RENAL HANDLING OF POLYAMINES

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By

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Dedicated to my beloved parents
for their continuous support, love
and inspiration during the entire
course of my studies and for
making my life worthwhile.

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ABSTRACT

Polyamines are aliphatic, organic cations distributed in all living cells. They play an important role in cell proliferation, cell growth and synthesis and metabolism of macromolecules like nucleic acids and proteins. The three principal polyamines in mammalian cells are putrescine, spermidine and spermine.

Polyamine excretion has been found to be increased in the urine of patients with various types of malignancies. Many workers have advocated the use of urinary polyamines as markers of malignancy and also for monitoring the effectiveness of cancer therapy. To use urinary polyamines for such a purpose it is important to understand how the kidney handles these substances, and to find out what is the source of the urinary polyamines. The present experiments were done to study these aspects of renal polyamine handling.

In both male and female rats, putrescine was the major polyamine excreted, followed by spermidine and smaller amounts of spermine and the acetylated polyamines. No cadaverine was detected in the urine of any of these rats on a polyamine-free diet. Equilibrium dialysis studies in vitro showed that about 8% of putrescine and 16% of spermidine are non-covalently bound to plasma proteins, therefore, most of the putrescine and spermidine should be filterable. When the excreted load of spermidine was compared to the probable filtered load, using

the free spermidine concentration, it was found that only 0.6-1.5% of the spermidine was excreted. The negligible arteriovenous difference in whole blood and plasma indicates that almost all of the spermidine entering the glomerulus is returned to the renal circulation. Thus reabsorption is the predominant fate of the spermidine filtered at the glomerular membrane. On the other hand, a higher proportion of the filtered putrescine was excreted, although this still only ranged from 14% (female) to 29% (male) of the probable filtered load in untreated rats. Much more putrescine is removed from the red blood cells as they pass through the kidney than what is excreted. The metabolic fate of this putrescine is not known.

In order to test whether reabsorption occurred in the proximal tubule, female rats were treated with maleate to produce an experimental Fanconi syndrome characterized by a generalized disruption of the reabsorption processes in the proximal renal tubule. Polyamine excretion was compared with that from saline-treated control rats. Maleate treatment for four hours produced polyuria, glucosuria and aminoaciduria. There was a significant drop in the plasma spermidine level and a significant decrease in the filtered load of spermidine. The plasma putrescine delivery and the plasma level remained unaltered due to maleate treatment. There was a significant decrease in the percentage reabsorption of both putrescine and spermidine. It was concluded that maleate interferes with

polyamine reabsorption as it does with reabsorption of amino acids and other substances by the proximal tubule. Spermidine reabsorption by the kidney may play an important role in maintaining its plasma level; an interference of reabsorption producing excess excretion causes a failure to maintain plasma spermidine levels. On the other hand, plasma putrescine level may have a tighter regulation, which prevents a decrease in plasma concentration even when its excretion is increased due to interference in reabsorption.

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LIST OF ABBREVIATIONS

ADH	Antidiuretic hormone
AdoDATO	S-adenosyl-1,8-diamino-3-thiooctane
AEC	Aminoethylcysteine
5'-AMP	Adenosine 5'-monophosphate
AG	Aminoguanidine
A-V	Arteriovenous difference
AVP	Arginine vasopressin
BLD	Below level of detection
BSA	Bovine serum albumin
CHO	Chinese hamster ovary cells
c-SAT	Acetyl CoA:spermidine/spermine N^1-acetyltransferase (cytosolic)
DAO	Diamine oxidase
DEAE	Diethylaminoethyl dextran
DeSAM	Decarboxylated S-adenosylmethionine
DFMO	D,L-αDifluoromethylornithine
GFR	Glomerular filtration rate
G-6-PDH	Glucose-6-phosphate dehydrogenase
HCG	Human chorionic gonadotropin
HPLC	High-pressure liquid chromatography
I.P.	Intraperitoneal
MGBG	Methylglyoxal-bis(guanylhydrazone)
MTA	5'-methylthioadenosine
n-SAT	Acetyl CoA:spermidine N^8-acetyltransferase (nuclear)
ODC	Ornithine decarboxylase
OPA	O-phthalaldehyde
OSA	Octanesulphonic acid
PCA	Perchloric acid
PIG	Phosphate-independent glutaminase
RBC	Red blood cells
RBF	Renal blood flow
REF	Rat embryo fibroblasts
RPF	Renal plasma flow
SAM	S-adenosylmethionine
SAMDC	S-adenosylmethionine decarboxylase
SSA	Sulfosalicylic acid
TCA	Tricarboxylic acid cycle
T_m	Transfer maximum or transport maximum
UFR	Urine flow rate
U/P	Urine-to-plasma ratio

AMINO ACIDS

TAU	Taurine	MET	Methionine
ASP	Aspartic acid	ILE	Isoleucine
HYP	Hydroxyproline	TYR	Tyrosine
THR	Threonine	PHE	Phenylalanine
SER	Serine	TRP	Tryptophan
ASN	Asparagine	ORN	Ornithine
GLU	Glutamic acid	LYS	Lysine
GLN	Glutamine	HIS	Histidine
PRO	Proline	ARG	Arginine
GLY	Glycine	ß-ALA	ß-alanine
ALA	Alanine	GABA	γ-aminobutyric acid
CIT	Citrulline		
VAL	Valine	1-METHIS	1-methylhistidine
CYS	Cystine	3-METHIS	3-methylhistidine
HYLYS	Hydroxylysine		

CHAPTER 1

INTRODUCTION TO POLYAMINES

I begin my discussion with the well-known tenet of Sir Hans Krebs, "almost all the properties of living matter have a function, i.e. if a substance or a process occurs it is likely to have a role in the life of the cell. This follows from the principle that in the course of evolution, non-functional properties do not, in general, survive" (Krebs, 1981). With this in mind, it is very easy to believe that the polyamines must play very important roles in the cell, since they are ubiquitous.

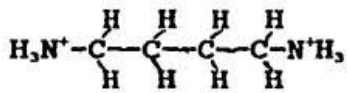
Chemically, polyamines are low molecular weight organic cations which are present in all bacteria, bacteriophages, plant and animal cells. At physiological pH each nitrogen carries a net positive charge. The principal polyamines in eukaryotic cells are putrescine, spermidine and spermine (their structures are presented in Figure 1.1). Polyamines play important roles in cellular functions and are required for optimal cell growth, cell division and differentiation (Bueb et al.,1992). Because of their cationic nature, polyamines can interact with anionic macromolecules such as DNA, RNA, phospholipids and proteins and also play a role in their metabolism.

1.1 THE METABOLISM OF POLYAMINES

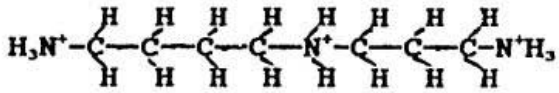
Polyamine metabolism is governed by two sequences of chemical reactions termed the interconversion pathway and

Figure 1.1

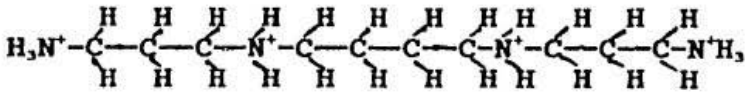
Structures of the principal polyamines.



PUTRESCINE



SPERMIDINE



SPERMINE

terminal catabolism (Seiler,1990). In the interconversion pathway, the biosynthetic reactions and a sequence of catabolic reactions are combined to form a cyclic process. The designation derives from the fact that one polyamine can be converted into another. In the catabolic branch of the interconversion cycle, spermine is degraded to spermidine, and spermidine to putrescine. The first step in this sequence is acetylation of the respective polyamine (spermine or spermidine) in the N^1 position followed by the oxidative splitting of the acetylated polyamine whereby the aminopropyl residues that originated from an aminopropyl donor (decarboxylated S-adenosylmethionine) are removed (Seiler, 1990). Reutilization of putrescine for spermidine biosynthesis in tissues like brain has been postulated to occur to a considerable extent since the putrescine moiety in spermidine and spermine apparently has a longer half-life than the aminopropyl moieties in spermidine or spermine (Seiler et al.,1985a). Seiler and Heby (1988) have proposed that putrescine reutilization has a general physiological significance. Terminal catabolism is a major fate in those selected tissues that contain appropriate Cu^{2+}-dependent amine oxidases because the products of these oxidases cannot be directly converted into polyamines, hence these reactions are called terminal catabolic reactions (Seiler et al.,1985a). The

small intestine is especially rich in these enzymes (Seiler et al.,1985a).

1.1.1 Polyamine biosynthesis

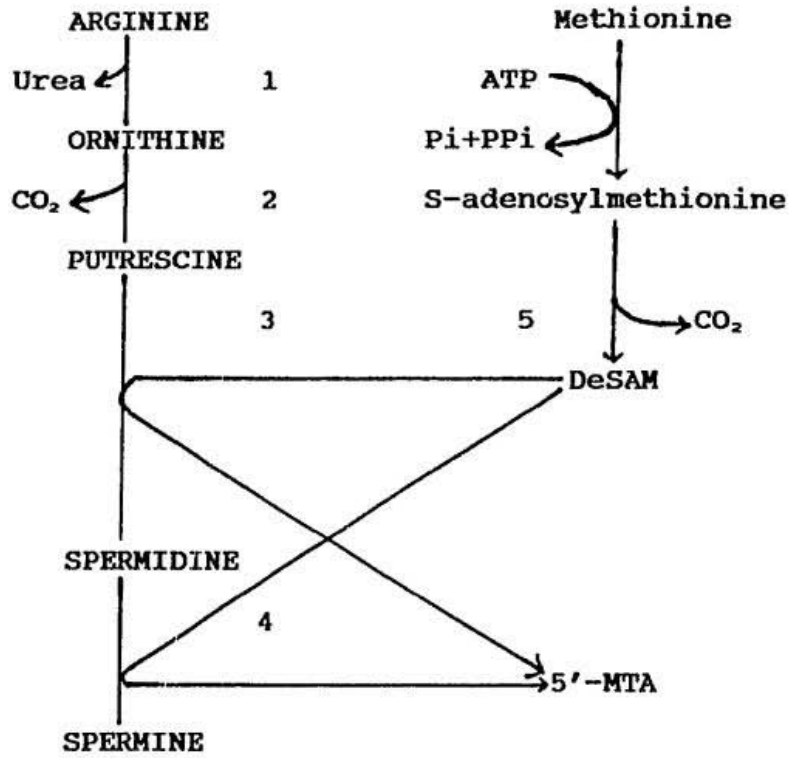
Cellular growth is associated with an increase in the biosynthesis of polyamines, hence the polyamine biosynthetic pathway is a potential target for therapeutic agents against various proliferative disorders (Persson et al.,1989). The polyamine biosynthetic pathway is presented in Figure 1.2.

The *de novo* synthesis of polyamines requires two amino acids, arginine and methionine. Many microorganisms and higher plants are able to produce putrescine from agmatine by the decarboxylation of arginine, but all mammalian cells and many lower eukaryotes lack arginine decarboxylase (Pegg and McCann, 1982). Putrescine, the precursor of spermidine and spermine, is formed in mammalian cells by the direct decarboxylation of ornithine (William-Ashman and Canellakis,1979) by ornithine decarboxylase (ODC). Ornithine may be available for these reactions from plasma and can also be formed by the action of the enzyme arginase. In most eukaryotic tissues, except plants, putrescine can be formed only from ornithine; hence the control of ornithine formation is most important in the regulation of putrescine formation (Pegg and McCann,1982).

Putrescine is converted to spermidine by the addition of

Figure 1.2

Polyamine biosynthetic pathway.



DeSAM-decarboxylated S-adenosylmethionine

MTA-methylthioadenosine

ENZYMES

1. Arginase
2. Ornithine decarboxylase
3. Spermidine synthase
4. Spermine synthase
5. S-adenosylmethionine decarboxylase.

an aminopropyl group. The source of the aminopropyl group is methionine which first has to be converted to S-adenosylmethionine and then decarboxylated by the action of the enzyme S-adenosylmethionine decarboxylase (SAMDC). Decarboxylated S-adenosylmethionine is used as an aminopropyl donor in an analogous manner to the use of S-adenosylmethionine itself as a methyl donor. Once decarboxylated, S-adenosylmethionine is committed to polyamine production. No other reactions utilizing decarboxylated S-adenosylmethionine at any physiologically significant rates are known (Pegg and McCann, 1982). Hence the production of this compound is coupled to polyamine biosynthesis.

Spermidine synthase and spermine synthase are the two aminopropyltransferases which catalyse the transfer of the aminopropyl moieties to putrescine and spermidine to form spermidine and spermine respectively (Heby and Persson,1990). The other product of the aminopropyltransferase reaction is 5'-methylthioadenosine (MTA). Though formed in stoichiometric amounts with the polyamines, its concentration in the cell is very low due to its rapid degradation by phosphorylase (more than 99% of MTA is rapidly degraded in the cells) producing adenine and 5'-methylthioribose-1-phosphate (Pegg and McCann, 1982). The adenine is then converted to 5'-AMP (adenosine 5'-monophosphate) by the action of adenine phosphoribosyl-

transferase and the 5'-methylthioribose-1-phosphate is converted back to methionine in a reaction that conserves the methyl group and all but the C-1 of the carbon atoms of this sugar. Therefore all of the decarboxylated S-adenosyl-methionine molecule not used for polyamine production is effectively salvaged. (Pegg and McCann, 1982). The enzymes of polyamine biosynthesis are discussed below.

1.1.1.1 Arginase

Arginase catalyses the conversion of arginine to urea and ornithine. The large negative free energy associated with this reaction makes it irreversible in vivo. The reported K_m values for arginase from rat liver range from 1-20 mM. The enzyme has an absolute requirement for Mn^{2+} (Garganta and Bond,1986). It is suggested that arginase plays a role in providing ornithine for putrescine synthesis (Klein and Morris,1978).

Arginase is more widely distributed compared to other enzymes of the urea cycle and is found in extrahepatic tissues like mammary gland (Oka and Perry,1974) and lymphocytes (Klein and Morris,1978). Though it is abundant in the liver primarily functioning in the formation of urea in ureotelic animals, it is also present in significant amounts in other tissues (Herzfeld and Raper,1976). Arginase in conjunction with ODC and SAMDC has been suggested to participate in spermidine biosynthesis in the mammary gland (Oka and Perry, 1974) during

lactation (Russell and McVicker,1972). The considerable formation of ornithine in the lactating mammary gland has been suggested to be due to the arginase activity (Yip and Knox, 1972). In lymphocytes activated by concavalin A, arginase has been seen to increase by nearly four-fold by 24 hours following stimulation (Klein and Morris,1978).

1.1.1.2 Ornithine decarboxylase (ODC)

ODC catalyses the formation of putrescine from ornithine. The enzyme is widely distributed. ODC is a key regulatory enzyme in the polyamine biosynthetic pathway supplying putrescine as a substrate for the *de novo* synthesis of spermidine and spermine.

ODC has the shortest half-life (10-30 min.) among the enzymes studied (Pegg and Williams-Ashman,1981). Inhibition of ODC by pharmaceutical means has been the goal of many investigators, because it is believed that complete inactivation of ODC would lead to the arrest of cell division and thus provide a new approach to the therapy of proliferative disorders (Seiler and Heby,1988). Among the potent inhibitors, D,L-α difluoromethylornithine (DFMO) is of prime importance because it is a specific and irreversible inhibitor of ODC activity in spite of certain disadvantages such as its low rate of uptake by the cells, a relatively high K_i (39 μM) and a rapid clearance from the body by excretion (Seiler and Heby,1988).

ODC is a pyridoxal phosphate-dependent enzyme. It is present in very small amounts in quiescent cells and its activity can be increased many-fold within a few hours of exposure to trophic stimuli such as hormones, drugs, tissue regeneration and growth factors. Even after such stimulation, ODC represents only a very small fraction of total cellular protein, ranging from 0.01% of the cytosolic protein in androgen-stimulated mouse kidneys to 0.00012% in thioacetamide stimulated rat liver (Pegg and McCann,1982).

The degradation of ODC is subject to control by the polyamines. Putrescine affects the degradation of ODC. The molecular mechanism of the polyamine-mediated degradation is not understood. Putrescine has been reported to induce the synthesis of an ODC inhibitory protein(s). Spermidine and spermine have also been reported to induce the synthesis of the inhibitory protein (Heller et al.,1976). This inhibitory protein named the antizyme binds non-covalently to ODC (Heby and Persson,1990). The binding (and inhibitory effect) of the antizyme to ODC is extremely strong with an equilibrium constant as high as $1.4 \times 10^{11} M^{-1}$ (Hayashi and Canellakis,1989). Since the ODC protein decreases more rapidly after exposure to polyamines than after inhibition of protein synthesis by cycloheximide, the antizyme may act as a targeting system for ODC degradation (Heby and Persson,1990).

1.1.1.3 S-adenosyl methionine decarboxylase (SAMDC)

SAMDC catalyses the decarboxylation of S-adenosyl-methionine to ensure the availability of this substrate for the *de novo* synthesis of spermidine and spermine respectively. Mammalian SAMDC is activated by putrescine and repressed by spermidine, thus linking the supply of decarboxylated S-adenosylmethionine to the need for spermidine and to the availability of the other substrate (putrescine) for spermidine synthesis (Pegg and McCann,1982).

SAMDC is also a key regulatory enzyme in the polyamine biosynthetic pathway. It has an enzyme-bound pyruvate as cofactor and in this respect it is different from other decarboxylases in which the usual cofactor is pyridoxal phosphate (Tabor and Tabor,1984). The enzyme is present in mammalian tissues in very small amounts equal to 0.015% of the soluble protein in the ventral prostrate to 0.0097% in liver. Its activity is regulated by many hormones and other growth-promoting stimuli (Pegg and McCann,1982). SAMDC has a relatively short half-life of 1-2 hours (Tabor and Tabor, 1984).

A number of structural analogues of SAM have been shown to be potent inhibitors of SAMDC in cell-free extracts (Kolb et al.,1982). But these inhibitors were not very useful in vivo presumably due to slow penetration through the cell

membrane. MGBG or methylglyoxal-bis(guanylhydrazone) has been used by most investigators. This compound was originally designed as an antileukemic drug but was found to be a reversible inhibitor of SAMDC. Owing to its structural similarity to spermidine, MGBG uses the same cell membrane transport system and may consequently interfere with spermidine uptake. 5'-deoxy-5'[N-methyl-N-(3-hydrazino-propyl)]aminoadenosine has been found to be a specific inhibitor of SAMDC and it also inhibits growth of DFMO resistant cells (Madhubala et al.,1987).

1.1.1.4 Spermidine synthase and spermine synthase (the aminopropyl transferases)

The two synthases are discrete enzymes each specific for its own substrate, though there is a similarity between the two reactions they catalyse. They are present in many cells in much greater amounts than the decarboxylases and are regulated by the availability of their substrates (Heby and Persson, 1990) particularly decarboxylated S-adenosylmethionine (Pegg and McCann,1982). The activity of spermidine synthase shows marked changes in response to hormones, tissue regeneration and cell growth factors (Pegg and McCann,1982).

The role of spermine in mammalian cell growth is not well understood. Treatment of cells with ODC inhibitors does not deplete the cells of spermine; it is generally understood that

the effect of ODC inhibitors would produce a cytostatic effect rather than a cytotoxic effect suggesting that the cells would remain in a quiescent but viable state even in the absence of a normal rate of polyamine synthesis (Pegg et al.,1986). Therefore the use of a specific inhibitor of spermine synthase would enable the study of the importance of spermine synthesis for the growth and viability of mammalian cells (Pegg et al., 1986).

A series of 5'-substituted adenosines have been reported to inhibit spermine synthase preferentially (Pegg et al., 1986). Spermidine synthase has been reported to be inhibited by MTA analogues such as 1-aminooxy-3-aminopropane and N-chlorosulfonyldicyclohexylamine (Seiler and Heby,1988). It is also inhibited by S-adenosyl-1,8-diamino-3-thiooctane (AdoDATO), a specific inhibitor for both mammalian and bacterial spermidine synthases (Seiler and Heby,1988).

1.1.2 Interconversion of Polyamines

It has been known for many years that the interconversion of polyamines for example, spermine to spermidine and spermidine to putrescine can occur in vivo. This interconversion takes place by the action of two enzymes namely, spermidine-N^1-acetyltransferase (or more specifically acetyl CoA:spermidine/spermine N^1-acetyltransferase or c-SAT; c

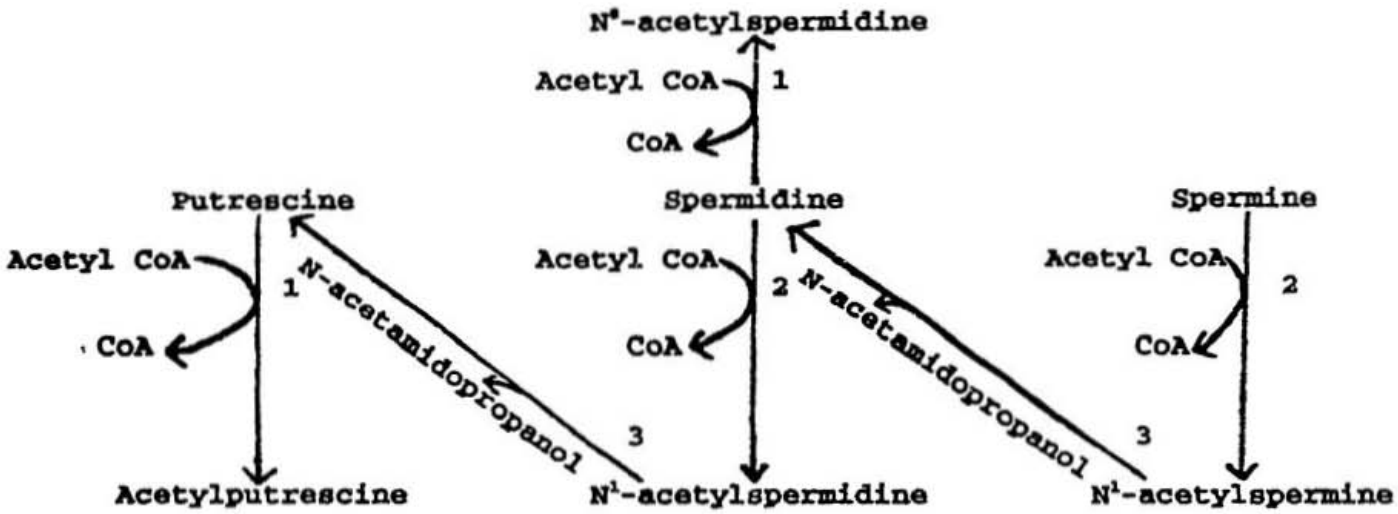
denotes cytosolic) and polyamine oxidase respectively. The interconversion pathway is given in Figure 1.3.

The cytosolic enzyme, c-SAT,(Seiler,1987) uses acetyl CoA as an acetyl donor to convert spermidine to N^1-acetylspermidine and will also acetylate spermine forming N^1-acetylspermine. Polyamine oxidase has a low K_m and high V_{max} for N^1-acetylspermidine and N^1-acetylspermine respectively (Tabor and Tabor,1984). Polyamine oxidase cleaves acetylspermidine or acetylspermine at the internal nitrogen to yield N-acetylpropionaldehyde and putrescine or spermidine depending on the substrate (Pegg and McCann,1982).

Only the primary amino group(s) of the polyamines is acetylated physiologically. Spermidine is not a symmetrical molecule, hence it has two monoacetyl derivatives, N^1 and N^8-acetylspermidine respectively. A nuclear acetylating enzyme namely acetyl CoA:spermidine N^8-acetyltransferase (n-SAT denoting a nuclear enzyme) is known to acetylate spermidine at the N^8 position. The only important metabolic fate of N^8-acetylspermidine is its hydrolysis to spermidine by a cytosolic enzyme known as N^8-acetylspermidine acetylhydrolase (Seiler,1987). The n-SAT also acetylates putrescine to monoacetylputrescine, which in turn is hydrolysed to putrescine by spermidine acetylhydrolase. Spermidine is its substrate as well (Seiler,1987). Acetylputrescine is present

Figure 1.3

Interconversion of polyamines



1. n-SAT or acetyl CoA:spermidine N^8-acetyltransferase (nuclear)
2. c-SAT or acetyl CoA:spermidine/spermine N^1-acetyltransferase (cytosolic)
3. Polyamine Oxidase

in animal tissues in small amounts and is thought to be an intermediate in the formation of GABA from putrescine (Seiler, 1980). In contrast, little or no acetylspermidine or acetylspermine is found in animal tissues with the exception of the epididymis, where in addition to putrescine, spermidine and spermine, N^1-acetylspermidine and N^1-acetylspermine were found (Matsuzaki et al.,1982). However, significant amounts of N^1 and N^8-acetylspermidine have been found to be present in urine (Tabor and Tabor,1984).

Acetylation of spermidine and spermine has been shown to be a limiting step in the interconversion pathway leading to the loss of the aminopropyl group forming putrescine and spermidine respectively. The polyamine metabolic pathway contains multiple enzymes catalysing polyamine acetylation and deacetylation respectively (Ragione and Pegg,1983). Though acetylated polyamines are found in small amounts in blood and urine, the importance of acetylation in permitting polyamine excretion is unclear (Pegg and McCann,1982). One possibility could be that acetylation could permit a rapid change in free polyamine concentration in the tissues. However, acetylation is a means by which the net cationic charge on a polyamine can be reduced (Seiler,1987).

Polyamine interconversion in tissues is greatly stimulated by exposure to toxic agents, but the process is

also induced by fasting and by exposure to excess spermidine. The physiological significance of this pathway may lie in preventing the levels of spermidine and spermine from exceeding certain limits within the cell (Pegg and McCann, 1982).

1.1.2.1 Spermidine/spermine N^1acetyltransferase (c-SAT) and spermidine N^8-acetyltransferase (n-SAT)

The c-SAT is a highly inducible enzyme, and can be induced by a number of stimuli, including hormones (eg. growth hormone, insulin, glucagon, secretin, parathyroid hormone), vitamins (1,25-hydroxy-vitamin D_3), toxic agents (eg. thioacetamide, carbon tetrachloride, MGBG, dialkylnitrosamines), physiologic or pathologic events (eg. injuries, tumor growth, partial hepatectomy) and polyamines (eg. spermidine, spermine) (Seiler,1987). In contrast, the n-SAT is induced by the growth hormone (Seiler,1987) and is not subject to induction by a variety of stimuli.

The c-SAT is highly specific for the acetylation of a primary amine group that is separated by a 3-carbon aliphatic chain from another nitrogen atom (Ragione and Pegg,1983). It exclusively forms the N^1-acetylated polyamine derivative when incubated with the polyamines and acetyl CoA (Matsui et al., 1981). The c-SAT does not acetylate histones. This enzyme is sensitive to coenzyme A and has a short half-life of 45 mins.

which makes it suitable as a regulatory enzyme (Seiler,1987). The n-SAT preparations from calf and rat liver nuclei have been shown to acetylate histones as well as polyamines (Libby,1978; Libby,1980).

N-[2-(S-CoA)acetyl]sym-norspermidine amide is a potent inhibitor of c-SAT but it is not specific because it also inhibits n-SAT and histone acetylases (Seiler and Heby,1988). However, induction of c-SAT by polyamine analogues like N^1N^{11}-bis(ethyl)spermidine that are not themselves able to substitute for the growth functions of the polyamines may have an important role in contributing to growth inhibition caused by these analogues (Erwin and Pegg,1986).

1.1.2.2 Polyamine oxidase

Polyamine oxidase is an FAD-dependent peroxisomal enzyme (Seiler,1990). N^1N^4-Bis-allenylputrescine and related compounds are very potent inactivators of polyamine oxidase.

1.1.3 Degradation of Polyamines

Each intermediate of the interconversion cycle can serve as a substrate for the Cu^{2+} containing amine oxidases (serum amine oxidase and diamine oxidase). The Cu^{2+} containing enzymes are unevenly distributed and show great interspecies variations. They catalyse the reactions of the terminal catabolism (Seiler and Heby,1988).

Putrescine can be oxidised by diamine oxidase (DAO) yielding γ-aminobutyraldehyde, which can be further oxidised to γ-aminobutyrate (GABA), or Δ^1-pyrroline, the spontaneously cyclized form of γ-aminobutyraldehyde; Δ^1-pyrroline may be converted to 2-pyrrolidone (Pegg and McCann,1982). Monoacetyl-putrescine can also be oxidised by monoamine oxidase. This pathway is important in the tissues like brain with little diamine oxidase for GABA formation (Seiler,1980).

Putrescine and probably also spermidine and spermine are natural substrates for DAO. DAO has high activity in tissues like small intestine and placenta but is lower in most other tissues and in rodents. The highest activity of serum amine oxidases is found in the blood of ruminants (Seiler and Heby, 1988).

1.1.3.1 Amine oxidases

Amine oxidases catalyse the interconversion of polyamines; each intermediate of the interconversion cycle or more specifically the N^1-acetyl derivatives of the polyamines can act as substrates for the Cu^{2+} containing amine oxidases, such as monoamine oxidase, diamine oxidase depending on what substrates they utilize. The distribution of these oxidases is not as abundant in all tissues as the other enzymes of the polyamine metabolic pathway. They may have some regulatory role in certain physiologic conditions like pregnancy. Liver

and kidney diamine oxidase increased dramatically from a low or undetectable level during the 2nd half of pregnancy in rat (Piacentini et al.,1986).

The most efficient inhibitor of polyamine oxidase and Cu^{2+} containing oxidases is aminoguanidine which apparently has no side effects when given to humans (Seiler and Heby,1988).

1.2 POLYAMINE TRANSPORT

Cationic amino acids (arginine, lysine) must be transported from the extracellular fluid by most tissues, but unlike them, polyamines can be synthesized by all tissues from the amino acids arginine and methionine. Acetylation of polyamines eliminates the positive charge associated with the acetylated nitrogen; acetyl polyamines can also be oxidised in the cells, thus rapidly decreasing the polyamine concentrations (Brosnan and Brosnan,1990).

Transport of polyamines can occur into or out of cells, and they are present in secretions such as semen or milk (Brosnan and Brosnan,1990). The cellular polyamine levels are normally a function of the rate of polyamine biosynthesis (Heby and Persson,1990) and degradation. The rate of exogenous polyamine uptake by the cells may also serve to regulate intracellular polyamine levels (Nicolet et al.,1990). The polyamine transport system has been studied in various

mammalian cell lines, and as Seiler and Dezeure (1990) have pointed out, none of these studies are complete. However, based on these studies, some of the characteristics of polyamine transport have been deduced (Seiler and Dezeure, 1990).

Byers and Pegg (1989) have demonstrated that at least two classes of transport systems can exist for the polyamines depending on their ability to recognize the terminal aminopropyl and aminobutyl moieties respectively. They have also observed putrescine uptake to be Na^+-dependent; spermine uptake is Na^+-independent. Nicolet et al (1990) have also shown the presence of two polyamine transporters in a rat tumoral cell line (AR4-2J) on the basis of their specificities towards the aminopropyl and aminobutyl groups. It was also shown that arginase deficient CHO (chinese hamster ovary) cells were unable to maintain cellular growth in the absence of ornithine, without a supply of exogenous polyamines (Hölltä and Pohjanpelto,1982).

Polyamine transport has been shown to be temperature-dependent (Kumagai and Johnson,1988; Nicolet et al.,1990; Pohjanpelto,1976), energy-dependent and saturable (Kumagai and Johnson,1988; Nicolet et al.,1990; Seiler and Dezeure,1990), suggesting that this transport is carrier-mediated (Seiler and Dezeure,1990). However, uptake of polyamines in ventilated

and perfused rat lung slices appears to be an exception, where neither energy-dependence nor Na^+-activation was observed. It was concluded that polyamine uptake occurred via diffusion in the lung slices (Rao and Mehendale,1988).

Polyamine uptake is not found to be inhibited by the amino acids asparagine, leucine or aminoisobutyrate in isolated rat villious enterocytes (Kumagai and Johnson,1988); uptake is not inhibited by lysine, leucine and asparagine (representing the three main transporters) in rat pancreatic acinar cell line (Nicolet et al.,1990). In another study with isolated rat enterocytes, kinetic data indicated that spermidine and spermine share a carrier that is distinct from the one mediating putrescine transport. Spermidine uptake was inhibited by ouabain (a cardiotonic steroid derived from plants and is a potent inhibitor of Na^+/K^+-ATPase) but not significantly depressed by the replacement of Na^+ by mannitol or sucrose. This suggested that spermidine uptake does not depend on Na^+-cotransport but it may be dependent on the electrical gradient established by the Na^+/K^+-ATPase (Kumagai et al.,1989). A recent study in human umbilical-vein endothelial cells (Morgan,1992) also shows characteristics of polyamine uptake to be time-, temperature- and concentration-dependent, energy-requiring, and saturable. The existence of two carriers is suggested; one carrier shared by spermidine

and spermine (which may suggest aminopropyl group specificity) and the other being capable of transporting all three principal polyamines (Morgan,1992).

Polyamine uptake studies have been carried out in in vitro systems. However, much remains to be studied in the realm of polyamine transport in the intact animal. This may possibly clarify more of the aspects of the regulation of polyamine concentration in physiological and pathological situations.

1.3 FUNCTIONS of POLYAMINES

Polyamines have important biochemical functions with respect to growth, interactions with various anionic macromolecules, effect on protein kinases, in the regulation of cell cycle etc. The major functions of polyamines are summarized in Table 1.1. The functions of polyamines are briefly discussed here.

1.3.1 Polyamines and growth

In most cells the requirement for polyamines is absolute. Studies have shown that rapidly growing cells have higher levels of putrescine and ODC than quiescent cells. Quiescent cells when stimulated have an increase of putrescine and ODC levels before there is an increase of DNA, RNA and protein

Table 1.1

Functions of Polyamines

1. Polyamines serve as essential factors of growth in all organisms.
2. Polyamines are cationic molecules; by virtue of their cationic charges they interact with and thereby influence protein, DNA and RNA metabolism.
3. Polyamines play an important role in the regulation of the various events of the cell cycle.

contents (Tabor and Tabor,1984).

1.3.2 Polyamine interactions with macromolecules

The polyamines due to their polycationic nature (Seiler,1987) bind strongly to polynucleotides and the differential binding to double-stranded and single-stranded species stabilizes the former to thermal melting (Bloomfield and Wilson,1981).

1.3.2.1 Polyamine interactions with DNA

Polyamines possess an ability to protect DNA from heat denaturation or damage from shearing in vitro (Tabor and Tabor,1984). It has been proposed that the polyamines act by a counterion effect rather than by any direct specificity of the carbon-nitrogen chain. This counterion effect is evident from the finding that inorganic trivalent cations such as $[Co^{3+}(NH_3)_6]$ (an inert metal-ion complex) have similar effects (Widom and Baldwin,1980). Polyvalent cations such as spermidine are required for DNA catenation (or interlocking) by the action of gyrase, a topoisomerase. Spermidine or $[Co^{3+}(NH_3)_6]$ were found to produce aggregation and catenation, as are required for the action of gyrase (Krasnow and Cozzarelli,1982). It has been suggested that compaction of DNA by polyvalent cations is required for several enzymatic reactions (Tabor and Tabor,1984).

The levels of ODC and polyamines have been found to be the highest at the end of the G_1 phase of the cell cycle in synchronised Chinese hamster ovary (CHO) cells (Heby et al.,1976). It has been suggested that the changes involved may be associated with the cell's preparation for DNA synthesis (Tabor and Tabor,1984).

1.3.2.2 Polyamine interactions with RNA

S.cerevisiae mutants lacking ODC activity have no spermidine or spermine. In these mutants, spermidine or spermine is required in the growth medium for the maintenance and replication of double-stranded RNA plasmids coding for a killer toxin (Cohn et al.,1978). A two-fold stimulation of rRNA (ribosomal RNA) synthesis was observed when ODC was microinjected into Xenopus oocytes; thus ODC can modify RNA synthesis (Russell,1983). Androgen administration to castrated male mice causes a 1000-fold stimulation of ODC in kidney along with a dramatic increase of RNA levels compared to a very small increase in DNA content (Henningsson et al.,1978). Two classes of spermidine binding to tRNA have been described, one which is low affinity and relatively non-specific, occurring only at low concentrations of other cations, and the other which is high affinity and quite specific even in the presence of higher concentrations of other cations (McMahon and Erdmann,1982). Like DNA, polyamine

binding to RNA is by the formation of a counter-ion atmosphere around the negatively charged macromolecule (Tabor and Tabor, 1984).

1.3.2.3 Polyamines and protein biosynthesis

It was observed that under conditions of polyamine starvation, chain elongation during the synthesis of ß-galactosidase was slowed down by 40%; therefore it was suggested that polyamines have a non-essential catalytic role in accelerating chain elongation (Loftfield et al.,1981). In the presence of suboptimal concentrations of Mg^{2+}, polyamines markedly stimulate several protein synthesizing systems in vitro. It is also known that polyamines as well as Mg^{2+} bind to ribosomes and facilitate the association of ribosomal subunits (Tabor and Tabor,1984).

1.3.3 Polyamines and the cell cycle

Stimulation of cell proliferation of a variety of cell types in vivo and in culture is accompanied by increased intracellular levels of the polyamines. When logarithmically growing rat embryo fibroblasts (REF) in culture are incubated with MGBG, cells continue to divide at about the normal rate for approximately one cycle and then there is an arrest in the G_1 phase of the cell cycle. This arrest is preceeded by a decrease in cellular spermidine and spermine levels,

suggesting their requirement for cell division. Replenishment of cellular pools of spermidine or spermine in these MGBG-arrested cells causes the cells to enter the S-phase about 12 hours later and divide (Rupniak and Paul,1981). Similar results were seen with CHO fibroblasts in which polyamines started to accumulate toward the end of the G_1 phase. The rate of synthesis of polyamines peaked as the cells started to synthesize DNA in the S-phase (the phase of DNA synthesis) and the highest polyamine content was seen at the beginning of the S-phase (Pegg and McCann,1982). From these studies and other studies, it becomes evident that DNA synthesis in the S-phase is preceeded by increased ODC and polyamine levels in the cells. There is a second increase of polyamine biosynthesis prior to cell division (mitosis) similar to the first rise at the G_1 phase prior to the S-phase. Thus there is a biphasic increase or that the polyamine peaks can be seen during late G_1-early S and in S-G_2, probably preparing the cell for DNA synthesis and division (Pegg and McCann,1982).

1.3.4 Effect of polyamines on Na^+/K^+-ATPase

Polyamines have been shown to stimulate the Na^+/K^+-ATPase activity, which is regulated by arginine vasopressin (AVP) in the rat renal medullary thick ascending limb of the loop of Henle. There is an increase in the activity of the enzyme

after stimulation with putrescine, spermidine and spermine (each 1 mmol/L) for 2.5, 2.0 and 1.5 mins. respectively. When spermidine and spermine synthases were inhibited by their respective inhibitors, stimulation of ATPase by AVP was inhibited. This inhibition was reversed by spermine. These findings suggest that polyamines may be involved in the stimulus-response coupling of the AVP-mediated response (Charlton and Baylis,1990).

1.4 POLYAMINES IN TUMOR TISSUES

Owing to the immense importance of polyamines in cell proliferation, a brief discussion of polyamines in tumor tissues seems relevant, tumors being a true representative of a proliferate disorder.

Stimulation of cell growth is accompanied by increases in the rates of polyamine biosynthesis and cellular polyamine levels (Morris,1978). In fact, a correlation of increased polyamine biosynthesis or accumulation with rapid cell proliferation is well established (Rupniak and Paul,1981). Owing to the properties of enhanced synthesis and accumulation in rapid growth systems, polyamines have been suggested as indicators of rapidly proliferating tumors (Russell,1971).

ODC and the polyamines putrescine, spermidine and spermine are crucial for cell proliferation. ODC activity and

the levels of polyamines putrescine, spermidine, spermine, cadaverine and acetylputrescine in human colonocytes isolated from cancerous areas was measured and compared to the normal colon tissue. It was found that colonocytes isolated from cancerous areas had significantly higher mean values of ODC activity (increased by 14.8 fold) and the putrescine, spermidine, spermine and cadaverine levels were elevated by 47%, 260%, 380% and 510% respectively compared to colonocytes isolated from normal colonic mucosa. No difference was found in acetylputrescine levels between cancerous and normal colonocytes (Elitsur et al.,1992).

Polyamine contents and the activity of ODC have been measured in patients with recurrent gliomas, meningiomas and pituitary adenomas. In benign tumors, ODC activity was less than 10 nmoles/gram/hour, whereas in malignant gliomas values upto 34 nmoles/gram/hour were observed. In rapidly growing tumors, pronounced heterogeniety was observed with ODC activity in solid tumor parts and low activity in necrotic areas. It is suggested that high ODC activity represents a reliable biochemical marker of malignancy in brain tumors, but low values do not prove benignity (Ernestus et al.,1992).

1.5 POLYAMINE EXCRETION PATTERN

The polyamine excretion pattern in urine varies somewhat

from one individual to the next, and is clearly different in men and women (Pöyhöenen et al.,1990). Rats and mice excrete significantly more non-conjugated polyamines than humans. However, among the conjugates, acetyl derivatives prevail in both humans and rodents. The fact that rodents do not excrete a considerable proportion of polyamines in the unconjugated form demonstrates that acetylation is not an absolute requirement for polyamine excretion (Seiler,1987). In a study in humans, a circadian pattern was observed in the excretion of the total polyamines and the two acetylspermidines. It was found that the excretion of these polyamines was highest in the morning and that the excretion of spermidine, N^8-acetyl-spermidine and spermine was significantly higher in men than in women. No correlations between polyamine excretion and age, or menstrual cycle (in women) were found (Pöyhöenen et al., 1990).

1.5.1 Clinical significance of polyamine excretion

Several diseases, both malignant and non-malignant, are associated with alterations of polyamine metabolism and excretion. Since Russell's observation (1971) of increased polyamine levels in the urine of patients with various kinds of metastatic cancer, much work has been done on the clinical importance of polyamines in the field of cancer. However, non-

malignant diseases like chronic obstructive lung disease, uremia, cirrhosis of liver, cystic fibrosis, and infections are associated with disturbed polyamine metabolism (Pöyhöenen et al.,1990). Regenerating tissues have a high demand for *de novo* polyamine formation (Seiler et al.,1992). Tissue regeneration and wound healing after an accident or surgical trauma is associated with increased urinary polyamine excretion (Russell and Durie,1978a).

1.5.2 Polyamine excretion in cancer patients

The observation that there is an increased polyamine excretion in patients with metastatic cancer (Russell,1971) raised a possibility for the use of extracellular polyamine levels as a test for the early detection of cancer and also for monitoring the changes in these levels in order to assess the effectiveness of therapy (Russell et al.,1971). A direct correlation was found between increases in [^{3}H]thymidine labeling index and an increase in urinary putrescine level. Putrescine and spermidine have been suggested to be useful tumor kinetic markers because changes in tumor growth and tumor cell death parameters closely parallel changes in the levels of putrescine and spermidine in the plasma and urine of cancer patients (Rosenblum et al.,1978).

Polyamine concentrations in blood and urine were measured

during chorionepithelioma (choriocarcinoma) in which there is an intensive growth process due to excessive secretion of the hormone HCG (human chorionic gonadotropin) secreted by the tumor itself. An increase in urinary free putrescine and spermine levels was observed in choriocarcinoma while there was a decrease in urinary spermidine levels. The free putrescine levels in urine increased and remained the same after consecutive chemotherapeutic treatments, while spermine, though it increased greatly, did not show any characteristic fluctuations (Kamininski,1983).

Urinary polyamines were found to be elevated in patients with breast cancer; but the elevation was restricted to only 15% in this study (Tormey et al.,1975). In another study it was observed that there was an elevation of spermine excretion in about 15 out of 30 patients (50%) studied. However, the effect was found to be absent in 30 patients with benign tumors. The irregular pattern of spermine excretion seen in some patients with malignant tumors raised a question as to why the spermine output would be increased in some while the effect would be absent in others (Chayen et al.,1983).

1.5.3 Polyamine excretion in non-malignant diseases

Cirrhotic patients were found to have increased urinary levels of free, monoacetylated and total polyamines. They also

showed an increase in the N^1-acetylspermidine to N^8-acetylspermidine molar ratio. Urinary polyamine excretion was not related to the severity of liver disease nor to the values of liver function tests. It is suggested that enhanced polyamine biosynthesis and catabolism, particularly N^1-acetylation, occur in cirrhotic patients, probably due to hepatic regeneration and/or increased levels of insulin and glucagon (Cecco et al.,1992). However, in patients with cerebro-hepato-renal syndrome of Zellweger, a condition characterised by lack of peroxisomes, polyamine excretion was found to be normal when compared to controls (Govaerts et al.,1990). In uremia, polyamines accumulate inside and outside the cell. There is an imbalance between polyamine production, degradation and excretion. A decreased polyamine synthesis and blunted polyamine pathway response suggest that polyamines may participate in cellular polyamine downregulation (Campbell, personal communication).

1.6 PROBLEM OF INVESTIGATION

Since the initial observation by Russell (1971) of elevated urinary polyamine levels in cancer patients harboring a variety of tumors, a considerable interest has grown among many workers at the prospect of using urinary polyamines as biochemical markers of malignancy. Many workers have implied

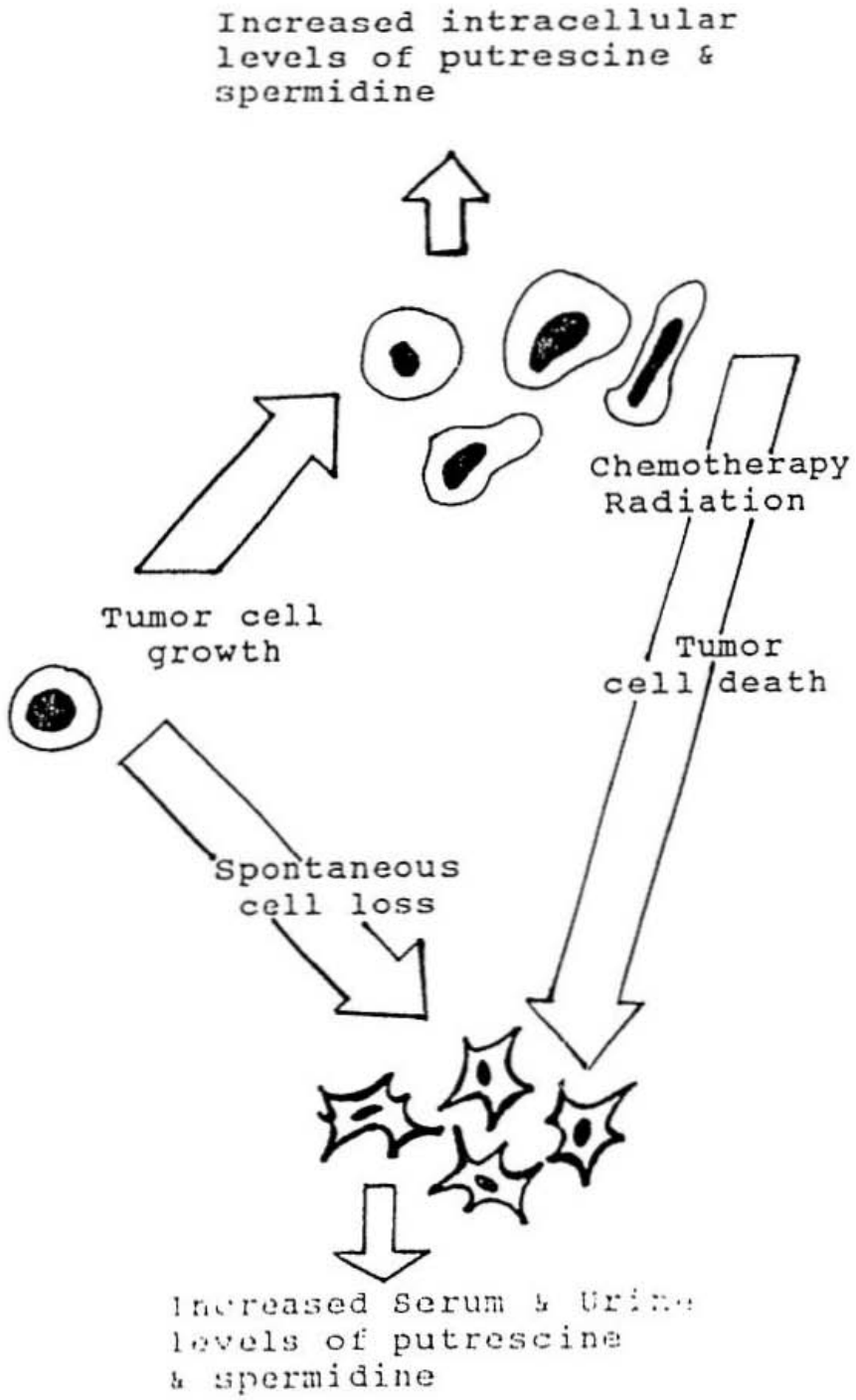
that an increased rate of polyamine synthesis, for instance in a tumor, will be reflected in the urinary excretion (Seiler et al.,1981). The results of several studies have shown that tumor cells usually contain higher intracellular concentrations of polyamines than their normal counterparts leading to the excessive excretion of polyamines (Rupniak and Paul, 1981). Based on animal studies of tumor growth and regression, spontaneously and in response to radiation and chemotherapy, a model (Figure 1.4) was proposed to summarise the potential role of polyamines as biochemical markers of human tumor cell growth and tumor cell death (Russell and Durie,1978b).

Polyamines have been used as tumor markers by many, but the correlation was not found to be satisfactory, although polyamine concentrations were found to be high in tumor tissues and may correlate in some cases with the degree of malignancy (Pöyhöenen et al.,1990). An understanding of the factors influencing polyamine excretion seems critically important in this respect. Seiler et al (1981) suggested that some of the factors which could influence the urinary pattern of polyamines are:

a) Rate of polyamine formation within the tissue
b) Metabolic activity within the tissue
c) Rate of transfer from the cells into the circulation
d) Catabolic activity within the circulation

Figure 1.4

Proposed model of polyamines in serum and urine as biochemical markers of cancer; modified and redrawn from Russell and Durie (1978b).


Increased intracellular
levels of putrescine &
spermidine
Chemotherapy
Radiation
Tumor cell
growth
Tumor
cell death
Spontaneous
cell loss
Increased Serum & Urine
levels of putrescine
& spermidine

e) Rate of cell death in various organs

f) Diet

The factors mentioned above do not consider any role for the kidneys in influencing the pattern of polyamine excretion. An understanding of renal handling of polyamines is important before an attempt is made to use urinary polyamines as markers of rapid growth situations.

The present studies are aimed at the understanding of how the kidneys handle the polyamines and how they themselves are involved in influencing the pattern of polyamine excretion. A number of questions are being addressed. To summarize, these are:

a) What percentage of the plasma polyamines that are delivered to the kidney are excreted ?

b) What is the renal arterio-venous difference for the polyamines ?

c) Are the polyamines freely filterable at the glomerulus or is a portion of them bound to the plasma proteins?

d) Are the filtered polyamines reabsorbed by the renal tubules ?

e) What happens to polyamine excretion if tubular functions are disrupted in disease or under experimental conditions ?

CHAPTER 2

MATERIALS AND METHODS

2.1 ANIMALS

Adult male Sprague-Dawley rats weighing 325-375 gms and adult female Sprague-Dawley rats weighing between 275-300 gms (Charles River, Montreal, Canada) were used for all *in vivo* experiments.

2.1.1 Standard conditions

Both male and female rats were housed singly in metabolic cages (Nalgene Plastic metabolism cages, Nalgene Company). The animals were adapted to the environment for 3 days after which the basal 24 hour urine collections were started. They were housed under the following standard conditions:

a) 20 ± 1° C temperature
b) 40% relative humidity
c) 12 hour light and 12 hour dark cycle (lights were off between 2000 hours and 0800 hours)
d) *ad libitum* access to food and water

2.1.2 Diet

It is important that the diet which the rats are fed contain adequate amounts of all nutrients but negligible amounts of polyamines since spillage of the diet could cause errors in urine polyamine estimation. Purina chow was found to contain large and variable amounts of polyamines (Table 2.1). Therefore, for all *in vivo* experiments, a 15% casein diet (a

Table 2.1

Free polyamines in feed Samples

Type of Feed	Putrescine	Cadaverine	Spermidine	Spermine
60% Casein diet	0.90	B.L.D.	1.30	2.00
15% Casein diet	0.90	B.L.D.	1.40	1.90
Chow diet	296.80	321.70	307.80	99.00

B.L.D.-below level of detection.

Values are expressed in nmoles/gm of diet.

Results in this table indicate analysis of one batch of diet.

high carbohydrate, moderate protein diet) was used. This diet was found to be a suitable alternative to rat chow, since it contains negligible amounts of polyamines (Table 2.1), but it does contain adequate nutrients for the rats (Biere et al., 1977). The diet composition is presented in Table 2.2.

2.1.3 Urine collection

For all experiments in vivo, two to three 24 hour basal urine collections were done. Urine samples were collected in plastic bottles (to prevent binding of polyamines to glass) (Campbell et al.,1981) in absolute ethanol to prevent bacterial growth. Freshly collected urine samples were frozen at -20°C in small aliquots and analysed as quickly as possible. In no case were samples stored longer than four days. Urine samples collected from ureter and bladder (sterile urine) also contained no traces of cadaverine. However, samples stored at -20°C for longer than one week usually did contain some cadaverine, especially if they were frozen and thawed more than once.

2.1.4 Maleate treatment

Animals received injections of a 5% maleate solution in 154 mM NaCl with the pH adjusted to 7.0 with 10 N NaOH. The dose given was 200 mg/kg body weight i.p. Control animals received the same dose of physiological saline (154 mM)

Table 2.2

Composition of the synthetic diet (15% casein diet)

Ingredient	gms/kg
Sucrose[1]	533.0
Casein[2]	148.5
L-methionine[1]	1.5
Corn starch[1]	17.0
α-Cellulose[2]	50.0
Corn oil[3]	50.0
Vitamin mixture[2] (AIN 76)	10.0
Mineral mixture[2] (AIN 76)	35.0
Choline bitartrate[2]	2.0
	1000.0

[1] Sigma chemicals, St.Louis,MO.

[2] ICN Biochemicals, Cleveland,OH.

[3] Best Foods Canada Inc., Etiobicoke,ON.

(Günther et al.,1979; Rosenberg and Segal,1964). The treated rats (n=4 for saline control and n=3 for maleate-treated) were operated under pentobarbital anaesthesia for renal studies (the surgical method is described later on in this chapter) at 3.6 ± 0.6 hours after injection. At this time there is no evidence of histological abnormalities in the kidney (Rosenberg and Segal,1964). For some animals, both saline and maleate treated, only blood and urine samples were collected without inulin infusion. Urine collections were done between the time of injection and the time of surgery or sacrifice. Aliquots of plasma samples (prepared from the abdominal aorta and left renal vein) were analysed for polyamines.

2.2 MEASUREMENT OF RENAL PARAMETERS

For the measurement of glomerular filtration rate (GFR), a substance has to have the following criteria (Pitts,1966a):

a) The mass of the substance should remain unaltered,
b) It is not reabsorbed or metabolized by the kidneys,
c) It is neither reabsorbed nor secreted by the kidney tubules to a significant extent,
d) It should be non-toxic and does not have any effect on renal function when infused in quantities which permit adequate quantification in plasma and urine,
e) It should be freely filterable through the glomerular capillary membrane i.e. not bound to plasma proteins, and

f) It can be quantified in plasma and urine with a high degree of accuracy.

Inulin, a fructose polysaccharide meets the above criteria and was therefore employed for the measurement of glomerular filtration rate. Since inulin is hydrolysed to fructose in the gastrointestinal tract and poorly absorbed from subcutaneous tissue or muscle it must be infused intravenously. Therefore its clearance is equal to the glomerular filtration rate (Pitts,1966a). An administration of a priming dose followed by a steady infusion will maintain a plateau concentration of inulin in plasma which depends on the rate at which inulin is excreted. It has been demonstrated that this infusion protocol maintains ^{14}C-inulin at plateau levels between 20-40 mins. (Dhanakoti,1991). In human studies, inulin is used only for the determination of GFR. In experimental animals, however, where it is possible to sample blood from the renal vein as well as an artery, it is possible to determine the filtration fraction of inulin across the kidney (the percent of plasma filtered). It is then possible to calculate the renal plasma flow (RPF) from GFR and filtration fraction. The renal blood flow (RBF) is calculated from the RPF provided one determines the hematocrit value. Therefore, by measuring the urine flow rate (UFR) and the concentration of inulin in arterial and renal venous plasma, it is possible to calculate the RPF, RBF and GFR.

2.2.1 The Surgical Method

The surgical technique used was as described by Lowry et al (1987) and the protocol is shown in Figure 2.1. The animal was anaesthesized with sodium pentobarbital (60 mg/kg body weight i.p.) and placed on a heating pad. The trachea was cannulated with a small piece (2.5 cm long x 2.5 mm I.D.) of polyethylene tubing. The right jugular vein was catheterized with PE-50 tubing (Clay-Adams, Parispany, New Jersey) for the infusion of inulin.

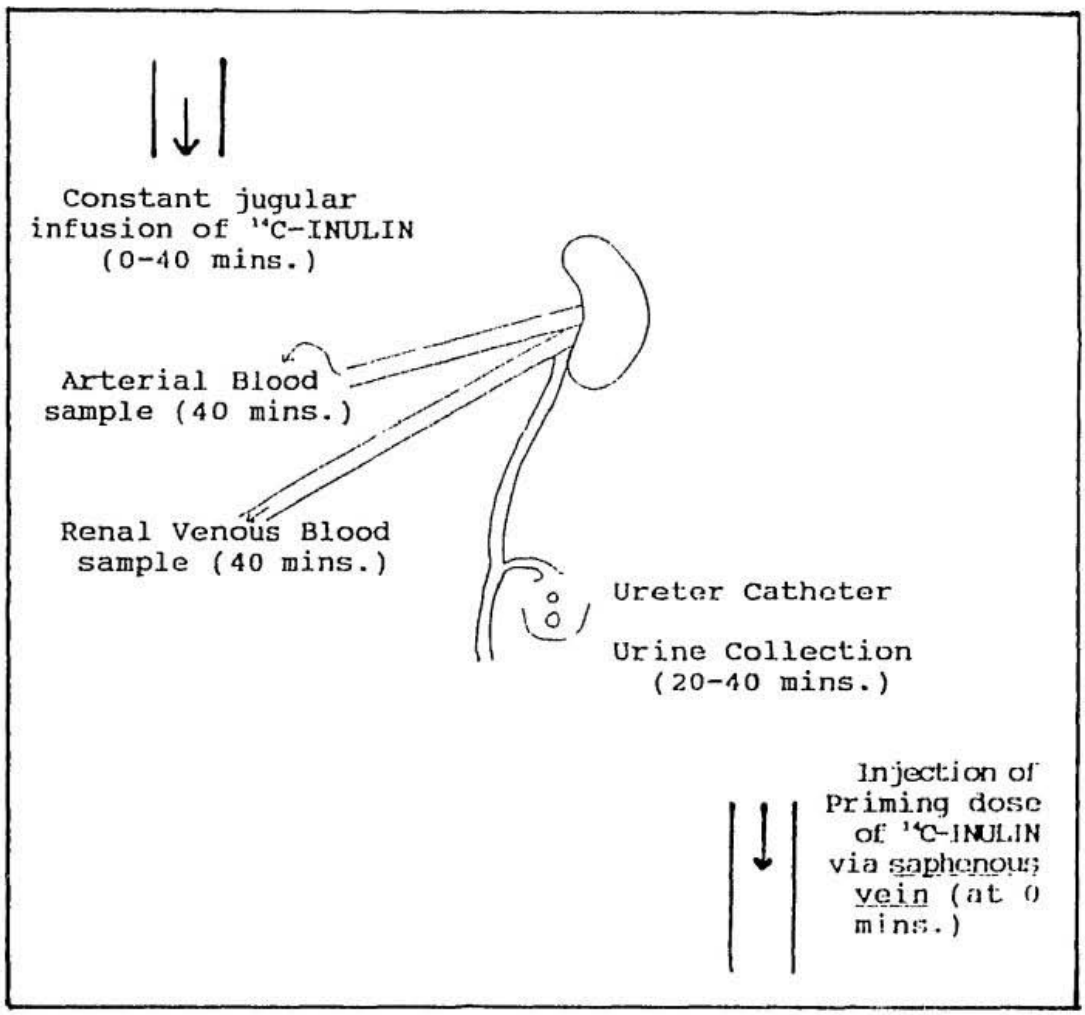
The rat was opened along its ventral aspect and a sample of bladder urine was collected. Then a priming dose of 1.75 µCi of [carboxyl-^{14}C] inulin (New England Nuclear, Lachine, Quebec) in 0.8 ml of 154 mM NaCl was given through the saphenous vein. This was followed by a continuous infusion of the same solution at a rate of 0.037 ml/min. using a Harvard Apparatus model 975 compact infusion pump. In studies with maleate treated animals and their corresponding saline treated controls, the prime and infusion solutions contained inulin in a 5% mannitol-0.45% NaCl solution. The mannitol present in the prime and infusion solutions acts as a diuretic and thus helps in urine collection. Mannitol does not affect the GFR.

The left ureter was then catheterized with a short piece of PE-10 tubing fitted inside a length of PE-50 tubing. After a 15 min. stabilization period, urine collection from the ureter was begun. Urine was collected between 20-40 mins. At

Figure 2.1

Surgical protocol used in rats.

Ureter urine was sampled between 20-40 mins. Whole blood was sampled from the abdominal aorta and left renal vein respectively at 40 mins. Injection of the priming dose of the solution of ^{14}C-inulin was given at 0 mins. through the saphenous vein followed by the constant infusion of the same solution through the right jugular vein between 0 to 40 mins.


Constant jugular infusion of ^{14}C-INULIN (0-40 mins.)
Arterial Blood sample (40 mins.)
Renal Venous Blood sample (40 mins.)
Ureter Catheter
Urine Collection (20-40 mins.)
Injection of Priming dose of ^{14}C-INULIN via saphenous vein (at 0 mins.)

the end of the urine collection period, 1 ml blood samples were drawn from the left renal vein and the abdominal aorta. Blood was drawn from the abdominal aorta rather than the renal artery because it is easier to sample and there are no differences in the blood composition of any artery. Whole blood samples were collected in heparinized syringes and centrifuged to obtain plasma. Aliquots of plasma (25 µl) and ureter urine (10 µl) were used for the determination of inulin radioactivity. Blood and urine samples were prepared for polyamine analysis.

2.2.2 Calculations

GFR was calculated from urinary inulin excretion in the 20 to 40 min. clearance period. RPF was calculated using the expression derived by Wolf (1941). The calculations that are involved in the measurement of RPF, RBF and GFR are as follows:

$$U/P_{(inulin)} = \frac{\text{Inulin counts in urine (dpm/ml)}}{\text{Inulin counts in arterial plasma (dpm/ml)}}$$

where $U/P_{(inulin)}$ is the urine-to-plasma ratio for inulin.

$$GFR\ (ml/min.) = U/P_{(inulin)} \times UFR\ (ml/min.)$$

RPF is calculated by determining the inulin counts in urine, in arterial and renal venous plasma and also urine flow rate

(UFR) (Wolf,1941).

$$\text{RPF (ml/min.)} = \frac{\text{Urine(dpm/ml)}}{\text{Arterial plasma-Renal venous plasma [(dpm/ml)]}} \times \text{UFR}$$

$$\text{RBF (ml/min.)} = \frac{\text{RPF}}{1 - (\text{Arterial blood Hematocrit}/100)}$$

The arterial hematocrit was taken as 45 and there is no difference between the arterial and the renal venous hematocrit values (Dhanakoti et al.,1990).

The values of GFR, RPF and RBF are multiplied by 2 for two kidneys and were expressed as ml/min./2 kidneys.

2.3 BIOCHEMICAL ANALYSES

2.3.1 Polyamines

Blood, plasma and urinary polyamines were determined by a HPLC (high-pressure liquid chromatography) method developed by Seiler and Knödgen (1985). Samples were deproteinized by the addition of perchloric acid (PCA) to a final concentration of 0.2 M PCA, the precipitated proteins were removed by centrifugation and the supernatants were used for polyamine analysis. The PCA extracts were filtered through a 0.45 µm filter before analysis (Dhanakoti et al.,1990). Polyamine standards were also prepared in 0.2 M PCA; the amount of compound in the working solution was of 1 nmole/0.1ml (Seiler and Knödgen,1985).

Polyamines do not exhibit any structural features that would allow their sensitive detection without derivatization (Seiler,1986), so all chemical methods of polyamine analysis require derivative formation, either before or after separation. Polyamines were converted to their ortho-phthalaldehyde derivatives after separation (post-column derivatization) (Seiler and Knödgen,1985). O-phthalaldehyde (OPA) in the presence of 2-mercaptoethanol, reacts with primary amines to form fluorescent products which can be easily detected (Benson and Hare,1975). The polyamines were separated on a Beckman Ultrasphere IP column (4.6 x 250 mm) with 5 µm spherical core with chemically bonded C_{18} groups (ion-pair analytical). The analytical column was protected by a Beckman Ultrasphere IP (2.1 x 70 mm) pre-column or guard column filled with pellicular ODS and chemically bonded C_{18} groups and 5 µm spherical core. The gradient composition used for polyamine separation is given in Table 2.3. The gradient system consisted of 0.1 M sodium acetate (pH 4.5) containing 10 mM octanesulphonic acid (OSA) and 10% methanol (buffer "a") and 0.2 M sodium acetate (pH 4.5) containing 10 mM OSA, 10:3 (v/v) acetonitrile and 10% methanol (buffer "b"). Post-column derivatization was done using O-phthalaldehyde/2-mercapto-ethanol reagent and the derivatives were detected using a fluorescence detector (excitation 345 nm, emission 455 nm) which had a 10 µl continuous flow cell. A flow-rate of 1

Table 2.3

Composition of gradient for separation of polyamines by HPLC

	Elution time (mins.)	Flow ml/min.	Percentage of solvent a	b
	Initial	1.00	100	0
	12.00	1.00	100	0
	16.00	1.00	60	40
	36.00	1.00	60	40
	48.00	1.00	0	100
End-time	65.00	1.00	100	0
Equilibration time	97.00	1.00	100	0

Solvent a: 0.1 M sodium acetate, 10 mM OSA (A); methanol (B); 9:1 (A+B=a).

Solvent b: 0.2 M sodium acetate, 10 mM OSA, acetonitrile 10:3 (v/v) (A); methanol (B); 9:1 (A+B=b).

This is the gradient of Seiler and Knödgen (1985).

ml/min. was maintained throughout. The time for separation of each sample was followed by a 32 min. washing (equilibration period) of the column with 100% buffer a. The identity of all polyamine peaks was established by spiking the PCA extracts with standard polyamines. A chromatographic profile of the separated polyamines is shown in Figure 2.2. Area under the curve was proportional to concentration (with a significant correlation; $p<0.05$) for all of the compounds of interest. The resultant standard curves for putrescine, spermidine and spermine are given in Figure 2.3. The recovery of the added polyamines was determined by adding the standard polyamine to the samples in a ratio of 1:1, such that the final concentration of the added polyamine in the sample-standard mixture was 0.5 nmoles/0.1 ml. The recovery of the added polyamines was about 92-94% (Table 2.4).

The water used for HPLC purposes was an 18 mega-ohm HPLC grade water. An ultrapure Bårnstead Reverse Osmosis system is coupled with organic removal, demineralization and submicron filtration to give Grade I quality water. This water is fed to a Nanopure II system with submicron filtration (0.2 μm) to give an 18 mega-ohm HPLC grade water.

2.3.2 Amino acid

Amino acids were analysed in plasma and urine samples. Aminoethylcysteine (AEC) was added as internal standard for

Figure 2.2

Separation of polyamines and related compounds by HPLC. Fluorescence intensity after reaction with o-pthalaldehyde/2-mercaptoethanol. Amount of each compound is 1.0 nmol per 0.1 ml. The following compounds are shown:

1] N-acetylputrescine, 2] putreanine, 3] putrescine, 4] cadaverine, 5] histamine, 6] N^1-acetylspermidine, 7] N^8-acetylspermidine, 8] spermidine, 9] N^1-acetylspermine, 10] spermine.

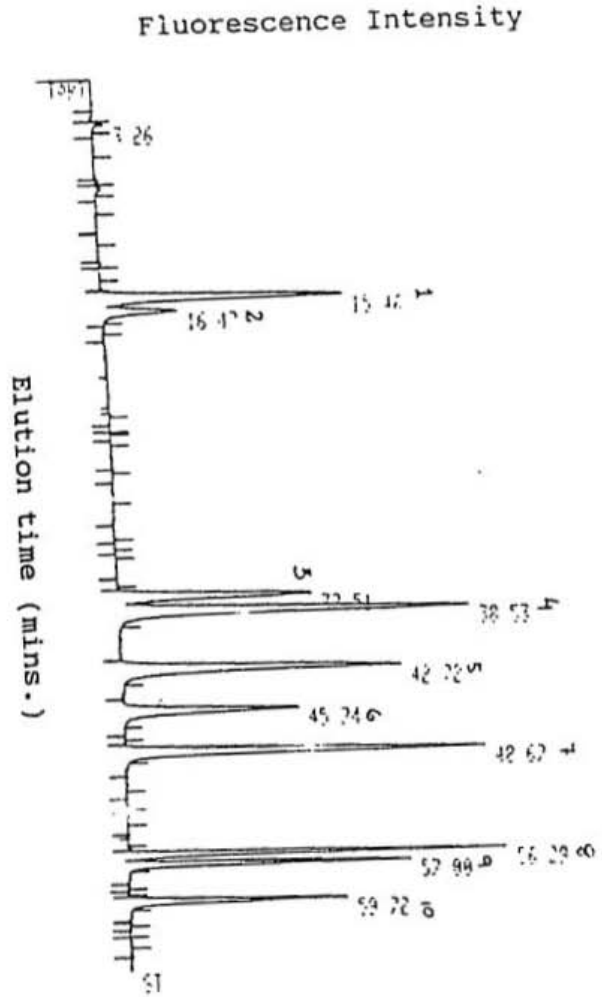

Fluorescence Intensity
Elution time (mins.)

Figure 2.3

Peak area as a function of polyamine concentration. Concentration of polyamines varied from 0.25 nmole to 1.0 nmole per 0.1 ml. Area calculated by the 3390A Reporting Integrator (Hewlett Packard).

1 Area count = 0.125 $\mu V/sec^{-1}$.

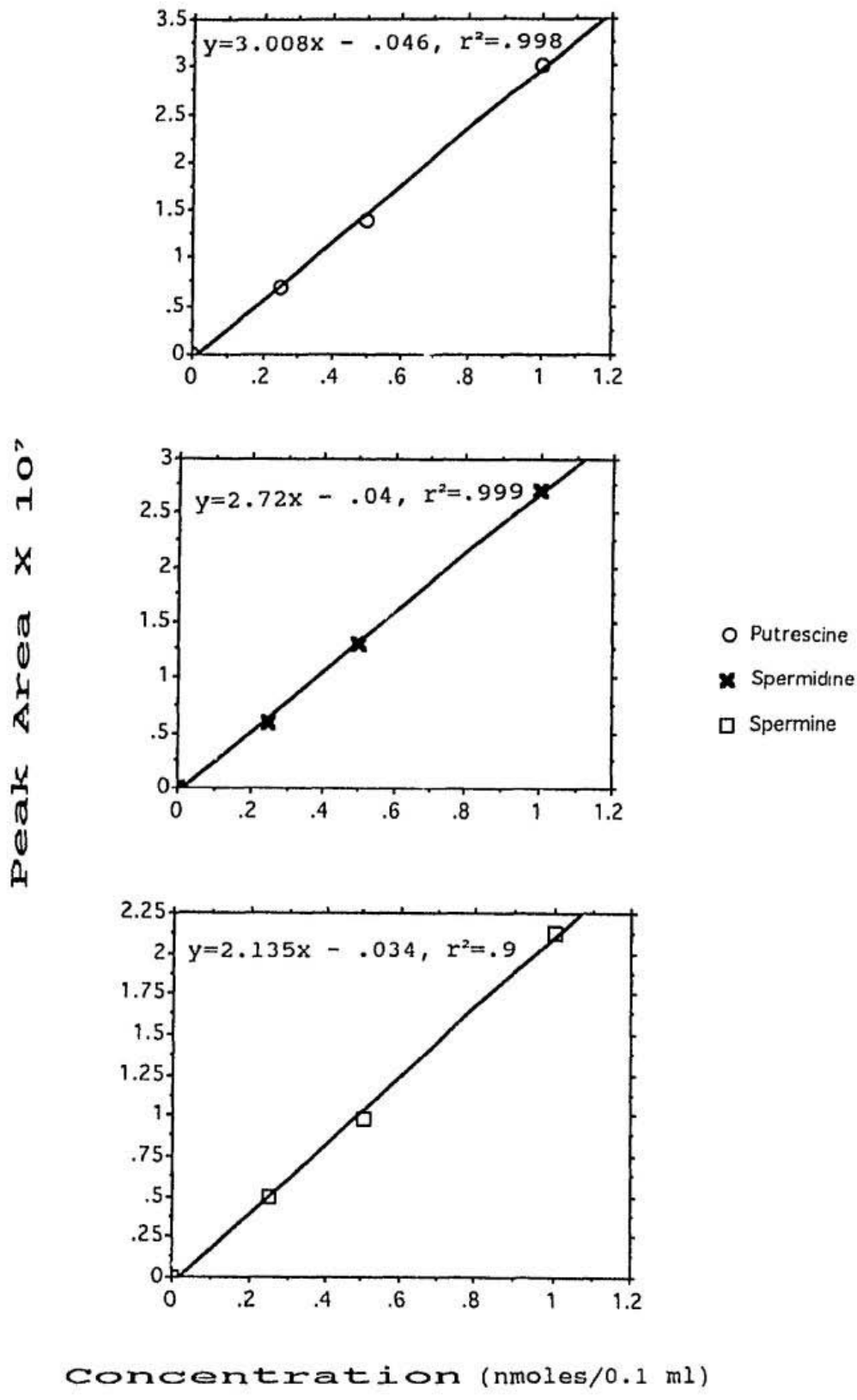

y=3.008x - .046, r²=.998
y=2.72x - .04, r²=.999
y=2.135x - .034, r²=.9
Peak Area X 10'
Concentration (nmoles/0.1 ml)
Putrescine
Spermidine
Spermine

Table 2.4

Percentage recovery of polyamines assayed by HPLC

Polyamines	Percentage recovery
Putrescine	93.8 ± 8.4
Spermidine	91.8 ± 3.7
Spermine	92.3 ± 4.3

Values are mean ± S.D. (n=5).

all analyses. The plasma samples containing 65 nmoles of AEC were deproteinized with 14% sulfosalicylic acid (SSA) and the pH of the deproteinized supernatants was adjusted to 2.2 ± 0.1 with concentrated hydrochloric acid (Dhanakoti et al.,1990). For the urine samples, 1.25 mM AEC was added to the samples, pH was adjusted between 12-14 with 3 M lithium hydroxide and deammoniated in a vacuum desiccator for 2-3 hours. Proteins were precipitated by adding 10% SSA and removed by centrifugation. The pH was adjusted to 2.2 ± 0.1 with concentrated hydrochloric acid and diluted with Li^+ citrate buffer (pH 2.2).

Deproteinized samples were analysed on a Beckman 121 MB amino acid analyser using Benson D-8.25 resin and a single column according to the three buffer lithium method (Brosnan et al.,1983).

2.3.3 Glucose

A highly specific enzymatic assay for glucose determination originally designed by Bonder and Mead (1974) was employed for all determinations. This method utilises the coupled enzyme reactions catalysed by hexokinase and glucose-6-phosphate dehydrogenase (G-6-PDH). The reactions are as follows:

Glucose + ATP $\xrightarrow{\text{HEXOKINASE}}$ G-6-phosphate + ADP

G-6-phosphate + NAD $\xrightarrow{\text{G-6-PDH}}$ 6-phosphogluconate + NADH

Glucose is phosphorylated by adenosine triphosphate (ATP) in a reaction catalysed by hexokinase to form glucose-6-phosphate. Glucose-6-phosphate is then oxidised to 6-phosphogluconate in the presence of nicotinamide adenine dinucleotide (NAD). This reaction is catalysed by G-6-PDH whereby NAD is reduced to NADH. Thus the consequent increase in absorbance at 340 nm is directly proportional to the glucose concentration over the range of the concentration used.

Glucose was determined in the urines of saline and maleate-treated rats. For the saline-treated rats, urine samples were directly used for analysis. For the maleate treated rats, urines were diluted appropriately with water to fit within the concentration range used. A sample-to-reagent ratio (glucose "Hexokinase" reagent for in vitro Diagnostic use, Sigma chemical company, St.Louis) of 1:100 was used for all determinations. Initial absorbance (A_1) was recorded with the glucose reagent. Samples were then added and placed in a water bath at 37°C for 5 mins. after which the final absorbance (A_2) was recorded. Glucose concentration was calculated from the differance in the two absorbances (ΔA). For each urine sample, a water blank and a saline blank (consisting of sample and 154 mM NaCl in 1:100 sample-to-reagent ratio) were done and subtracted from each sample to get the sample glucose concentration. The saline blank was

used to account for any turbidity in the sample.

2.3.4 Creatinine

Creatinine is removed from plasma by glomerular filtration and is then excreted in the urine without being reabsorbed by the tubules to any significant extent. This results in a relatively high clearance rate for creatinine as compared with urea (125 versus 70 ml per min.). In addition when plasma levels increase above the normal, the kidney can also secrete creatinine through the tubules. Since the excretion of creatinine in one given person or animal is relatively constant (because of the constancy of the daily creatinine production), 24 hour urine creatinine levels are used as a check on the completeness of a urine collection (Schuster and Seldin,1985).

Creatinine assay is based on the Jaffé reaction; addition of an alkaline picrate solution to the sample produces a yellow/orange colour due to the formation of a tautomer of creatinine picrate. Jaffé reaction is not specific; a number of non-specific chromogens may interfere (DiGiorgio,1974). Hence, a method with improved specificity was developed by Slot (1965) who noted that under acid conditions, the creatinine-picrate colour faded faster than the interfering chromogens. Thus the colour intensity measured at 500 nm before and after acidification is proportional to the

creatinine concentration.

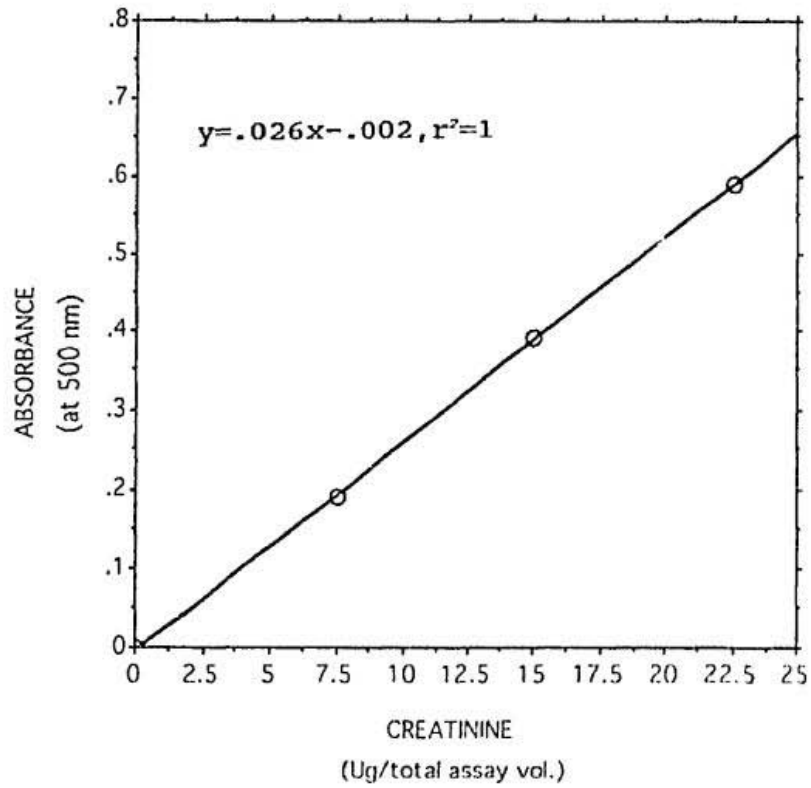
All urine samples were diluted (1:10) with water to fit into the range of the standard calibration curve (Figure 2.4) and mixed with freshly prepared alkaline picrate reagent and an initial absorbance was recorded at 500 nm. A final absorbance was recorded after the addition of the acid reagent. The difference between the two absorbances is proportional to the creatinine concentration.

2.4. STUDIES IN VITRO-Equilibrium Dialysis

Equilibrium dialysis was performed using double-chamber plexiglass dialysis units, as described by Dalgleish and Parker (1980). Sepraphor dialysis tubing with a molecular weight cutoff of 10,000 daltons was boiled in 0.1 M $NaHCO_3$, slit and used as the dialysis membrane separating the chambers. One ml of a solution of physiological saline (154 mM NaCl) containing ^{14}C-putrescine (5 µM) or spermidine (20 or 40 µM) was placed in one chamber, while 1 ml of plasma was placed in the other. The tightly sealed dialysis units were incubated at 25°C in a shaking water bath. Control chambers contained saline or BSA (5 gm/100ml saline) in place of plasma. At the completion of the incubation period, aliquots were taken from the saline and plasma compartments for determination of spermidine or putrescine radioactivity and protein (U-V absorption at 260 nm and 280 nm). In the first

Figure 2.4

Standard curve of creatinine (absorbance versus concentration).


y=.026x-.002, r²=1
ABSORBANCE
(at 500 nm)
.8
.7
.6
.5
.4
.3
.2
.1
0
0
2.5
5
7.5
10
12.5
15
17.5
20
22.5
25
CREATININE
(Ug/total assay vol.)

set of experiments, radioactivity was determined in separate dialysis chambers from 4 to 26 hours. Dialysis reached equilibrium by 20 hours after which no further change was seen, so subsequent experiments were carried out for 20 or 26 hours (to ensure equilibrium in all cases). Samples from each chamber were confirmed by HPLC analysis. At no time could protein be detected in the saline compartment. Thus the concentration of polyamine in the saline compartment represents free polyamine. Since the concentration of free polyamine will be the same in each compartment, the bound polyamine is given by,

$[\text{polyamine}]_{\text{protein compartment}} - [\text{polyamine}]_{\text{saline compartment}}$

Bound polyamines were expressed as a percentage of free polyamines.

2.5 CHEMICALS

Chemicals used for this study are listed in Table 2.5.

2.6 STATISTICAL ANALYSIS

All results are expressed as mean ± S.D. Significant differences between individual means were determined by unpaired t test or by paired t test as appropriate, as indicated in results. Differences were considered to be significant if $p<0.05$.

Table 2.5

List of Chemicals

Chemicals	Company
Polyamine standards	Sigma chemical company (St.Louis,USA)
Glucose(HK) assay kit	"
Creatinine assay kit	"
Maleic acid	"
Brij 35 solution	"
Mercaptoethanol	"
AEC	"
Lithium hydroxide	"
70% PCA	BDH (Merck) Inc. (Toronto,Ontario)
Acetonitrile(gradient grade)	"
OSA	"
Methanol (HPLC grade)	Fisher chemical (Fair Lawn,NJ,USA)
Sodium acetate (HPLC grade)	"
Potassium hydroxide	"
Boric acid	"
[^{14}C] carboxylinulin	New England Nuclear, (Lachine,Quebec)
[^{14}C] putrescine 2HCl	Amersham International (Amersham,UK)
[^{14}C] spermidine 3HCl	"

Chemicals used for the 15% casein diet are listed in Table 2.2.

CHAPTER 3

ARE PLASMA POLYAMINES BOUND TO MACROMOLECULES?

3.1 INTRODUCTION

Filtration of plasma is the initial step in the formation of urine. Plasma spermidine represents only 10% of the total blood spermidine as compared to 76% of total circulating spermidine present in red cells (Lundgren and Oka,1978). The amounts of spermine present in RBC are equivalent to about 80% of that in circulating whole blood (Uehara et al.,1980) and 95% or more of spermine in whole blood can be accounted for in the blood cells (Takami et al.,1979). Polyamine uptake into red cells depends on the polyamine concentration in plasma, but efflux of spermidine and spermine from these cells is very slow (Moulinoux et al.,1984). In spite of the high percentage of polyamines in blood cells, it is the plasma which represents the filterable fraction of the blood, and therefore, to assess the filterability of the polyamines, the plasma is considered as the compartment of blood for study. Therefore in vitro experiments were designed to determine the relative percentage of free polyamines and of polyamines non-covalently bound to macromolecules in plasma.

3.2 OBJECTIVES

To assess the percentage of bound and unbound polyamines in plasma.

3.3 RESULTS-Equilibrium dialysis

Freshly drawn plasma (or albumin/saline) was dialysed against 154 mM saline containing ^{14}C-polyamine from 4-24 hours. Spermidine represents the the major circulating polyamine in rat blood (Lundgren and Oka,1978) and putrescine is detectable in plasma, therefore both these amines were used to study polyamine binding in plasma. Spermine was not detectable in plasma within the limits of our assay system (<0.5 nmoles/ 1.0 ml) and was therefore not considered for the present studies. Plasma contains a number of proteins, but albumin is by far the most abundant, so spermidine binding to albumin was studied using bovine serum albumin (BSA). Figures 3.1 and 3.2 express spermidine as a percentage of the total spermidine in the plasma/albumin and the saline compartments. From Figures 3.1 and 3.2 it can be seen that there is a steady decline in the percentage of spermidine in the saline compartment; a plateau was reached by 20-26 hours for spermidine added to plasma or BSA. It was found that when saline was dialysed against saline with added ^{14}C-spermidine (no protein), equilibrium was reached by 24 hours (free concentration was the same in the two compartments). Spermidine dialysis was then carried out with a physiological spermidine concentration (20 µM) for 26 hours. It was also found that by 20 hours equilibrium was reached for added ^{14}C-putrescine in saline

Figure 3.1

Total spermidine in the plasma and saline compartments during the period of plasma dialysis from 0-26 hours; (spermidine 40 µM, n=3).

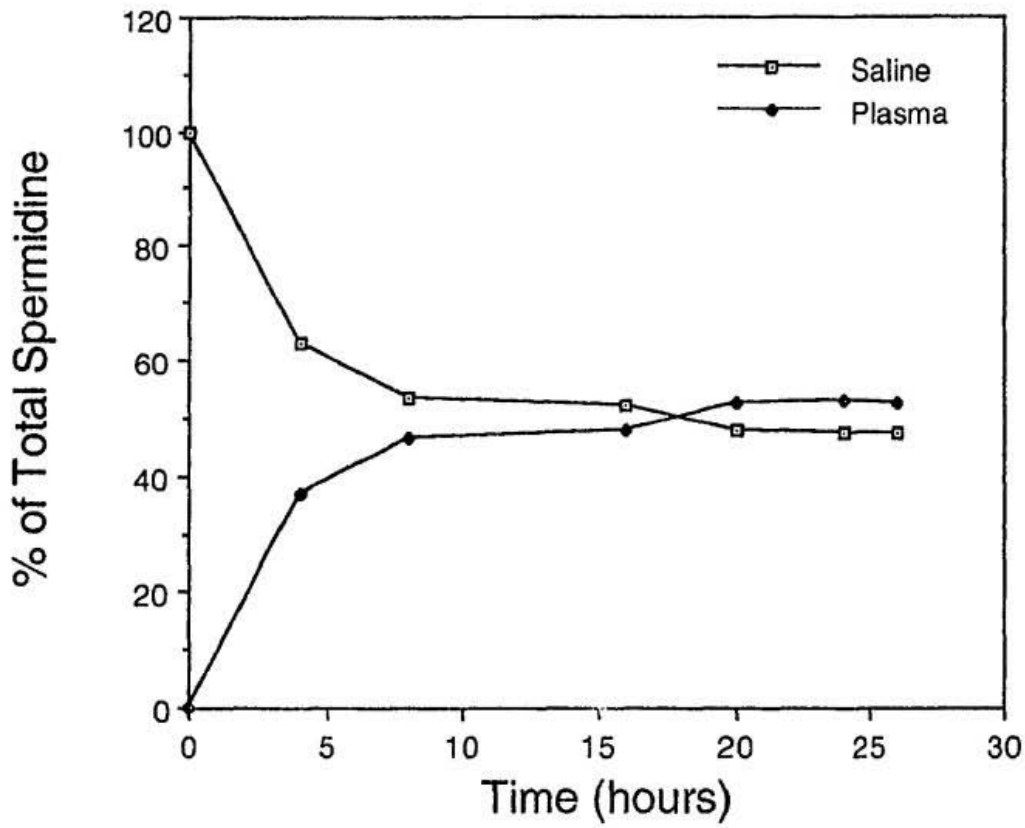
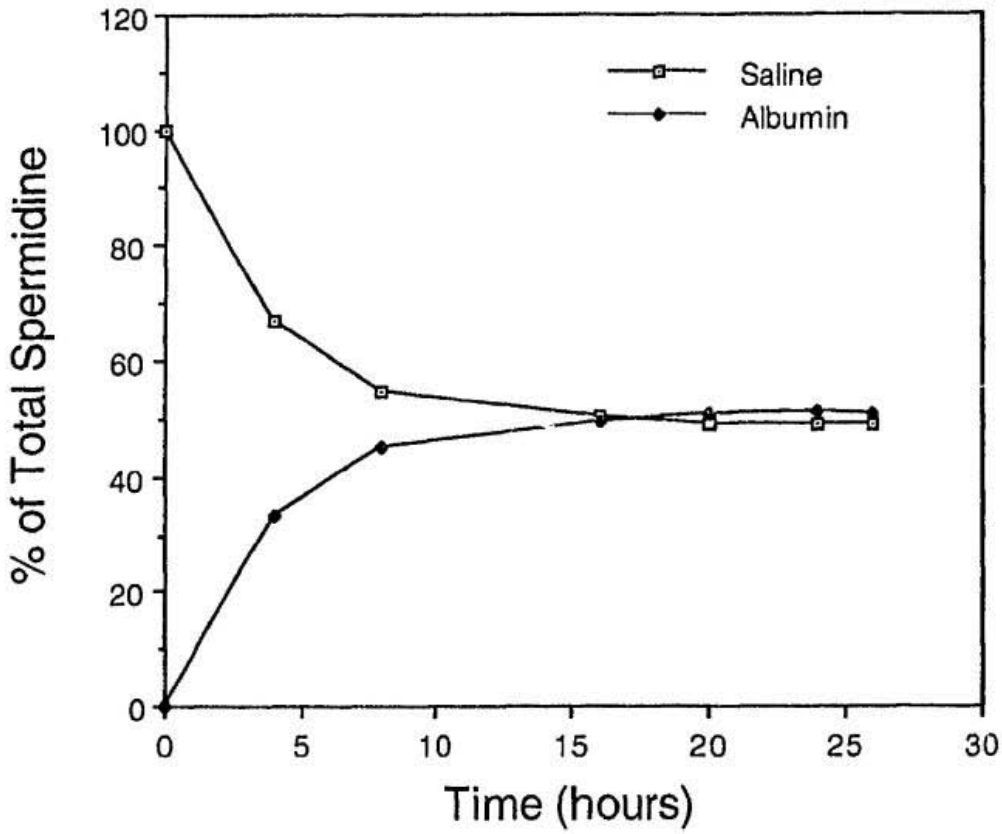

120
100
80
60
40
20
0
% of Total Spermidine
0
5
10
15
20
25
30
Time (hours)
Saline
Plasma

Figure 3.2

Total spermidine in the albumin and saline compartments during the period of dialysis from 0-26 hours; (spermidine 40 μM, n=3).


120
100
80
60
40
20
0
% of Total Spermidine
0
5
10
15
20
25
30
Time (hours)
Saline
Albumin

(with no protein). Saline to which ^{14}C-putrescine (with a physiological putrescine concentration of 5 μM) was added was then dialysed with plasma for 20 hours. Aliquots from the saline and the plasma compartments were analysed for their protein contents. No proteins were found in the saline compartment, which means that proteins are held back in the plasma compartment by the semi-permeable membrane. The recoveries of both putrescine and spermidine were always found to be above 90% at the end of the dialysis period. It was found that about 8% of plasma putrescine and 16% of the plasma spermidine were bound (Table 3.1) at physiological putrescine (5 μM) and spermidine (20 μM) concentrations. Albumin binding was only about 2%. Thus albumin is probably responsible for some polyamine binding in plasma.

3.4 DISCUSSION

The glomerular membrane of dog kidney is predicted to have glomerular pores with a diameter of 75Å, which permit the filtration of only a minute fraction of the circulating plasma albumin molecules (Pitts,1966b). Besides pore size, molecular charge is also an important determinant of glomerular filtration. In Munich-Wistar rats, the fractional clearance of dextran sulphate, an anionic polymer, is reduced relative to that of neutral dextran, a molecule with the same molecular

Table 3.1

Equilibrium dialysis of plasma with saline with added spermidine/putrescine at physiological concentrations

Polyamine	Saline	Protein	% Bound
Putrescine (20 hours)	2.2 ± 0.0	2.4 ± 0.0	8.0
Spermidine (26 hours)	8.9 ± 0.1	10.6 ± 0.2	16.0

Values are mean ± S.D. (n=3), values are expressed in μM; (hours of dialysis).

size and structure (Chang et al.,1975; Bohrer et al.,1977); the clearance of negatively charged albumin is also less than that of its neutral counterpart of the same molecular weight (Skorecki et al.,1986). Studies have shown that the filtration of the circulating cationic macromolecules is enhanced relative to their neutral counterparts (Skorecki et al.,1986). For example, it has been shown that the fractional clearance of the cationic dextran derivative, diethylaminoethyl dextran (DEAE dextran) is greatly enhanced relative to neutral dextran (Bohrer et al.,1978).

The present studies have shown that most putrescine and spermidine should be filterable at the glomerular membrane since they are not associated with non-diffusible macromolecules. Filterable polyamines may be in the free form and/or in the form of derivatives like the acetyl polyamines. However, our present methods are not sensitive enough to detect acetylated polyamines in plasma, if indeed they are present. Very low levels (nM) of acetylspermidines recently were observed in human plasma (Scheurmann et al.,1991), so it is likely they are also present in rat plasma. Analysis of non-hydrolysed rat urines show more polyamines in the free form than in the form of their acetylated derivatives (Seiler et al.,1981); this pattern of polyamine excretion has also been found in the present studies (chapter 4).

From the experiments described, it can be concluded that about 8% of putrescine and 16% of spermidine in plasma is bound non-covalently to non-diffusible macromolecules. Albumin may be responsible for some binding in plasma. For our remaining studies, it will be assumed that about 92% of putrescine and 84% of spermidine can be filtered at the glomerular membrane.

CHAPTER 4

PHYSIOLOGY OF POLYAMINE EXCRETION

4.1 INTRODUCTION

The kidneys are highly vascularized organs responsible for the maintenance of the body's internal environment. The kidneys receive the greatest blood flow, in proportion to weight, of any organ of the body. At rest, renal blood flow amounts to 20-25% of cardiac output; renal weight amounts to only 0.4% of body weight. High flow is probably related to the function of regulating the composition of body fluids (Pitts, 1966c). The nephron is the basic structural and functional unit of the kidney; it is involved in the complex interplay between tubular and vascular components resulting in the excretion of fluids and solutes. The regulation of body fluids and solutes is governed by the processes of filtration, reabsorption and secretion; unwanted substances are excreted from the body as urine (Chmielewski,1992).

4.1.1 The Nephron

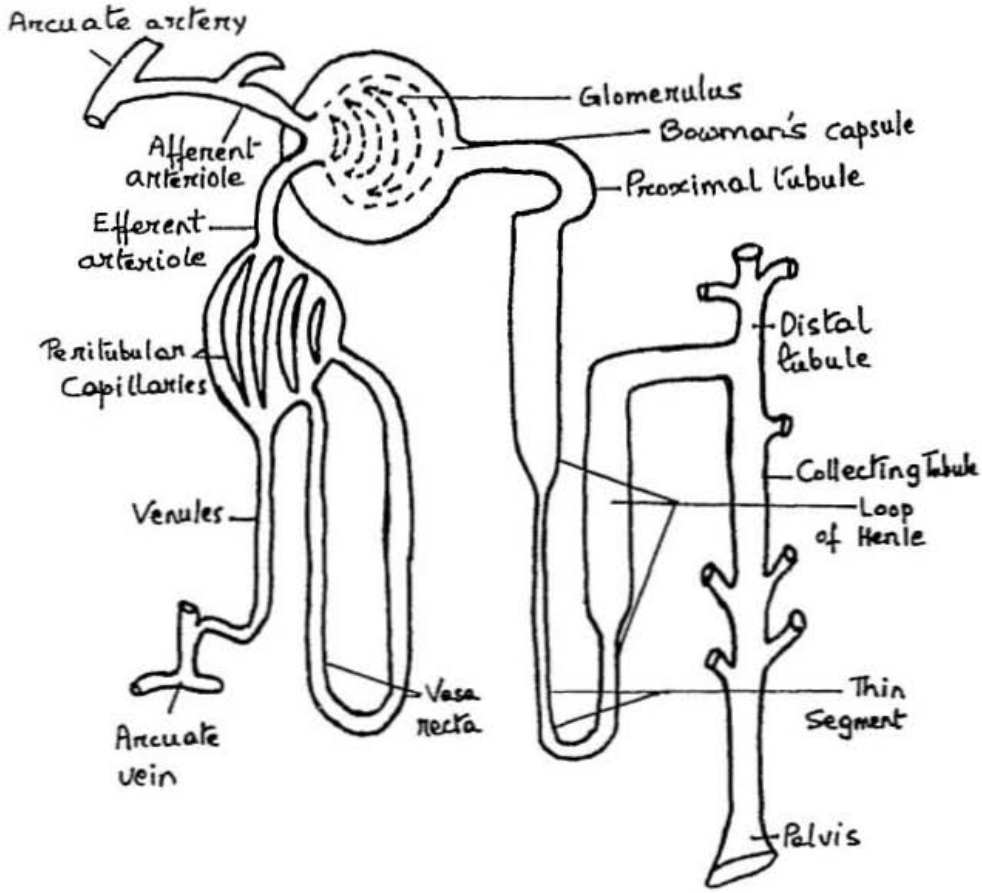
The two kidneys contain about 2,400,000 nephrons, each of which is capable of forming urine by itself. The nephron is composed of a) a glomerulus from which fluid is filtered, and b) a long tubule in which the filtered fluid is converted into urine on its way to the pelvis of the kidney. Blood enters the glomerulus through the afferent arteriole and leaves through the efferent arteriole. The glomerulus is a network of up to 50 parallel capillaries covered by epithelial cells and en-

cased in Bowman's capsule. Pressure of the blood in the glomerulus causes fluid to filter into the proximal tubule that lies in the cortex of the kidney along with the glomerulus. Fluid then passes through the loop of Henle and then to the distal tubule. Finally, the fluid flows to the collecting duct or tubule which collects fluid from several nephrons. As the glomerular filtrate flows through the tubules, most of its water and varying amounts of solutes are reabsorbed into the peritubular capillaries (Guyton,1976a). Figure 4.1 illustrates the structure of the nephron.

The function of kidney is to "clean" or "clear" plasma of unwanted substances as it passes through the kidney. The substances that must be cleared include the end-products of metabolism such as urea, creatinine, uric acid and urates. In addition, substances like sodium ions, potassium ions, chloride ions, phosphate ions and hydrogen ions tend to accumulate in the body in excess quantities; it is the function of the nephron to clear the plasma of the excesses. The glomerulus filters a large proportion of the plasma (filtration fraction) into the tubules, the unwanted substances fail to be reabsorbed while the wanted substances such as water and electrolytes are reabsorbed back into the plasma of the peritubular capillaries. The kidney also secretes unwanted substances through the epithelial cells lining the tubules directly into the tubular fluid. The urine that is formed is composed of

Figure 4.1

Structure of a nephron (redrawn from Guyton,1976a)


Arcuate artery
Glomerulus
Bowman's capsule
Afferent arteriole
Proximal tubule
Efferent arteriole
Distal tubule
Peritubular Capillaries
Collecting Tubule
Loop of Henle
Venules
Vasa recta
Thin Segment
Arcuate vein
Pelvis

filtered substances and secreted substances (Guyton,1976a).

The "renal clearance" is defined as the number of millilitres of plasma completely cleared of a substance in one minute. If the substance is freely filterable through the glomerular capillaries, if it is neither actively nor passively reabsorbed or secreted, if it is inert per se, exerts no effect on renal function and if it can be accurately quantified in the plasma and urine, then its clearance will be a valid measure of the rate of glomerular filtration. Good evidence exists that inulin (a fructose polysaccharide) exhibits these properties (details are described in chapter 2) and therefore "inulin clearance" is a measure of the volume of plasma filtered through the glomeruli. Similarly, renal clearance can be calculated for any other substance and related to the GFR. If the substance is reabsorbed, its renal clearance will be less than the GFR, whereas if it is secreted the renal clearance will be greater than the GFR (Pitts,1966a).

4.2 OBJECTIVES

Experiments in vivo were carried out to determine what percentage of the polyamines filtered from plasma are excreted. Clearance of polyamines was calculated to determine whether the polyamines are being reabsorbed or secreted in the renal tubules during the process of their excretion.

4.3 CALCULATIONS

The expressions for the calculations of the renal hemodynamic parameters have been described in chapter 2. The values of GFR and RBF were used to determine the following parameters:

a) renal polyamine flux across the kidneys
b) the filtered load and the excreted load (rate of polyamine excretion)
c) the renal clearance of polyamines

The "model calculations" are shown below:

Renal polyamine flux (nmoles/min.) in whole blood is given by,

RBF (ml/min.) x whole blood polyamine A-V (nmoles/ml)

Net removal of polyamines across the kidney can be determined by calculating the difference between the arterial concentration entering the kidney and the venous concentration leaving it (A-V) using a paired t-test. A qualitative picture may be obtained from an A-V difference measurement, but the product of the A-V and RBF gives a quantitative measure of the removal of the substance/substances under study (Dhanakoti, 1991).

Plasma is filtered and the cells are retained at the glomerulus. Therefore, plasma polyamines are those potentially available for filtration. However, it is important to consider

that there may be a redistribution of polyamines between the blood cells and plasma during their transit through the kidneys. The difference in concentration (A-V) of whole blood polyamines would indicate whether there is a removal.

Filtered load of polyamines (nmoles/min.) is given by,

GFR (ml/min.) x free arterial plasma polyamine concentration (nmoles/min.).

Excreted load of polyamines or the rate of polyamine excretion (nmoles/min.) is given by,

urine flow rate (ml/min.) x urinary polyamine concentration (nmoles/ml).

The urine flow rate is calculated from the 24 hour basal urine collection.

Renal clearance of a polyamine (C_p in ml/min.) is equal to the concentration of polyamine (nmoles/ml) in the urine times the urine volume (U_v) voided per min. (ml/min.) divided by the concentration of the polyamine in plasma (P_p in nmoles/ml). It is expressed as,

$$C_p = \frac{U_p \text{ (nmoles/ml)} \times U_v \text{ (ml/min.)}}{P_p \text{ (nmoles/ml)}}$$

4.4 RESULTS

Renal hemodynamic parameters in male and female rats are presented in Table 4.1. These values are in good agreement with similar studies in the literature (Brosnan and Hall,1989; Dhanakoti *et al.*,1990).

Whole blood and plasma polyamine concentrations in male and female rats are given in Table 4.2. While the plasma putrescine concentration is not significantly different in male and female animals, spermidine levels are significantly higher in female animals than in males. Plasma spermine levels are below the levels of detection of our method (<0.5 nmoles /1.0 ml). Whole blood putrescine and spermine levels are however, significantly higher in males than in females. Acetylated polyamines could not be detected using our HPLC method in plasma or whole blood.

The arteriovenous difference of the polyamines across the kidneys in female rats is presented in Table 4.3. The A-V difference of whole blood and plasma polyamines (spermidine and spermine) across the kidneys is not significantly different from zero ($p>0.05$; paired t-test). This indicates that the same amount of plasma polyamines going to the kidneys is returned to the blood stream. Our reproducibility is probably ± 5%, so at least a 10% difference in A-V is needed to observe a change. Therefore spermidine and spermine removal is definitely less than 10% of the delivered load. Also no

Table 4.1

Basic physiological data for male and female rats

Parameter	Male (n=7)	Female (n=4)
Urine flow rate (µl/min.)	4.7±2.7	4.3±1.7
U/P inulin	365.8±167.5	331.3±104.8
Glomerular filtration rate (ml/min./2 kidneys)	2.9±1.2	2.6±0.6
Renal plasma flow (ml/min./2 kidneys)	9.5±5.0	6.9±2.5
Renal blood flow (ml/min./2 kidneys)	17.3±9.2	12.7±4.7
Creatinine excretion (mg/24 hrs)	14.5±2.6[1]	11.4±0.6

Values are mean ± S.D.

[1] Value significantly higher in males than females ($p<0.05$).

Table 4.2

Polyamine concentrations in whole blood and plasma in male and female rats

	Whole blood		Plasma	
Polyamines	Male (n=3)	Female (n=3)	Male (n=7)	Female (n=4)
Putrescine	6.40±0.58[1]	2.50±0.87	0.99±0.18	1.26±0.40
Spermidine	31.92±3.33	25.98±2.84	5.27±1.40[1]	10.13±0.97
Spermine	8.06±0.90[1]	3.41±0.66	BLD	BLD

Values are mean ± S.D. All values are expressed in nmoles/ml.

[1] Male values significantly different from females ($p<0.05$).

Table 4.3

A-V differences for polyamines in whole blood and plasma in female rats

	Whole blood (n=3)			Plasma (n=4)		
Polyamines	Arterial	Venous	A-V	Arterial	Venous	A-V
Putrescine	2.50±0.87	1.69±0.76	0.81±0.13[1]	1.26±0.40	1.07±0.30	0.19±0.58
Spermidine	25.98±2.84	27.50±3.88	-1.50±6.65	10.13±0.97	9.81±1.23	0.32±1.41
Spermine	3.41±0.66	3.22±0.38	0.19±0.97	BLD	BLD	BLD

Values are mean ± S.D. All values are expressed as nmoles/ml.

[1] Significantly different from zero ($p<0.05$; paired t test).

Plasma spermine values are BLD.

spermidine and spermine are removed from the red cells as blood passes through the kidney (this is evident because there is no A-V difference in whole blood). To detect a change using our methods, we estimate that at least a 1.0 nmol/ml difference would be required, but the excretion is only 0.1 nmol/min.

The diamine, putrescine, presents a different picture. There is a significant removal of putrescine from whole blood as it passes through the kidneys (Table 4.3). The putrescine removed must be coming from the RBC, since no A-V difference is seen in plasma (Table 4.3), and the total putrescine removed (Table 4.3) is almost equal to the total plasma concentration in female rats (Table 4.2). The total flux of putrescine across the kidney for female rats is 10.32 ± 1.60 nmol/min. (Table 4.4).

The basal polyamine excretion data in male and female animals are presented in Table 4.5. The data are expressed as µmoles/24 hours. Male animals excrete slightly larger amounts of polyamines than females. It is relevant to mention that the male urine contains the secretions of the other glands like the prostate and Cowper's gland (the secretion of these glands and the spermatozoa together constitute the semen) and is known to be rich especially in spermine along with small amount of other polyamines (Leone et al.,1981). The low levels of spermine in this study make it unlikely that contamination

Table 4.4

Renal blood flow, whole blood A-V difference of putrescine across the kidney and the net renal flux of putrescine in female rats

Parameter	Female
RBF (ml/min./2 kidneys)	12.70±4.68 (4)
A-V (nmoles/ml)	0.81±0.13 (3)
Net renal flux (nmoles/min.)	10.32±1.60 (3)

Values are mean ± S.D. (n rats).

Table 4.5

Basal polyamine excretion in male and female rats

Polyamines	Male (n=7)	Female (n=4)
N-acetylputrescine	0.11±0.01	0.08±0.02
Putrescine	1.07±0.23	0.82±0.14
N^1-acetylspermidine	0.14±0.02	0.09±0.02
N^8-acetylspermidine	0.08±0.02	0.07±0.006
Spermidine	0.28±0.05	0.19±0.03
N^1-acetylspermine	0.09±0.02	0.07±0.01
Spermine	0.11±0.03	0.09±0.02

Values are means ± S.D. Values are expressed in µmoles/24 hours. Results for each rat were averaged over two-24 hour periods. Cadaverine was not detected in any of these urine samples.

with seminal fluid occurred. The urogenital system of the females is completely separated as compared to the males, thus making the female system more ideal for study. For this reason female animals were used for the latter part of the study.

The major "polyamine" excreted by both male and female rats was the diamine, putrescine. Spermidine excretion was only about one-quarter that of putrescine, although the plasma level of spermidine was much higher than putrescine. Small amounts of spermine and of the acetylated polyamines, N-acetylputrescine, N^1-acetylspermidine, N^8-acetylspermidine and N^1-acetylspermine also appeared in the urine. This general pattern of polyamine excretion was similar to that reported by Seiler et al (1985a) with the important exception of cadaverine. The rats in this study, on a polyamine free diet, did not show any cadaverine excretion, thus suggesting a dietary origin of the cadaverine reported by Seiler et al (1985a).

The filtered load and the excreted load of polyamines are presented in Table 4.6 assuming that about 92% of putrescine and 84% of spermidine are filterable. This is a predicted free concentration of putrescine and spermidine concluded from the results in chapter 3. It can be seen that only a small portion of the filtered polyamines is excreted. Table 4.7 shows the polyamine clearance with the free (predicted) in male and female rats. From Tables 4.6 and 4.7 it can be seen that i) the excreted load of putrescine and spermidine is very small

Table 4.6

Filtered load, excreted load and percent excretion in male and female rats

Polyamines	Filtered load (nmoles/min.)		Excreted load (nmoles/min.)		% excreted	
	Male[1]	Female[2]	Male	Female	Male	Female
Putrescine	2.6±0.4	3.9±0.9	0.7±0.1	0.6±0.1	29.2	14.6
Spermidine	12.9±3.4	21.2±3.1[3]	0.2±0.04	0.1±0.03	1.5	0.6

[1] n=7

[2] n=4

[3] Female values significantly higher than males ($p<0.05$).

Table 4.7

Renal clearance of putrescine and spermidine in male and female rats

	Plasma polyamine concentration (free) (nmoles/ml)		Renal polyamine clearance (ml/min.)		GFR (ml/min./2kidneys)
	Putrescine	Spermidine	Putrescine	Spermidine	
Male[1]	0.9±0.2	4.7±1.3	0.9±0.3	0.04±0.008	2.9±1.2
Female[2]	1.8±0.8	8.7±1.3	0.4±0.1	0.02±0.005	2.6±0.6

Values are mean ± S.D.

[1] n=7

[2] n=4

compared to the filtered loads, and ii) the clearance values of these polyamines are far lower than their GFR values. When clearance is lower than GFR, the substance is reabsorbed. The small A-V difference and the excreted load of polyamines are further evidences of reabsorption being the predominant fate of the filtered polyamines.

4.5 DISCUSSION

The purpose of the studies *in vivo* was to look into the basic mechanisms underlying the renal handling of polyamines during the process of their excretion. For this purpose the whole blood, plasma and the excreted polyamine concentrations were determined. The basic renal parameters were also determined.

4.5.1 Spermidine

The very low renal clearance of spermidine indicates that only a small proportion of the filtered spermidine reaching the kidney is being excreted under physiological conditions. Since the clearance is much lower than the GFR and the A-V difference is negligible (Table 4.5), it can be said that the predominant fate of the filtered spermidine is reabsorption.

Spermidine is the major circulating polyamine. However, plasma spermidine represents only 10% or less of the total blood spermidine as compared to 76% of the total circulating

spermidine found in the red cells (Lundgren and Oka,1978). In the present study, there is no evidence for movement of spermidine from the cells to the plasma as blood passes through the kidneys; this is evident from the A-V difference obtained from the whole blood and plasma (Table 4.3). Moulinoux et al (1984) have reported that the efflux of spermidine from red cells is very slow. Thus spermidine entering the glomerular filtrate should be derived only from the plasma compartment.

4.5.2 Putrescine

Unlike spermidine, putrescine is significantly removed from blood as it passes through the kidney. The source of putrescine is the blood cells since no A-V is found in plasma. In contrast to spermidine, putrescine has been shown to be readily transported out of red blood cells (Moulinoux et al., 1984). Our data do not allow us to determine whether this additional putrescine is also filtered at the glomerulus and reabsorbed into the kidney cells for further metabolism, or whether the kidney cells take up putrescine from the peritubular circulation. From the renal putrescine flux in females (Table 4.4), it can be calculated that the rate of putrescine removal would be much greater than the urinary excretion of putrescine. Therefore, putrescine entering the kidney must be converted to some other substance(s). It could be oxidized by DAO (E.C.1.4.3.6) (Quash et al.,1979) to give

aminobutyraldehyde, which could be further metabolized through the TCA (tricarboxylic acid cycle) cycle or could be excreted in the urine (Seiler and Eichentopf,1975). Aminoguanidine sulfate (AG), an inhibitor of DAO has been frequently used to inhibit oxidative deaminations of polyamines *in vivo* (Seiler *et al.*, 1985b). Administration of AG by i.p. injection in rats did increase the excretion of putrescine in the urine (Seiler *et al.*, 1985b), but Seiler did not determine the flux of putrescine across the kidneys of AG-treated animals. It could be acetylated by n-SAT, which uses acetyl CoA (Seiler and Al-Therib, 1974) to give N-acetylputrescine, which can be oxidized by monoamine oxidase (Seiler and Eichentopf, 1975), or it can be excreted (Seiler *et al.*, 1981). In our rats, urinary excretion of N-acetylputrescine was much less than the renal flux of putrescine, so this is unlikely to be the explanation. Extracellular putrescine could also serve as a precursor for spermidine or spermine synthesis in kidney cells (Seiler, 1987). Whatever the fate of putrescine, the kidney is removing significant amounts of putrescine, but not much of that putrescine is appearing in urine despite the fact that putrescine constitutes more than half of the "polyamines" in male and female rat urine.

4.5.3 N-acetylpolyamines

The N-acetylpolyamines, N-acetylputrescine, N^1 and N^8-

acetylspermidine and N^1-acetylspermine are present in small amounts in rat urine (Table 4.5 and Sarhan et al.,1991). There is no information available, however, on the source of the urinary acetylpolyamines. They could be filtered from the blood in the glomerulus, as are the polyamines, they could be synthesized in the tubular lumen by acetylation of filtered polyamines, or they could be synthesized by kidney cells and transported into the tubular lumen. If acetylated polyamines are present in plasma, their concentration is below the limits of the available assays (<0.5 nmol/ml plasma in our assay). If N^1-acetylspermidine were filtered and 90% of it reabsorbed, then the filtered load in the male rat would be about 1 nmol/min., and the plasma concentration would be about 0.3 nmol/ml (using data from Tables 4.1 and 4.5). Using similar calculations, it could be seen that concentrations of the other acetylated polyamines would be slightly lower than this. These values would be below the limits of detection with our method. Seiler (1987) has proposed that acetylated polyamines could be transported out of cells into the plasma. Thus it is quite conceivable that acetylated polyamines are handled by the kidney as are the polyamines themselves. It is very unlikely that acetylation of polyamines occurs in the tubular lumen since all of the known polyamine acetylases require acetyl CoA as acetyl group donor (Seiler,1987). Kidney does contain polyamine acetylases, so we cannot rule out the

possibility that acetylation occurs in some type(s) of kidney cells, which then transports the acetylated polyamines into the tubular lumen.

4.5.4 Polyamine excretion in disease

Radioactive polyamines added to the plasma of normal and cancer patients have been shown to be rapidly cleared from plasma (Russell and Durie,1978a). It has been tacitly assumed therefore that polyamines added to blood would be removed in the urine without any active intervention of the kidney. Thus it was proposed that increased urinary polyamine excretion would be a good marker for tumor growth and regression (Durie et al.,1977). The present study has shown that this is an oversimplification. Polyamine filtration should be a function of the polyamine concentration of plasma. If the rate of reabsorption is unchanged, the polyamine excretion should parallel polyamine concentration in plasma. However, any factor(s) which affect GFR or tubular reabsorption mechanisms could be expected to alter the renal excretion of polyamines. In addition, any aberrant binding of polyamines to macromolecules in plasma, affecting their filtration, would also alter urinary polyamine excretion. Thus it is not possible to draw conclusions about changes in polyamine excretion in the absence of information on plasma free polyamine concentration and renal function. Since some cancer therapies can cause

nephrotoxicity (Mercatello et al.,1991), and some cancers can cause tubular disfunction (De Fronzo and Their,1986), this is a very important point to remember.

Hyperpolyaminemia is also not unique to the malignant state. Non-malignant diseases including renal diseases like uremia are associated with increased plasma polyamines. Uremia is seen in total renal failure. In the absence of major disposal routes for polyamines (because of very little filtration), the extracellular levels are elevated (Campbell et al.,1978). Renal abnormalities (primary) may be due to inflammatory and degenerative diseases or due to defects in the transport mechanisms in the renal tubule; renal functions are also affected (secondary) due to other "non-renal" factor(s) like hormones, injuries or renal insult caused by nephrotoxic substances.

In glomerulonephritis (a diffuse inflammatory disease) there is a loss of glomerular functions and a degeneration of the conjoined tubules; there is proteinuria, hematuria (loss of red cells in urine), hypertension and edema in acute (sudden) and chronic (progressive loss of renal functions) states of the disease. Nephrotic syndrome is characterised by degenerative changes in the tubular epithelium, but the primary defect lies in the capillary basement membrane, therefore protein escapes into the glomerular filtrate. Anuria (complete stoppage of urine formation) results from acute

renal failure; the damage may be caused by injuries or due to prolonged hypotension, which may lead to renal ischaemia (Straffon,1973). A number of inborn errors (cystinuria, Fanconi syndrome, which may also be acquired) of transport which are renal in origin are responsible for defects in the excretion pattern of amino acids, glucose, phosphate etc. Excessive losses of polyamines in cystinuria (Bender,1985) and in Fanconi syndrome (Berry et al.,1978) have been reported.

If there is a renal malfunction in diseases which may be renal or non-renal in origin, it can affect prerenal homeostasis of calcium, or spermidine and many other substances. Polyamine excretion by a patient will be a function of a number of variables, including rate of release from tissues or tumors, oxidation, uptake by other tissues (Seiler et al., 1981), and renal filtration and reabsorption (this thesis).

CHAPTER 5

EFFECT OF A GENERALIZED DISRUPTION OF TUBULAR FUNCTIONS ON POLYAMINE EXCRETION

5.1 INTRODUCTION

In the discussion of the previous chapter, it was concluded that plasma polyamines, filtered at the glomerular membrane, are almost completely reabsorbed from the glomerular filtrate. This is similar to the situation for glucose, amino acids, and ions such as sodium and calcium (Pitts,1966d). For an understanding of the mechanisms of reabsorption, amino acid and glucose transport are discussed below. Polyamine transport is also discussed in this context.

5.1.1 Transcellular reabsorption mechanisms for amino acids, glucose and polyamines

For amino acids to be reabsorbed from the glomerular filtrate, they must be transported across the luminal or brush-border membrane of the proximal tubule (Silbernagl, 1985), and then across the basolateral membrane into the blood in the juxtaglomerular capillary bed. A number of transport systems have been recognised, each specific for one, more, and often several, structurally-related amino acids (Bergeron and Scriver,1985). The transporters that are best characterized are described in Table 5.1. Transport at the brush-border membrane is active, allowing the cell to concentrate amino acids, which can then passively move down their concentration gradient into the blood, crossing the basolateral membrane with the aid of specific carriers (facilitated diffusion)

Table 5.1

Renal amino acid transporters and their nature

Transporter	Amino acid
Na⁺-dependent	Zwitterionic amino acids
System Glycine	Glycine
System A	Most zwitterionic amino acids; tolerates N-methyl group
System ASC (protonated system accepts certain anionic amino acids)	Most amino acids, but not N-methyl substituted; but tolerates proline
ß-system	ß-alanine, GABA and taurine
Imino acids Low K_m system	Proline, hydroxyproline, L-alanine, L-phenylalanine and other N-substituted amino acids
High K_m system	Proline, hydroxyproline and other N-substituted amino acids like sarcosine (methylglycine) and N-methyl L-alanine
Na⁺-independent	
System L	Ubiquitous; preference for branched non-polar side-chain amino acids
Na⁺-independent	Cationic amino acids
System y^+	Ubiquitous
Na⁺-dependent	Anionic amino acids
System X^-_{AG}	Similar reactivity with aspartate and glutamate
System X^-_A	Aspartate and its analogues
System X^-_G	Glutamate and its analogues

Bender, 1985, Christensen, 1984 and Silbernagl, 1985.

(Silbernagl,1985). Most of the brush-border membrane transporters are sodium-dependent, in which the sodium gradient across the brush-border membrane is the major driving force behind the concentrative uptake of amino acids into the tubule cell. The sodium gradient is maintained by the Na^+/K^+ ATPase on the basolateral membrane which pumps sodium into the blood (Ullrich,1979).

D-glucose is also co-transported with Na^+ at the brush border membrane, combined with Na^+ in a transporter complex (Baeyer and Deetjen,1985). Thus glucose transport is also secondary active transport (Guyton,1976b). The glucose, once inside the tubular cell, diffuses down its concentration gradient into the plasma, with the assistance of a carrier in the basolateral membrane. Therefore, an important requirement of this co-transport is that the intracellular D-glucose concentration should be higher than the concentration in the peritubular space; this co-transport system is capable of building up an ample D-glucose concentration in the cytosol, which permits its transfer into the interstitial compartment (Baeyer and Deetjen,1985).

Most, if not all, mammalian cells contain a transport system for polyamines (Byers and Pegg,1989), which can be induced by depleting the cells of endogenous polyamines (Alhonen-Hongisto *et al.*,1980). Induction of transport is particularly impressive in the presence of various growth

stimuli (eg. fetal-calf serum, epidermal growth factor) (Byers and Pegg,1989). No information is available on the trans-cellular transport of polyamines, but polyamine transport into epithelial cells of small intestine (Kumagai et al.,1989) and in kidney (Parys et al.,1990) has been characterized. These cells might be expected to have a mechanism for transcellular polyamine transport for absorption of polyamines from the luminal side to blood, which might differ from the cell's uptake mechanism from blood for the cell's own requirements. In the case of intestine, isolated villous enterocytes transport putrescine by an active, Na^+-independent process against a concentration-gradient (Kumagai and Johnson,1988). Spermidine is transported on a different carrier, which is also Na^+-independent, but which apparently requires the activity of Na^+/K^+-ATPase (Kumagai et al.,1989). The experimental system used would not allow the authors to determine transcellular transport, but only uptake. DeSmedt and his collegues (1989) studied a renal epithelial cell line, LLC-PK_1, derived from proximal tubule of pig kidney. The cells grown to confluence on nitrocellulose filters showed polarised expression of some membrane proteins, so the authors could study uptake from the apical (luminal) or the basolateral side. In all experiments, however, the authors studied the cells after total depletion of intracellular putrescine and spermidine by DFMO treatment for several days followed by

addition of fetal-calf serum as a growth stimulus (Parys et al.,1990). These investigators identified two active transport systems for putrescine, a high-affinity Na^+-independent system which was exclusively on the basolateral side in the cultured cells and a low-affinity Na^+-independent system which showed no specific localization (van den Bosch et al.,1990). Putrescine was concentrated in the cells, but no attempt was made to study release in the opposite compartment, nor were transport of spermidine nor spermine studied. The authors suggest that the localization of transporters they reported "would favour transepithelial secretion of polyamines" (Van den Bosch et al.,1990).

5.1.2 Disorders of renal transport

Genetic disorders of transport of one or more substances are recognised. Some of these inborn errors of transport and their pathological effects are summarized in Table 5.2 (Bergeron and Scriver,1985). Diseases characterized by aminoacidurias (and also defects in the transport of other substances) are common inborn errors of transport. One such disease is cystinuria in which not only cystine but other basic amino acids like lysine, ornithine and arginine are also excreted in excess, and the concentrations of these amino acids in plasma are normal or lower than normal (Bender,1985). Basic amino acid transport seems to be defective both in the

Table 5.2

Inborn errors of transport and their effects

Inborn Errors	Effects
Mendelian disorders of D-glucose transport	Renal glucosuria type A and type B involves defects of D-glucose transport; a third one involves glucose-galactose malabsorption; predominantly an intestinal disorder.
Hartnup disorder	A brush border carrier for a large group of neutral amino acids is affected; transepithelial transport in kidney and intestine is impaired.
Cystinuria	Transport of amino acids cystine, lysine, ornithine and arginine is affected; a specific cystine-lysine-arginine-ornithinuria is identifiable.
Lysine-protein intolerance or dibasic aminoaciduriaII	Deficiency of dibasic amino acid transport only at the basal lateral membrane.
Renal hypertaurinuria	Characterized in certain strains of mice; decreased permeability at the basal lateral membrane associated with increased cellular taurine content.
Lowe's disease*	Characterized by a generalized aminoaciduria, glycosuria and proteinuria.
Fanconi syndrome	Generalized disruption of tubular transport activities; excessive excretion of amino acids, glucose and water.

* Berry et al., 1978.

Other disorders from Bergeron and Scriver, 1985.

intestinal mucosa and in the renal tubule. Interestingly, high urinary levels of putrescine, cadaverine and spermidine have been reported in these patients (Bender,1985). Hartnup disease is also characterized by a generalized aminoaciduria in which there is excessive excretion of tryptophan and other neutral amino acids. The defect appears to lie in the L-system in both the intestinal mucosa and the kidney and possibly in the carrier at the blood-brain barrier. The L-system is responsible for transporting large neutral amino acids (Bender,1985). Fanconi syndrome or Lignac-de Toni-Debre-Fanconi syndrome presents as a generalized disruption of tubular transport activities in which monosaccharides, amino acids, electrolytes, urate, protein and water are excreted in large excesses (Bergeron and Scriver,1985). Fanconi syndrome can be inherited or it can be acquired by heavy metal poisoning caused by inhalation or ingestion of cadmium, uranium, lead or mercury, or damage caused by chemical toxins like nitrobenzene, lysol, outdated tetracycline and salicylate (Bergeron and Scriver,1985). Since these insults could happen to the general population, they may be responsible for unsuspected reabsorptive problems. Nutritional deficiences like scurvy, rickets and pernicious anemia (Scriver and Rosenberg,1973a), as well as diseases like multiple myeloma (Engle and Wallis, 1957) and acute tubular necrosis (Smith et al.,1956) have also been described to be associated with

aminoaciduria (Scriver and Rosenberg,1973a). There has been an isolated report on an elevated polyamine excretion (putrescine and cadaverine) in patients with Fanconi syndrome (Berry et al.,1978). This observation agrees with our conclusion that polyamines are reabsorbed in the kidney (chapter 4), and in addition it points to the proximal tubules as the site of transport.

A transient Fanconi-like syndrome can be produced in animals by the administration of maleate, a *cis*-isomer of fumarate. Berlinger et al (1950) first showed that the administration of maleate in dogs produced a transient Fanconi-like syndrome. This procedure was used to study the effect of a generalized disruption of proximal tubular function on polyamine excretion. Blood and urine samples were taken from female animals treated with maleate and the effects of treatment on glomerular filtration rate (GFR) and renal plasma flow (RPF) were studied.

5.2 OBJECTIVES

To induce a defect in the proximal tubular cells which interferes with reabsorption in the proximal tubule.

5.3 RESULTS

The basic physiological parameters in control and maleate-treated rats are given in Table 5.3. Comparison of the

Table 5.3

Basic physiological data in saline and maleate-treated rats

Parameter	Saline (n=4)	Maleate (n=3)
U/P inulin	117.1±93.4	15.1±4.2[a]
Glomerular filtration rate (ml/min./2 kidneys)	1.6±0.9	0.9±0.4
Renal plasma flow (ml/min./2 kidneys)	4.4±3.1	4.0±1.3

Values are mean ± S.D.

[a] Significantly different from saline controls ($p<0.05$).

results in Table 5.3 show that the mean GFR 4 hours after maleate treatment was about 60% of that from control rats, although the difference was not statistically significant in this study. Günther et al (1979) have reported that the GFR values were reduced to a third of the control values in their study of maleate treatment of male rats. No change was noted in the renal plasma flow due to maleate treatment. Maleate produced diuresis and marked increase in the excretion of glucose as shown in Table 5.4. Glucosuria is a characteristic response of rats to maleate treatment (Harrison and Harrison,1954). The increased urine volume is required for the increased solute load (Rosenberg and Segal, 1964).

Plasma and urinary amino acid levels of control and maleate-treated rats are given in Table 5.5. Excretion of all amino acids except glutamine was increased in response to maleate injection. The generalized aminoaciduria indicates that amino acids representing the acidic, neutral and basic groups are affected (Wyss et al.,1992). The most marked absolute increases occurred in valine, isoleucine, glutamate and leucine, whereas some of the smallest changes occurred in those amino acids transported by the ß-system, taurine, ß-alanine and GABA. Rosenberg and Segal (1964) reported increased excretion of taurine, serine, threonine, glutamate, glycine, alanine, valine, lysine and histidine during the

Table 5.4

Urine volume and glucose excretion in saline and maleate-treated rats

	Urine vol.(ml/4 hrs)	Glucose (µmoles/hr)
Saline (n=4)	3.25±1.32	1.32±0.74
Maleate (n=6)	6.00±1.30	5.84±1.23

Values are mean ± S.D.

All parameters are significantly higher in maleate-treated animals than their saline controls ($p<0.05$).

Table 5.5

Plasma and urinary amino acid levels in saline and maleate-treated rats

AMINO ACIDS	Plasma (nmoles/ml) Saline (n=4)	Plasma (nmoles/ml) Maleate (n=4)	Urine (nmoles/hour) Saline (n=4)	Urine (nmoles/hour) Maleate (n=5)
TAU	226.5 ± 35.1	232.9 ± 35.7	1863.0 ± 659.0	3348.0 ± 286.0
ASP	15.5 ± 1.6	15.6 ± 1.9	45.9 ± 11.0	401.6 ± 89.8
HYP	17.9 ± 2.9	23.2 ± 7.1	12.8 ± 6.2	178.5 ± 54.1
THR	808.2 ± 217.4	702.2 ± 247.8	94.2 ± 16.1	1407.5 ± 407.3
SER	240.1 ± 34.7	258.3 ± 14.2	39.8 ± 16.2	619.7 ± 145.8
ASN	72.8 ± 14.1	66.9 ± 17.7	27.6 ± 8.9	190.7 ± 54.8
GLU	106.6 ± 4.7	120.2 ± 39.9	33.0 ± 16.3	2378.3 ± 485.3
GLN	593.4 ± 68.2	663.9 ± 64.1	107.0 ± 50.8	151.1 ± 66.2[1]
PRO	399.1 ± 36.2	323.1 ± 161.0	29.4 ± 9.5	504.9 ± 120.3
GLY	61.3 ± 8.6	71.4 ± 10.1	91.6 ± 21.6	1986.8 ± 521.7
ALA	930.2 ± 88.8	812.9 ± 308.2	75.5 ± 18.9	1075.0 ± 392.5
CIT	86.1 ± 10.2	94.4 ± 5.2	2.3 ± 0.9	59.2 ± 7.0
VAL	333.4 ± 42.0	283.9 ± 40.3	6.0 ± 1.2	648.0 ± 102.4
CYS	8.7 ± 2.8	7.9 ± 1.4	9.8 ± 2.4	65.9 ± 9.6
MET	76.7 ± 5.6	82.2 ± 15.3	32.5 ± 13.3	283.6 ± 95.3
ILE	140.4 ± 22.2	136.9 ± 10.1	3.1 ± 1.5	279.2 ± 50.6
LEU	225.3 ± 32.4	226.5 ± 18.6	9.8 ± 3.7	402.0 ± 83.8
TYR	72.7 ± 6.1	87.0 ± 18.9	9.1 ± 3.3	155.2 ± 30.6
PHE	80.9 ± 5.7	91.5 ± 9.4	25.3 ± 6.1	162.7 ± 41.7
TRP	107.8 ± 9.3	86.0 ± 10.5[1]	3.2 ± 1.0	21.8 ± 5.0
ORN	39.4 ± 7.2	38.1 ± 7.9	5.4 ± 4.7	14.6 ± 2.1
LYS	901.0 ± 138.3	647.3 ± 98.5[1]	63.5 ± 20.8	1199.4 ± 154.5
HIS	81.8 ± 5.8	85.8 ± 10.6	25.9 ± 11.5	236.5 ± 36.0
ARG	135.0 ± 22.8	140.2 ± 19.3	13.8 ± 7.7	57.8 ± 17.7
ß-ALA	<1.5	<1.5	26.6 ± 14.2	48.9 ± 14.6
γABA	<0.3	<0.3	9.3 ± 3.6	27.3 ± 13.1
1-METHIS	5.3 ± 2.0	11.0 ± 4.5	19.4 ± 13.7	106.3 ± 34.8
3-METHIS	<0.5	<0.5	9.8 ± 3.9	47.3 ± 14.8
HYLYS	<0.3	<0.3	6.1 ± 2.3	9.9 ± 3.1

All values are mean ± S.D.

[1] Significantly different in maleate-treated rats from saline-treated controls ($p<0.05$).

[2] Excretion of glutamine in maleate-treated rats is not significantly different from saline controls ($p>0.05$). All other values are significantly higher in maleate-treated rats ($p<0.05$).

first 12 hours after maleate injection in rats. Günther et al (1979) also reported the increased excretion of the four amino acids they studied, namely glycine, alanine, aspartate and taurine. From Table 5.5, it can be seen that the concentrations of most of the amino acids in plasma of maleate-treated rats were not significantly different from saline controls. The exceptions were the essential amino acids, tryptophan and lysine whose concentrations decreased. The plasma levels of GABA, ß-alanine, hydroxyproline and 3-methylhistidine were below the limits of detection of our assay in the presence or absence of maleate. Table 5.6 shows the filtered and excreted loads of amino acids in maleate and saline-treated rats. The filtered load of all amino acids with the exception of 1-methylhistidine was decreased significantly. The percentage reabsorption of all amino acids except glutamine was decreased due to maleate treatment. The most dramatic decreases occurred in taurine, aspartate, hydroxyproline, glutamate, glycine, cystine and 1-methylhistidine. This lowering of percent reabsorption in taurine, glycine and aspartate in our studies is comparable to decreases reported by Günther et al (1979) for these amino acids.

Table 5.7 shows the polyamine excretion pattern in saline and maleate treated rats. Four hours of maleate treatment caused a mixed effect on polyamine excretion. There was a

Table 5.6

Filtered load, excreted load and percentage reabsorption of amino acids and polyamines in saline and maleate-treated rats

AMINO ACIDS	Filtered load (nmoles/min.)		Excreted load (nmoles/min.)		% reabsorption	
	Saline	Maleate	Saline	Maleate	Saline	Maleate
TAU	362.4	209.6	31.1	55.8	91.4	73.4
ASP	24.8	14.0	0.7	6.7	97.2	52.1
HYP	28.6	20.9	0.2	3.0	99.3	85.6
THR	1293.1	631.9	1.6	23.4	99.9	96.3
SER	384.1	232.5	0.6	10.3	99.9	95.6
ASN	116.5	60.2	0.4	3.2	99.7	97.0
GLU	170.5	106.2	0.5	39.6	99.7	62.7
GLN	949.4	597.5	1.8	2.5	99.8	99.6
PRO	638.5	290.8	0.5	8.4	99.9	97.1
GLY	98.1	64.2	1.5	33.1	98.5	48.5
ALA	1408.0	731.6	1.2	17.9	99.9	97.6
CIT	137.7	85.0	0.04	0.9	99.97	98.9
VAL	533.4	255.5	0.1	10.8	99.98	95.8
CYS	13.9	7.1	0.1	1.1	99.3	84.5
MET	122.7	74.0	0.5	4.7	99.96	93.7
ILE	224.7	123.2	0.05	4.6	99.98	96.3
LEU	360.5	203.9	0.1	6.7	99.97	96.7
TYR	116.3	78.3	0.1	2.6	99.9	96.7
PHE	129.4	82.4	0.4	2.7	99.7	96.7
TRP	172.5	77.4	0.05	0.3	99.97	99.6
ORN	63.0	34.3	0.09	0.2	99.9	99.4
LYS	1441.6	582.6	1.1	20.0	99.9	96.6
HIS	130.9	77.2	0.4	3.9	99.7	95.0
ARG	216.0	126.2	0.2	0.9	99.9	99.3
1-METHIS	8.5	9.9[1]	0.3	1.8	96.5	81.8
POLYAMINES						
PUTRESCINE	2.0	1.0[1]	0.8	0.6	60.8	35.3
SPERMIDINE	13.4	3.9	0.2	0.3	98.1	92.0

Data for amino acids were calculated from Tables 5.3 and 5.5, and data for polyamines were calculated from Tables 5.3 and 5.7 respectively.

[1] Values are not significantly different from controls ($p>0.05$); all other values are significantly different in maleate-treated rats from the saline controls ($p<0.05$) and the mean and S.D. were calculated from individual filtered load values, ($n=4$, for saline and maleate-treated rats for amino acids, for polyamines $n=7$ for saline controls and maleate-treated rats).

Table 5.7

Plasma and urinary polyamine levels in saline and maleate-treated rats

Polyamines	Plasma (nmoles/ml) Saline (n=7)	Plasma (nmoles/ml) Maleate (n=7)	Urine (nmoles/hour) Saline (n=5)	Urine (nmoles/hour) Maleate (n=5)
Putrescine	1.2 ± 0.5	1.1 ± 0.9	46.4 ± 11.8	39.2 ± 11.1
Spermidine	8.4 ± 1.9	4.3 ± 1.0[a]	14.5 ± 5.2	18.5 ± 8.4
Spermine	BLD	BLD	5.7 ± 1.8	0.9 ± 0.6[a,1]
N-acetylputrescine	BLD	BLD	4.3 ± 1.3	10.6 ± 5.1[a]
N^1-acetylspermidine	BLD	BLD	4.9 ± 1.1	5.7 ± 1.3
N^8-acetylspermidine	BLD	BLD	3.3 ± 0.7	3.0 ± 0.4
N^1-acetylspermine	BLD	BLD	3.3 ± 0.9	0.3 ± 0.4[a,1]

Values are mean ± S.D.

[a] Values are significantly different from controls ($p<0.05$).

[1] In some urines N^1-acetylspermine and/or spermine were BLD (< 0.5 nmoles/ml). For these samples, zero was used for the calculation.

significant decrease in spermine excretion, while putrescine and spermidine excretion were similar to controls. Among the acetyl derivatives, the excretion of N-acetylputrescine increased significantly, while N^1-acetylspermine decreased significantly. The levels of N^1 and N^8-acetylspermidines in urine were unchanged due to treatment. Plasma and whole blood polyamines are presented in Tables 5.7 and Table 5.8 respectively. It is interesting to note that plasma putrescine remains unchanged due to maleate treatment, but there was a significant decrease in plasma spermidine level. Despite this decrease, blood spermidine level remained unchanged, indicating again the sluggishness of spermidine efflux from red cells. The net plasma polyamine delivery to the kidney (RPF X plasma concentration) was significantly decreased for spermidine due to maleate treatment, but putrescine delivery remained almost unchanged after maleate treatment.

The calculated filtered loads for putrescine and spermidine are given in Table 5.6. There is a significant decrease in the filtered load of spermidine due to the decrease in GFR and plasma level of spermidine. When polyamine excretion is compared to the filtered load (Table 5.6), it can be seen that a significant decrease in the percentage of putrescine and spermidine reabsorption occurred due to maleate treatment with respect to the controls. Spermidine reabsorption dropped from 98 to 92% in this study and putrescine

Table 5.8

Whole blood polyamines in saline and maleate-treated rats

Polyamines	Saline	Maleate
Putrescine	2.57±0.72	2.19±0.60
Spermidine	28.09±4.82	26.98±2.68
Spermine	3.31±0.58	2.72±0.44

Values are expressed in nmoles/ml and are mean ± S.D.(n=4). None of the values in maleate-treated animals are significantly different from saline-treated controls ($p>0.05$).

reabsorption dropped from 60% to 35%. These changes in reabsorption are of similar magnitude to those seen for methionine and taurine respectively in our study. Saline treatment caused a slight increase in the excretion of polyamines (not significant; $p>0.05$) when compared to the untreated female rats (chapter 4).

5.4 DISCUSSION

Maleate causes a disruption of proximal tubule functions, as shown by glucosuria and aminoaciduria, but the exact mechanism of the disturbance is still not understood. Harrison and Harrison (1954) and Rosenberg and Segal (1964) have reported that urinary losses of glucose, phosphate and amino acids are not caused by increases in the plasma levels of these solutes. Our results indicate that the plasma levels of most amino acids are tightly regulated and are prevented from falling significantly even when the urinary losses are high due to maleate treatment. Lysine and tryptophan, two of the dietary essential amino acids, seem to be an exception because their plasma levels are not prevented from falling. This decrease may be due to a depletion of total pools in response to an accelerated loss (Wyss et al.,1992). Despite their lower plasma levels, their excretion was still increased by maleate. Reabsorption thus plays a major role in conserving these amino acids to prevent their loss from the body.

From our results it can be seen that there is a disruption in both the Na^+-dependent and the Na^+-independent transport systems. For some amino acids, for example, glutamic acid (system X^-_G) excretion was increased dramatically by seventy two-fold, valine (system L) by one hundred and eight-fold and lysine (system y^+) by almost nineteen-fold (Table 5.5). The most striking decreases in percent reabsorption occurred in amino acids like glycine, aspartate, taurine, hydroxyproline, cystine and 1-methylhistidine (Table 5.6). However, even a three to four percent decrease in reabsorption caused a very high urinary excretion of some amino acids, as in the case of valine whose excretion was markedly increased, when there was only a four percent decrease in the reabsorption.

Therefore, in maleate-induced Fanconi syndrome, other components of the transport mechanism are thought to be affected rather than the carriers per se. By microperfusing kidneys with amino acids with related structure, it was shown by Bergeron et al (1976) that the carrier sites appeared to be intact in maleate-treated kidneys, because competition still existed on the luminal side of the membrane. Robson and Rose (1957) showed that arginine reabsorption is further impaired in the Fanconi syndrome when the concentration of lysine, a competitive inhibitor of arginine transport, is increased in glomerular filtrate. It was shown by Scriver et al (1964) that

glycine transport is inhibited normally by proline in patients with the syndrome, because glycine is also transported by a specific imino-glycine carrier, responsible for carrying proline and hydroxyproline (Bender,1985). Rosenberg and Segal (1964) have also shown that maleic acid inhibited amino acid transport non-competitively, therefore indicating that the affinity of the amino acids for their carriers was not altered by the inhibitor. It is generally believed that the transfer process rather that the substrate binding processes is compromised (Bergeron and Scriver,1985).

In Fanconi syndrome tubular reabsorption may be impaired due to an interference with the ion-dependent or energy-requiring steps in the transport processes. Aminoaciduria (generalized) is accompanied by excessive losses of other substances that are also reabsorbed (Scriver and Rosenberg, 1973b). Incubation of tubule suspensions with cystine dimethyl ester (for cellular cystine loading) causes an inhibition of transport in the proximal convoluted tubules; in this *in vitro* model of Fanconi syndrome there is inhibition of glucose and bicarbonate transport and water reabsorption due to a decrease in the intracellular ATP concentration. Addition of exogenous ATP to the cystine-loaded tubules caused a return of the cellular ATP levels to normal and also attenuated the inhibition of water absorption (Coor *et al.*,1991). Inhibition by other substances like salicylate (Segal and Blair,1963)

suggest that they may limit transport by interference with energy-production; however, they do not give any information about the specific mechanisms of such an interference (Rosenberg and Segal,1964).

Glutamine excretion was not increased after maleate treatment. The possible reason is likely to be the activation of the phosphate-independent glutaminase (PIG). This enzyme has been shown to be an artificial activity of γ-glutamyl-transpeptidase, localized in the straight proximal tubule. Maleate activates the glutaminase activity of this protein, but blocks the γ-glutamyltranspeptidase activity (Curthoys and Kuhlenschmidt,1975). Maleate treatment produces a decrease in the reabsorption of amino acids, and we hypothesize that this is true of glutamine. As the glutamine in the tubular fluid enters the proximal straight tubule without being reabsorbed, it is converted to glutamate by PIG. The location of PIG on the external surface of the brush-border membrane of the proximal tubular cells (Curthoys and Kuhlenschmidt,1975) would ensure that glutamine in the tubular fluid in the proximal straight tubule would be available for conversion to glutamate in the presence of maleate. This would account for the great increase (seventy two-fold increase, Table 5.5) in glutamate excretion seen in maleate-treated rats.

It is worthwhile to note that the excretion of 3-methylhistidine is increased by five-fold (Table 5.5).

Excretion of 3-methylhistidine has been used to quantitate muscle protein breakdown (Wei et al.,1991) on the assumption that its excretion is quantitative (Young et al.,1972). The increased urinary excretion of this compound after maleate is more likely to be associated with the interference with the renal transport mechanisms. Recently Hoffer (1990) has reported that 3-methylhistidine clearance in humans is less than creatinine clearance, indicating that tubular reabsorption also occurs in that species. Thus it is important to consider changes in kidney functions when comparing 3-methylhistidine excretion in different conditions.

In chapter 4, it was concluded from the findings that 99% of the filtered spermidine and 86% of the filtered putrescine in female rats were reabsorbed. If this reabsorption occurred in the proximal tubule by an energy-dependent mechanism, it would be probable that maleate treatment would cause a disruption of polyamine reabsorption. This is what we observed (Table 5.6). Clinical evidence of disruption in polyamine reabsorption has been observed in two cases of inborn errors of transport. In patients with cystinuria, putrescine, cadaverine and spermidine are excreted in large excess (Bender,1985). In patients with Fanconi syndrome, putrescine and cadaverine have been reported to be excreted in large excesses (Berry et al.,1978). Thus it appears that the reabsorption of putrescine and spermidine occurs in the

proximal tubule cell, and that it is subject to disruptions as are the transport processes for amino acids, glucose etc. It could even be predicted that specific inborn errors of polyamine transport could exist, although none have been reported to date. There has been one report of the presence of a polyamine transporter on the apical membrane of the proximal tubule cell (van den Bosch et al.,1990). These authors proposed that their transporter could be involved in polyamine secretion to rid the body of the polyamines it could not oxidize, but we have no evidence for polyamine secretion in our present study. It is thus possible that this Na^+-independent putrescine transporter is involved in putrescine reabsorption in the kidney. The Na^+-dependent transporter, on the basolateral membrane, reported by the same authors, might be involved in the removal of putrescine from blood as it crosses the kidney. We did observe a positive A-V for putrescine in whole blood as it passed through the kidney, although we do not have any information on its fate.

It is interesting that decreased reabsorption of spermidine was associated with a fall in the plasma levels of spermidine. It appears that the plasma concentration of spermidine is not tightly regulated at low concentrations. It is not known what the T_m for spermidine transport in kidney is, so we cannot predict at what concentration spermidine excretion would markedly increase. Increased spermidine

concentration in cells also induces the spermidine N^1-acetyl-transferase (Pegg and Erwin,1985) and this increases the capacity of cells for spermidine oxidation. Thus increases in plasma spermidine concentration should be better defended against than decreases appear to be.

CHAPTER 6

SUMMARY AND CONCLUSIONS

SUMMARY

It is important to understand what factors maintain polyamine concentrations in circulation. Like any other substance(s), polyamine input into circulation will be from the gastrointestinal tract, release from the cells and reabsorption from the kidney. Regulation of the polyamine levels in blood will be achieved by polyamine oxidation, cellular uptake, and urinary excretion.

In our studies we have shown that plasma polyamine clearance is much lower than GFR. This is indicative of reabsorption of polyamines in the kidney tubules. There is no significant A-V difference of polyamines across the kidneys. This indicates that not only are the polyamines reabsorbed by the kidneys under conditions of intact reabsorptive functions in the renal tubules, but reabsorption also contributes in regulating plasma polyamine concentrations.

From the results presented in this thesis it may be said that i) reabsorption of spermidine by the kidney and its return to the renal venous circulation is a major contributing factor in the regulation of plasma spermidine concentration, and ii) the regulation of spermidine level in the plasma at the lower concentrations does not appear to be strict. If there was a tight regulation then any interference with the reabsorptive mechanisms of the kidney would trigger its replenishment through other sources; in that case the plasma

spermidine level would not drop below normal. Plasma putrescine level on the other hand appears to be tightly regulated because a significant increase in its percentage excretion in maleate treated rats does not cause an alteration in the plasma levels. There may be replenishment of putrescine from other sources or decreased oxidation of putrescine when there is an interference in putrescine reabsorption.

From our studies it is evident that the kidney plays a major role in handling polyamines. The plasma clearance of polyamines is lower than the GFR, which indicates reabsorption. When there is interference with reabsorption, there is an alteration in the pre-renal homeostasis. Therefore urinary polyamines cannot be used as biochemical markers of neoplasia in the absence of information on their renal handling.

CONCLUSIONS

The investigations conducted in the present study reveal the following major novel findings:

1. Approximately 8% of plasma putrescine and 16% of plasma spermidine is bound non-covalently to non-diffusible macromolecules.
2. Spermidine binding to albumin is only about 2%, thus it is likely that albumin is responsible for a small proportion of polyamine binding in plasma.
3. The very low renal clearance for spermidine indicates that only a small proportion of the filtered spermidine reaching the kidney is being excreted under physiological conditions. Since the clearance is much lower than the GFR and the A-V difference is negligible, the predominant fate of the filtered spermidine is reabsorption.
4. Only 14% (female) to 29% (male) of the putrescine which is filtered at the glomerulus is excreted in the urine. Much more putrescine, however is removed from the red blood cells as they pass through the kidney. The metabolic fate of this putrescine is not known.
5. Maleate treatment, which disrupts proximal tubule functions, produced polyuria, glucosuria, and amino-aciduria. Fractional reabsorption of most amino acids and of putrescine and spermidine was decreased. Thus it is probable that filtered polyamines are reabsorbed in the

proximal tubule, as are amino acids and glucose.

6. Decreased reabsorption of spermidine was associated with a fall in the plasma concentration of spermidine. It appears that the plasma concentration of spermidine is not tightly regulated at low concentrations. Our studies have also shown that most of the plasma amino acid concentrations are tightly regulated and are not altered due to maleate treatment.
7. It is impossible to draw meaningful comparisons of renal polyamine excretion in different physiological or pathological conditions without information on renal function.

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