection and Immunity*, 55, 2461-2464.

Kim, B.G., Sobota, A., Bitonti, A.J., McCann, P.P. & Byers, T.J. (1987). Polyamine metabolism in *Acanthamoeba*: polyamine content and synthesis of ornithine, putrescine, and diaminopropane. *Journal of Protozoology*, 34, 278-284.

Kingman, S. (1991). South America declares war on Chagas disease. *New Scientist*, 1791, 16-17.

Kramer, D.L., Miller, J.T., Bergeron, R.J., Khomutov, R., Khomutov, A. & Porter, C.W. (1993). Regulation of polyamine transport by polyamines and polyamine analogs. *Journal of Cellular Physiology*, 155, 399-407.

Kumagai, J., Jain, R. & Johnson, L.R. (1989). Characteristics of spermidine uptake by isolated rat enterocytes. *American Journal of Physiology*, 256, G905-G910.

- Kumagai, J. & Johnson, L.R. (1988). Characteristics of putrescine uptake in isolated rat enterocytes. *American Journal of Physiology*, **254**, G81-G86.
- Kuzoe, F.A.S. (1993). Current situation of African trypanosomiasis. *Acta Tropica*, **54**, 153-162.
- Lanham, S.M. (1968). Separation of trypanosomes from the blood of infected rats and mice by anion-exchangers. *Nature*, **218**, 1273-1274.
- Lewenhoeck, D.A. (1678). Observationes D.Anthonii Lewenhoeck, de natia à semine genitali afimalculis. *Philosophical Transactions of the Royal Society London*, **12**, 1040-1043.
- Li, X. & Coffino, P. (1992). Regulated degradation of ornithine decarboxylase requires interaction with the polyamine-inducible protein antizyme. *Molecular and Cellular Biology*, **12**, 3556-3562.
- Li, X. & Coffino, P. (1993). Degradation of ornithine decarboxylase: Exposure of the C-terminal target by a polyamine-inducible inhibitory protein. *Molecular and Cellular Biology*, **13**, 2377-2383.
- Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*, **193**, 265-275.
- Majumder, S. & Kierszenbaum, F. (1993a). *N,N'*-Thiophene-substituted polyamine analogs inhibit mammalian host cell invasion and intracellular multiplication of *Trypanosoma cruzi*. *Molecular and Biochemical Parasitology*, **60**, 231-240.
- Majumder, S. & Kierszenbaum, F. (1993b). Inhibition of host cell invasion and intracellular replication of *Trypanosoma cruzi* by *N,N'*-bis(benzyl)-substituted polyamine analogs. *Antimicrobial Agents and Chemotherapy*, **37**, 2235-2238.

- Majumder, S., Wirth, J.J., Bitonti, A.J., McCann, P.P. & Kierzenbaum, F. (1992). Biochemical evidence for the presence of arginine decarboxylase activity in *Trypanosoma cruzi*. *Journal of Parasitology*, 78, 371-374.
- Marr, J.J., Berens, R.L. & Nelson, D.J. (1978). Antitrypanosomal effect of allopurinol: conversion *in vivo* to aminopyrazolopyrimidine nucleotides by *Trypanosoma cruzi*. *Science*, 201, 1018-1020.
- Martin, R.L., Ilett, K.F. & Minchin, R.F. (1990). Characterization of putrescine uptake by cultured adult mouse hepatocytes. *Biochimica et Biophysica Acta*, 1051, 52-59.
- Martin, R.L., Ilett, K.F. & Minchin, R.F. (1991). Cell cycle-dependent uptake of putrescine and its importance in regulating cell cycle phase transition in cultured adult mouse hepatocytes. *Hepatology*, 14, 1243-1250.
- Marton, L.J. & Morris, D.R. (1987). Molecular and cellular functions of the polyamines. In: *Inhibition of Polyamine Metabolism: Biological Significance and Basis for New Therapies*, McCann, P.P., Pegg, A.E. & Sjoerdama, A., (editors). Orlando: Academic press, pp. 79-105.
- McCann, P.P., Bacchi, C.J., Bitonti, A.J., Kierzenbaum, F. & Sjoerdama, A. (1988). Inhibition of ornithine or arginine decarboxylase as an experimental approach to African or American trypanosomiasis. In: *Progress in Polyamine Research*, Zappia, V. & Pegg, A.E., (editors). Plenum Publishing Corporation, pp. 727-735.
- McCann, P.P. & Pegg, A.E. (1992). Ornithine decarboxylase as an enzyme target for therapy. *Pharmacology & Therapeutics*, 54, 195-215.
- Meng, S.Y. & Bennett, G.N. (1992). Nucleotide sequence of the *Escherichia coli* cad operon: a system for neutralization of low extracellular pH. *Journal of Bacteriology*, 174, 2659-2669.

- Minchin, R.F., Martin, R.L., Summers, L.A. & Ilett, K.F. (1989). Inhibition of putrescine uptake by polypyridinium quaternary salts in B16 melanoma cells treated with difluoromethylornithine. *Biochemical Journal*, 262, 391-395.
- Minchin, R.F., Raso, A., Martin, R.L. & Ilett, K.F. (1991). Evidence for the existence of distinct transporters for the polyamines putrescine and spermidine in B16 melanoma cells. *European Journal of Biochemistry*, 200, 457-462.
- Mitchell, J.L.A., Diveley, R.R., Jr. & BareyalLeyser, A. (1992). Feedback repression of polyamine uptake into mammalian cells requires active protein synthesis. *Biochemical and Biophysical Research Communications*, 186, 81-88.
- Mitchell, J.L.A., Judd, G.G., BareyalLeyser, A. & Ling SuY. (1994). Feedback repression of polyamine transport is mediated by antizyme in mammalian tissue-culture cells. *Biochemical Journal*, 299, 19-22.
- Miyahira, Y. & Dvorak, J.A. (1994). Kinetoplastidae display naturally occurring ancillary DNA- containing structures. *Molecular and Biochemical Parasitology*, 65, 339-349.
- Moncayo, A. (1992). Chagas disease: epidemiology and prospects for interruption of transmission in the Americas. *World Health Organization - World Health Statistics Quarterly*, 45, 276-279.
- Mondovi, B., Riccio, P. & Agostinelli, E. (1988). The biological functions of amine oxidases and their reaction products: an overview. *Advances in Experimental Medicine and Biology*, 250, 147-177.
- Moreno, S.N.J., Carnieri, E.G.S. & Docampo, R. (1994). Inhibition of *Trypanosoma cruzi* trypanothione reductase by crystal violet. *Molecular and Biochemical Parasitology*, 67, 313-320.



- Morgan, D.M.L. (1985). Polyamine oxidases. *Biochemical Society Transactions*, 13, 322-326.
- Morgan, D.M.L. (1990a). Polyamine uptake by human vascular endothelial cells. *Biochemical Society Transactions*, 18, 1221-1222.
- Morgan, D.M.L. (1990b). Polyamines and cellular regulation: Perspectives. *Biochemical Society Transactions*, 18, 1080-1084.
- Morgan, D.M.L. (1992). Uptake of polyamines by human endothelial cells. Characterization and lack of effect of agonists of endothelial function. *Biochemical Journal*, 286, 413-417.
- Morgan, D.M.L., Bachrach, U., Assaraf, Y.G., Harari, E. & Golenser, J. (1986). The effect of purified aminoaldehydes produced by polyamine oxidation on the development *in vitro* of *Plasmodium falciparum* in normal and glucose-6-phosphate-dehydrogenase-deficient erythrocytes. *Biochemical Journal*, 236, 97-101.
- Morris, D.R. (1991). A new perspective on ornithine decarboxylase regulation: prevention of polyamine toxicity is the overriding theme. *Journal of Cellular Biochemistry*, 46, 102-105.
- Morrow, C.D., Flory, B. & Krassner, S.M. (1980). Polyamines in the hemoflagellate, *Leishmania donovani*: evidence for spermine in the amastigote stage. *Comparative Biochemistry and Physiology*, 66B, 307-311.
- Moulinoux, J.-P., Le Calve, M., Quemener, V. & Quash, G. (1984). *In vitro* studies on the entry of polyamines into normal red blood cells. *Biochimie*, 66, 385-393.
- Munro, G.F., Bell, C.A. & Lederman, M. (1974). Multiple transport components for putrescine in *Escherichia coli*. *Journal of Bacteriology*, 118, 952-963.

- Munro, G.F., Hercules, K., Morgan, J. & Sauerbier, W. (1972). Dependence of the putrescine content of *Escherichia coli* on the osmotic strength of the medium. *Journal of Biological Chemistry*, **247**, 1272-1280.
- Murakami, Y., Matsufuji, S., Kameji, T., Hayaashi, S., Igarashi, K., Tamura, T., Tanaka, K. & Ichihara, A. (1992). Ornithine decarboxylase is degraded by the 26S proteasome without ubiquitination. *Nature*, **360**, 597-599.
- Myung Hee Park, , Wolff, E.C. & Folk, J.E. (1993). Hypusine: Its post-translational formation in eukaryotic initiation factor 5A and its potential role in cellular regulation. *BioFactors*, **4**, 95-104.
- Nathan, H.C., Bacchi, C.J., Hutner, S.H., Rescigno, D. & Sjoerdama, A. (1981). Antagonism by polyamines of the curative effects of  $\alpha$ -difluoromethylornithine in *Trypanosoma brucei* infections. *Biochemical Pharmacology*, **30**, 3010-3013.
- Newton, B.A. & Le Page, R.W.F. (1968). Interaction of Berenil with trypanosome DNA. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **62**, 131-132.
- Nicolet, T.G., Scemama, J.L., Pradayrol, L., Seva, C. & Vaysse, N. (1990). Characterization of putrescine- and spermidine-transport systems of a rat pancreatic acinar tumoral cell line (AR4-2J). *Biochemical Journal*, **269**, 629-632.
- Nicolet, T.G., Scemama, J.L., Pradayrol, L., Bethelmy, P., Seva, C. & Vaysse, N. (1991). Régulation du système de transport des polyamines par la prolifération et la différenciation cellulaire. *Medicine et Chirurgie Digestives*, **20**, 45-46.
- Nuttall, M.E., Byers, T.L. & Pegg, A.E. (1990). Evidence for the existence of multiple polyamine uptake systems in transport deficient Chinese hamster ovary cell lines expressing a human gene from HT-29 cells. *Journal of Cellular Biochemistry (supplements)*, **14C**, 106. (Abstract)

- O'Sullivan, M.C., Golding, B.T., Smith, L.L. & Wyatt, I. (1991). Molecular features necessary for the uptake of diamines and related compounds by the polyamine receptor of rat lung slices. *Biochemical Pharmacology*, **41**, 1839-1848.
- Pajunen, A., Crozat, A., Janne, O.A., Ihalaenen, R., Laitinen, P.H., Stanley, B., Madhubala, R. & Pegg, A.E. (1988). Structure and regulation of mammalian S-adenosylmethionine decarboxylase. *Journal of Biological Chemistry*, **263**, 17040-17049.
- Park, M.H., Wolff, E.C., Smit-McBride, Z., Hershey, J.W. & Folk, J.E. (1991). Comparison of the activities of variant forms of eIF-4D. The requirement for hypusine or deoxyhypusine. *Journal of Biological Chemistry*, **266**, 7988-7994.
- Pascal, R.A., Le Trang, N., Cerami, A. & Walsh, C. (1983). Purification and properties of dihydroorotate oxidase from *Crithidia fasciculata* and *Trypanosoma brucei*. *Biochemistry*, **22**, 171-178.
- Paulin, L., Vehmaanperä, J., Nykänen, I. & Pöös, H. (1983). GTP-insensitive ornithine decarboxylase in *Acetobacteria* able to synthesize spermine. *Biochemical and Biophysical Research Communications*, **114**, 779-784.
- Pegg, A.E. (1984). The role of polyamine depletion and accumulation of decarboxylated S-adenosylmethionine in the inhibition of growth of SV-3T3 cells treated with  $\alpha$ -difluoromethylornithine. *Biochemical Journal*, **224**, 29-38.
- Pegg, A.E. (1986). Recent advances in the biochemistry of polyamines in eukaryotes. *Biochemical Journal*, **234**, 249-262.
- Pegg, A.E. (1988). Polyamine metabolism and its importance in neoplastic growth and as a target for chemotherapy. *Cancer Research*, **48**, 759-774.
- Pegg, A.E. & McCann, P.P. (1988). Polyamine metabolism and function in mammalian cells and protozoans. *ISI Atlas of Science: Biochemistry*, 11-18.

Pegg, A.E. & McCann, P.P. (1992). S-Adenosylmethionine decarboxylase as an enzyme target for therapy. *Pharmacology and Therapeutics*, **56**, 359-377.

Pereira, M.E. & Hoff, R. (1986). Heterogeneous distribution of neuraminidase activity in strains and clones of *Trypanosoma cruzi* and its possible association with parasite myototropism. *Molecular and Biochemical Parasitology*, **20**, 183-189.

Persson, L. & Pegg, A.E. (1984). Studies of the induction of spermidine/spermine *N*<sup>1</sup>-acetyltransferase using a specific antiserum. *Journal of Biological Chemistry*, **259**, 12364-12367.

Persson, L., Stjernborg, L., Holm, I. & Heby, O. (1989). Polyamine-mediated control of mammalian S-adenosyl-L-methionine decarboxylase expression: effects on the content and translational efficiency of the mRNA. *Biochemical and Biophysical Research Communications*, **160**, 1196-1202.

Peterson, G.L. (1977). A simplification of the protein assay method of Lowry *et al.* which is more generally applicable. *Analytical Biochemistry*, **83**, 346-356.

Petry, K. & Eisen, H. (1989). Chagas disease: A model for the study of autoimmune diseases. *Parasitology Today*, **5**, 111-116.

Phillips, M.A., Coffino, P. & Wang, C.C. (1987). Cloning and sequencing of the ornithine decarboxylase gene from *Trypanosoma brucei*. Implications for enzyme turnover and selective difluoromethylornithine inhibition. *Journal of Biological Chemistry*, **262**, 8721-8727.

Pistocchi, R., Kashiwagi, K., Miyamoto, S., Nukui, E., Sadakata, Y., Kobayashi, H. & Igarashi, K. (1993). Characteristics of the operon for a putrescine transport system that maps at 19 minutes on the *Escherichia coli* chromosome. *Journal of Biological Chemistry*, **268**, 146-152.

- Pohjanpelto, P. (1976). Putrescine transport is greatly increased in human fibroblasts initiated to proliferate. *Journal of Cell Biology*, **68**, 512-520.
- Pohjanpelto, P., Holttä, E. & Janne, O.A. (1985a). Mutant strain of Chinese-hamster ovary cells with no detectable ornithine decarboxylase activity. *Molecular and Cellular Biology*, **5**, 1385-1390.
- Pohjanpelto, P., Holttä, E., Janne, O.A., Knuutila, S. & Alitalo, K. (1985b). Amplification of ornithine decarboxylase gene in response to polyamine deprivation in Chinese-hamster ovary cells. *Journal of Biological Chemistry*, **260**, 8532-8537.
- Pohjanpelto, P., Virtanen, I. & Holttä, E. (1981). Polyamine starvation causes disappearance of actin filaments and microtubules in polyamine-auxotrophic CHO cells. *Nature*, **293**, 475-477.
- Porter, C.W., Bergeron, R.J. & Stolowich, N.J. (1982). Biological properties of N<sup>4</sup>-spermidine derivatives and their potential in anticancer chemotherapy. *Cancer Research*, **42**, 4072-4078.
- Porter, C.W. & Bergeron, R.J. (1983). Spermidine requirement for cell proliferation in eukaryotic cells: structural specificity and quantitation. *Science*, **219**, 1083-1085.
- Porter, C.W. & Bergeron, R.J. (1988). Regulation of polyamine biosynthetic activity by spermidine and spermine analogs—a novel antiproliferative strategy. *Advances in Experimental Medicine and Biology*, **250**, 677-690.
- Porter, C.W. & Janne, J. (1987). Modulation of antineoplastic drug action by inhibitors of polyamine biosynthesis. In: *Inhibition of Polyamine Metabolism: Biological Significance and Basis for New Therapies*, McCann, P.P., Pegg, A.E. & Sjoerdama, A., (editors). Orlando: Academic press. pp. 203-243.
- Porter, C.W., Miller, J. & Bergeron, R.J. (1984). Aliphatic chain length specificity of

the polyamine transport system in ascites L1210 leukemia cells. *Cancer Research*, **44**, 126-128.

Porter, C.W. & Sufrin, J.R. (1986). Interference with polyamine biosynthesis and/or function by analogs of polyamines or methionine as a potential anticancer chemotherapeutic strategy. *Anticancer Research*, **6**, 525-542.

Poulin, R., Wechter, R.S. & Pegg, A.E. (1991). An early enlargement of the putrescine pool is required for growth in L1210 mouse leukemia cells under hypotonic stress. *Journal of Biological Chemistry*, **266**, 6142-6151.

Pressman, B.C. (1976). Biological applications of ionophores. *Annual Review of Biochemistry*, **45**, 501-530.

Rajanasayagam, O., Mannan, K. & Morgan, D.M.L. (1992). Inhibition of endothelial cell polyamine uptake by aliphatic diamines is dependent on chain length. *Biochemical Society Transactions*, **20**, 250S.

Rannels, D.E., Kameji, R., Pegg, A.E. & Rannels, S.R. (1989). Spermidine uptake by type II pneumocytes: Interactions of amine uptake pathways. *American Journal of Physiology*, **257**, L346-L353.

Richman, T.B. & Kerdel, F.A. (1989). Amebiasis and trypanosomiasis. *Dermatologic Clinics*, **7**, 301-311.

Rinehart, C.A. & Chen, K.Y. (1984). Characterization of the polyamine transport system in mouse neuroblastoma cells - effects of sodium and system A amino acids. *Journal of Biological Chemistry*, **259**, 4750-4756.

Rogers, S., Wells, R. & Rechsteiner, M. (1986). Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis. *Science*, **234**, 364-368.

- Rothstein, A. (1970). Sulfhydryl groups in membrane structure and function. *Current Topics in Membranes and Transport*, 1, 135-176.
- Saunders, N.A., Ilett, K.P. & Minchin, R.F. (1989). Pulmonary alveolar macrophages express a polyamine transport system. *Journal of Cellular Physiology*, 139, 624-631.
- Schochter, P.J., Barlow, J.L.R. & Sjoerdama, A. (1987). Clinical aspects of inhibition of ornithine decarboxylase with emphasis on therapeutic trials of Eflornithine (DFMO) in cancer and protozoan diseases. In: *Inhibition of Polyamine Metabolism: Biological Significance and Basis for New Therapies*, McCann, P.P., Pegg, A.E. & Sjoerdama, A., (editors). Orlando, Florida: Academic Press, pp. 345-364.
- Schochter, P.J. & Sjoerdama, A. (1986). Difluoromethylornithine in the treatment of African trypanosomiasis. *Parasitology Today*, 2, 223-224.
- Schofield, C.J., Minter, D.M. & Tonn, R.J. (1987). XIV. The triatomine bugs - biology and control. *World Health Organization, WHO/VBC/87.941*, 1-39.
- Schwarcz de Tarlovsky, M.N., Hernandez, S.M., Bedoya, A.M., Lammé, E.M. & Isola, E.L.D. (1993). Polyamines in *Trypanosoma cruzi*. *Biochemistry and Molecular Biology International*, 30, 547-558.
- Seiler, N. (1987a). Inhibition of enzymes oxidizing polyamines. In: *Inhibition of Polyamine Metabolism: Biological Significance and Basis for New Therapies*, McCann, P.P., Pegg, A.E. & Sjoerdama, A., (editors). Orlando, Florida: Academic Press, pp. 49-77.
- Seiler, N. (1987b). Functions of polyamine acetylation. *Canadian Journal Of Physiology And Pharmacology*, 65, 2024-2035.
- Seiler, N. (1988). Potential roles of polyamine interconversion in the mammalian organism. *Advances in Experimental Medicine and Biology*, 250, 127-145.

- Seiler, N. & Dezeure, F. (1990). Polyamine transport in mammalian cells. *International Journal of Biochemistry*, **22**, 211-218.
- Seyfang, A. & Duszenko, M. (1993). Functional reconstitution of the *Trypanosoma brucei* plasma-membrane D-glucose transporter. *European Journal of Biochemistry*, **214**, 593-597.
- Shames, S.L., Fairlamb, A.H., Cerami, A. & Walsh, C.T. (1986). Purification and characterization of trypanothione reductase from *Crithidia fasciculata*, a newly discovered member of the family of disulphide-containing flavoprotein reductases. *Biochemistry*, **25**, 3519-3526.
- Shantz, L.M., Holm, I., Janne, O.A. & Pegg, A.E. (1992). Regulation of S-adenosylmethionine decarboxylase activity by alterations in the intracellular polyamine content. *Biochemical Journal*, **288**, 511-518.
- Shim, H. & Fairlamb, A.H. (1988). Levels of polyamines, glutathione and glutathione-spermidine conjugates during growth of the insect trypanosomatid *Crithidia fasciculata*. *Journal of General Microbiology*, **134**, 807-817.
- Slater, E.C. (1967). Application of inhibitors and uncouplers for a study of oxidative phosphorylation. *Methods in Enzymology*, **10**, 48-57.
- Smith, T.A. (1985). The di-amine and poly-amine oxidases of higher-plants. *Biochemical Society Transactions*, **13**, 319-322.
- Snyder, R.D., Beach, D.C. & Loudy, D.E. (1994). Anti-mitochondrial effects of bisethyl polyamines in mammalian cells. *Anticancer Research*, **14**, 347-356.
- Snyder, S.H. & Hendley, E.D. (1968). A simple and sensitive fluorescence assay for monoamine oxidase and diamine oxidase. *Journal of Pharmacology and Experimental*



*Therapeutics*, 163, 386-392.

Spathas, D.H., Pateman, J.A. & Clutterbuck, A.J. (1982). Polyamine transport in *Aspergillus nidulans*. *Journal of General Microbiology*, 128, 557-563.

Steglich, C. & Scheffler, I.E. (1982). An ornithine decarboxylase-deficient mutant of Chinese hamster ovary cells. *Journal of Biological Chemistry*, 257, 4603-4609.

Stüber, E., Alves, F., Höcker, M. & Fölsch, U.R. (1993). Putrescine uptake in rat pancreatic acini: effect of secretagogues and growth factors. *Pancreas*, 8, 433-439.

Tabor, C.W. & Dobbs, L.G. (1970). Metabolism of 1,4-diaminobutane and spermidine in *Escherichia coli*: the effects of low temperature during storage and harvesting of cultures. *Journal of Biological Chemistry*, 245, 2086-2091.

Tabor, C.W. & Tabor, H. (1966). Transport systems for 1,4-diaminobutane, spermidine and spermine in *Escherichia coli*. *Journal of Biological Chemistry*, 241, 3714-3723.

Tabor, C.W. & Tabor, H. (1984). Polyamines. *Annual Review of Biochemistry*, 53, 749-790.

Tabor, C.W. & Tabor, H. (1985). Polyamines in microorganisms. *Microbiological Reviews*, 49, 81-99.

Tekwani, B.L., Bacchi, C.J. & Pegg, A.E. (1992). Putrescine activated S-adenosylmethionine decarboxylase from *Trypanosoma brucei brucei*. *Molecular and Cellular Biochemistry*, 117, 53-61.

ter Kuile, B.H. (1993). Glucose and proline transport in Kinetoplastids. *Parasitology Today*, 9, 206-210.

Turner, R., North, M.J. & Harwood, J.M. (1979). Putrescine uptake by the cellular

slime mould *Dictyostellium discoideum*. *Biochemical Journal*, **180**, 119-127.

Ueland, P.M. (1982). Pharmacological and biochemical aspects of S-adenosylhomocysteine and S-adenosylhomocysteine hydrolyase. *Pharmacological Reviews*, **34**, 223-253.

Van Nieuwenhove, S. (1992). Advances in sleeping sickness therapy. *Annales de la Societe Belge de Medecine Tropicale*, **72**, 39-51.

Van Nieuwenhove, S., Schochter, P.J., Declercq, J., Bone, G., Burke, J. & Sjoerdama, A. (1985). Treatment of gambiense sleeping sickness in the Sudan with oral DFMO (DL- $\alpha$ -difluoromethylornithine), an inhibitor of ornithine decarboxylase; first field trial. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **79**, 692-698.

Van-Voorhis, W.C. (1990). Therapy and prophylaxis of systemic protozoan infections. *Drugs*, **40**, 176-202.

Villanueva, V.R., Adlakha, R.C. & Calvayrac, R. (1980). Cell cycle related changes in polyamine content in *Euglena*. *Phytochemistry*, **19**, 962-964.

Wallace, H.M. & Keir, H.M. (1981). Uptake and excretion of polyamines from baby hamster kidney cells (BHK-21/C13) the effect of serum on confluent cell cultures. *Biochimica et Biophysica Acta*, **676**, 25-30.

Walsh, C.T., Bradley, M. & Nadeau, K. (1991). Molecular studies on trypanothione reductase, a target for antiparasitic drugs. *Trends in Biochemical Sciences*, **16**, 305-309.

Wang, C.C. (1991). The ornithine decarboxylase of *Trypanosoma brucei*: a stable protein in vivo. In: *Molecular and Immunological Aspects of Parasitism*, Wang, C.C., (editor). Washington, D.C.: American Association for the Advancement of Science, pp. 87-94.

- Watanabe, S.I., Kusama-Eguchi, K., Kobayashi, H. & Igarashi, K. (1991). Estimation of polyamine binding to macromolecules and ATP in bovine lymphocytes and rat liver. *Journal of Biological Chemistry*, **266**, 20803-20809.
- Weigle, M., Debernado, S. & Leimgruber, W. (1973). Fluorometric assay of secondary amino acids. *Biochemical and Biophysical Research Communications*, **50**, 352-356.
- Westhall, F. & Hesser, H. (1974). Fifteen minute acid hydrolysis of peptides. *Analytical Biochemistry*, **61**, 610-613.
- White, M.W., Degnin, C., Hill, J. & Morris, D.R. (1990). Specific regulation by endogenous polyamines of translational initiation of *S*-adenosylmethionine decarboxylase mRNA in Swiss 3T3 fibroblasts. *Biochemical Journal*, **268**, 657-660.
- WHO, (1992). World malaria situation 1990. Division of control of tropical diseases. *World Health Organization - World Health Statistics Quarterly*, **45**, 257-266.
- WHO, *Tropical Disease Research: progress 1991-92: eleventh programme report of the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases*, Geneva:World Health Organization, 1993.
- Williams, K. (1994). Mechanisms influencing stimulatory effects of spermine at recombinant *N*-methyl-D-aspartate receptors. *Molecular Pharmacology*, **46**, 161-168.
- Williams-Ashman, H.G. & Schenone, A. (1972). Methyl glyoxal bis(guanyldiazide) as a potent inhibitor of mammalian and yeast *S*-adenosylmethionine decarboxylases. *Biochemical and Biophysical Research Communications*, **46**, 288-295.
- Williams-Ashman, H.G. & Seidenfeld, J. (1986). Aspects of the biochemical

- pharmacology of methyl glyoxal bis(guanyldrazone). *Biochemical Pharmacology*, **38**, 1217-1225.
- Wohlhueter, R.M. & Plagemann, P.G.W. (1989). Measurement of transport versus metabolism in cultured cells. *Methods in Enzymology*, **173**, 714-732.
- Wyatt, I., Soames, A.R., Clay, M.F. & Smith, L.L. (1988). The accumulation and localisation of putrescine, spermidine, spermine and paraquat in the rat lung. *In vitro* and *in vivo* studies. *Biochemical Pharmacology*, **37**, 1909-1918.
- Yakubu, M.A., Basso, B. & Kierszenbaum, F. (1992). DL- $\alpha$ -difluoromethylarginine inhibits intracellular *Trypanosoma cruzi* multiplication by affecting cell division but not trypomastigote-amastigote transformation. *Journal of Parasitology*, **78**, 414-419.
- Yakubu, M.A., Majumder, S. & Kierszenbaum, F. (1993). Inhibition of S-adenosyl-L-methionine (AdoMet) decarboxylase by the decarboxylated AdoMet analog 5'-((Z)-4-amino-2-butenyl) methylamino)-5'-deoxyadenosine (MDL 73811) decreases the capacities of *Trypanosoma cruzi* to infect and multiply within a mammalian host cell. *Journal of Parasitology*, **79**, 525-532.
- Yamakawa, T. (1986). The biochemistry of polyamines (IV). *Chemical Times*, No.1, 2172-2180.
- Yamamoto, S., Imamura, T., Kusaba, K. & Shinoda, S. (1991). Purification and some properties of inducible lysine decarboxylase from *Vibrio parahaemolyticus*. *Chemical and Pharmaceutical Bulletin (Tokyo)*, **39**, 3067-3070.
- Yarlett, N. & Bacchi, C.J. (1987). Effect of DL- $\alpha$ -difluoromethylornithine on methionine cycle intermediates in *Trypanosoma brucei brucei*. *Molecular and Biochemical Parasitology*, **27**, 1-10.
- Yarlett, N., Garofalo, J., Goldberg, B., Ciminelli, M.A., Ruggiero, V., Sufrin, J.R. &

- Bacchi, C.J. (1993). S-Adenosylmethionine synthetase in bloodstream *Trypanosoma brucei*. *Biochimica et Biophysica Acta*, **1181**, 68-76.
- Yarlett, N., Quamina, A. & Bacchi, C.J. (1991). Protein methylases in *Trypanosoma brucei brucei*: activities and response to DL- $\alpha$ -difluoromethylornithine. *Journal of General Microbiology*, **137**, 717-724.
- Zappla, V. and Pegg, A.E., ed. *Progress in Polyamine Research: Novel Biochemical, Pharmacological, and Clinical Aspects*, New York: Plenum Press, 1988.
- Zilberstein, D. (1993). Transport of nutrients and ions across membranes of trypanosomatid parasites. *Advances in parasitology*, **32**, 261-291.

