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Review

Analysis of polyamines as markers of (patho)physiological conditions

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Abstract

The aliphatic polyamines, putrescine, spermidine and spermine, are normal cell constituents that play important roles in cell proliferation and differentiation. The equilibrium between cellular uptake and release and the balanced activities of biosynthetic and catabolic enzymes of polyamines are essential for normal homeostasis in the proliferation and functions of cells and tissues. However, the intracellular polyamine content increases in hyperplastic or neoplastic growth. Although the involvement of polyamines in physiological and pathological cell proliferation and differentiation has been well established, the role they play is quite different in relation to cell systems and animal models and is dependent on inducer agents and stimuli. Also, the experimental procedures used to deplete polyamines have been shown to influence the cell responses. In this paper, the assay methods currently in use for polyamines are reviewed and compared with respect to sensitivity, reproducibility and applicability to routine analysis. The relevance of polyamine metabolism and the uptake/release process in many physiological and pathological processes is highlighted, and the cellular polyamine pathways are discussed in relation to the possible diagnostic and therapeutic significance of these mediators.
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Keywords: Reviews; Pathophysiological conditions; Polyamines

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1. Introduction

The polyamines, putrescine, spermidine and spermine, widespread in all organisms, have been shown to play a significant role in the regulation of cell growth and differentiation [1–4]. At optimal and physiological concentrations they regulate a variety of cell activities involving DNA replication, gene expression, protein synthesis and cell surface receptor functions [4–7]. But in many pathological conditions polyamine concentrations may increase drastically by different mechanisms involving the activation of biosynthetic pathways, decreased release from the cells or uptake from the extracellular environment.

High polyamine levels are toxic to cells and facilitate cell death mainly by oxidative mechanisms [8,9]. The possible role of polyamines as clinical biochemical markers for malignancy was first suggested in a report indicating that polyamines are present in increased amounts in the urine of cancer patients [10]. Since then, many papers and reviews on the relationship between polyamines and cancer and the clinical utility of polyamine determination and the inhibition of their synthesis have been published [2,11–25]. Recently, Sugimoto et al. [26] and Hiramatsu et al. [27] demonstrated the common occurrence in neoplasia of urinary diacetylated polyamines N^1,N^{12}-diacetylspermine and N^1,N^8-diacetylspermidine, pointing out their relevance as tumor markers.

For a long time, the monitoring of polyamines and their conjugates and metabolites in biological fluids has been used for estimating the extent of tumor-cell death induced by chemotherapy or radiotherapy in cancer patients [28] and to obtain useful information about the kinetics of therapeutically-induced recruitment of cells moving from the non-proliferating to the proliferating cell compartment [28,29].

Polyamine concentrations have been employed as biochemical markers of tumor kinetics and related to rapid cell growth, spontaneous cell death, or both [29].

Elevated levels of polyamines have been found in active proliferating cells and in pathological conditions other than cancer, such as infections [30,31], psoriasis [32–35], polycythemia rubra vera [36], systemic lupus erythematous [37–41], uremia [42,43], chronic nephritis [44], liver cirrhosis [45–49], cystic fibrosis [50–54], insulin-dependent diabetes mellitus [55–59], muscular dystrophy [60–63] and Alzheimer's disease [64–66].

Widespread interest in the polyamines has led over the years to the development of several assay methods, which in time have become more rapid and sensitive. They include thin-layer chromatography (TLC) [67–72], gas chromatography (GC) [73–94], high-performance liquid chromatography (HPLC) [95–136], gas chromatography with mass spectrometry (GC–MS) [137–150], electrophoretic techniques [151–163], radioimmunoassay (RIA) [164–166] and enzyme-linked immunosorbent assay (ELISA) [167–170].

The availability of sensitive and reproducible techniques has allowed the identification and measurement of polyamines in a wide range of biological

specimens. The detection of these mediators in urine [68–70, 78, 79, 81, 83, 84, 87, 88, 91, 92, 103, 111, 115, 120,124,126,139,143,145,47], plasma [82,85,86, 96,126,138,140] seminal fluid [122], cerebrospinal fluid [108,109], bile [171], colostrum [172], prostate tissue [128], brain tissue [144,173,174], hair [150], and saliva [136] has provided useful information about the pathogenesis of several diseases and contributed to clarify the cellular pathways involved in organ and systemic pathological conditions.

The aim of the present review is to update the reader about the advances and improvements made in the last 20 years in the detection of polyamines and the clinical usefulness of their determination.

2. Polyamine metabolism

2.1. Chemical structures

The characterization of the chemical structure of polyamines has shown that putrescine and cadaverine are primary diamines (1,4-diaminobutane and 1,5-diaminopentane, respectively), spermidine is a triamine (mono-*N*-3-aminopropyl-1,4-diaminobutane or 1,8-diamino-4-azaoctane) and spermine is a tetramine (bis-*N*-3-aminopropyl-1,4-diaminobutane or 1,12-diamino-4,9-diazadodecane): all contain primary or secondary amino groups (Table 1).

The designation of these compounds as ''polyamines'' is evidently not quite appropriate, but the better denomination as ''oligoamines'' has not achieved wide acceptance, probably because the term polyamine is brief and able to define a class of biological, basic, low-molecular-mass aliphatic non-protein nitrogenous substances.

Table 1
Structures of biogenic polyamines

No.	Name	Structure
1	Putrescine	$H_2N(CH_2)_4NH_2$
2	Cadaverine	$H_2N(CH_2)_5NH_2$
3	Spermidine	$H_2N(CH_2)_3NH(CH_2)_4NH_2$
4	Spermine	$H_2N(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$

2.2. Biosynthesis

Polyamines are synthesized in the body by de novo synthesis, by retroconversion or by gastrointestinal flora able to metabolize dietary amino acids. Cheese, beer, sauerkraut, yeast extracts, fermented soy sauce, potatoes and canned and frozen vegetables are particularly rich in polyamines and contribute to the whole body polyamine content.

2.2.1. De novo synthesis

The main precursor of polyamines is the amino acid ornithine, which is synthesized mainly in mitochondria from glutamate through the acetylation of the amino group, phosphorylation and reduction of the acetylated derivative to the *N*-acetylglutamic-γ-semialdehyde. A subsequent transamination produces α-*N*-acetylornithine that, after release of the acetyl group, forms ornithine and regenerated *N*-acetylglutamate. In liver mitochondria, ornithine may enter the urea cycle and react with carbamyl phosphate to form citrulline, as follows:

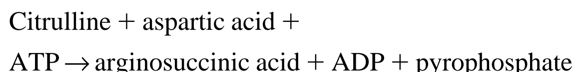
$$\text{Ornithine} + NH_2CO{-}OPO_3H_2 \rightarrow H_3PO_4 + \text{citrulline}$$

Carbamyl phosphate can be synthesized through three different reactions, but the following is the most involved in the biosynthesis of polyamines:

$$NH_3 + CO_2 + \text{ATP} \rightarrow NH_2CO{-}OPO_3H_2 + \text{ADP}$$

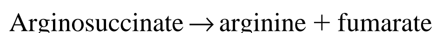
This reaction occurs in the mitochondria and is regulated by the enzyme carbamyl phosphate synthase, designated as CPS-I.

Newly formed citrulline is transferred to the cytosol where it is converted to the amino acid arginine in two steps. The first requires ATP-dependent condensation with aspartate to form arginosuccinic acid:



$$\text{Citrulline} + \text{aspartic acid} + \text{ATP} \rightarrow \text{arginosuccinic acid} + \text{ADP} + \text{pyrophosphate}$$

The second, regulated by arginine succinate lyase, leads to the formation of arginine and fumarate:



$$\text{Arginosuccinate} \rightarrow \text{arginine} + \text{fumarate}$$

Fumarate is a component of the citric acid cycle

and is converted to malate and then to oxaloacetate, which in turn is transaminated to form aspartate, which provides the nitrogen for the conversion of the ureido-containing citrulline to the guanidine-containing arginine. Arginine is successively degraded by arginase to form urea and ornithine.

Cytosolic ornithine may be degraded to putrescine via ornithine decarboxylase (ODC) or may be converted to proline via glutamic γ-semialdehyde. Unmetabolized cytosolic ornithine may also return to the mitochondrial compartment.

ODC appears to have the most rapid rate of synthesis and degradation among mammalian enzymes [175] and to be a multifunctional protein [176]. Its activity is regulated by complex mechanisms involving a variety of post translational modifications [177] and its own product, putrescine, acts either by feedback inhibition or by inducing the synthesis of an antizyme, which, combining stoichiometrically with the enzyme, blocks its activity [178]. A regulatory factor, the ''antizyme inhibitor'', specifically inhibits antizyme and reactivates ODC [178].

Putrescine is converted to spermidine by attachment of an aminopropyl moiety catalyzed by spermidine synthase. A second propylamine residue is added through spermine synthase to the primary nitrogen N8 of the diaminobutane-part of spermidine producing spermine. This biosynthetic pathway involves *S*-adenosylmethionine (SAM) as a donor of two propylamine residues. SAM is formed from methionine by SAM synthetase. SAM decarboxylase (SAMDC) cleaves the SAM carboxyl residue producing decarboxylated SAM, which retains the methyl group usually involved in SAM methyltransferase activity. Mammalian SAMDC is differently regulated by its products and is allosterically stimulated by putrescine or spermidine, but not by spermine. In fact, spermine inhibits the enhancement exerted by putrescine and does not require Mg^{2+} [179].

2.2.2. Retroconversion

Polyamines may be synthesized by retroconversion [180] of spermidine to putrescine and spermine to spermidine through the initial N^1-acetylation of spermidine and spermine to N^1-acetylspermidine and N^1-acetylspermine, respectively. The latter can be converted to N^1,N^{12}-diacetylspermine. These reactions are catalysed by the cytosolic spermidine/spermine N^1-acetyltransferase, using acetyl-CoA as co-substrate. Polyamine oxidase (PAO) releases acetamidopropyl moieties from N^1-acetylspermidine, N^1-acetylspermine and N^1,N^{12}-diacetylspermine to yield putrescine, spermidine and N^1-acetylspermidine, respectively. Nuclear acetyltransferase converts putrescine to *N*-acetylputrescine and spermidine to N^8-acetylspermidine, which may subsequently be retroconverted to putrescine and spermidine, respectively, by acetylhydrolase.

2.2.3. Polyamine synthesis by bacteria in the gastrointestinal tract

Bacteria in the gastrointestinal tract, such as Bacillus, Clostridium, Enterobacteriaceae, Enterococcus, Klebsiella, Morganella and Proteus organisms, decarboxylate a number of amino acids to biogenic amines, yielding, for example, histamine from histidine, putrescine from ornithine and cadaverine from lysine. Bacteria may also produce putrescine by initial decarboxylation of arginine to agmatine, which is catalyzed by arginine decarboxylase. Agmatine deamination produces putrescine and urea under the control of agmatine amidinohydrolase.

Polyamines from bacterial and of dietary origin are partly absorbed in the gastrointestinal tract and may subsequently be transported into cells by carrier-mediated mechanisms.

2.3. Catabolism

Terminal degradation, consisting of an oxidative deamination of primary amino groups of polyamines and their *N*-acetyl derivatives, is catalysed by copper-containing amine oxidases, like diamine oxidase and spermine oxidase. The derived aldehydes are oxidated by aldehyde dehydrogenase to form the corresponding acids, so putrescine is converted to γ-aminobutyric acid (GABA); spermidine to isoputreanine, putreanine and spermidic acid-2; N^1 acetylspermidine to *N*-acetylisoputreanine; spermine to spermic acid-1 and spermic acid-2; and N^1-acetylspermine to N^1-acetylspermic acid. The oxidative deamination of GABA produces succinic acid, whose shunting into the citric acid cycle finally

forms carbon dioxide, water and urea. Polyamine degradation in mammals also involves the production of 1,3-diaminopropane (DAP), *N*-acetyl-1,3-DAP, β-alanine, 2-hydroxyputrescine and the alcoholic counterparts of the *n*-α-amino acids [181].

3. Chromatographic methods

3.1. Thin-layer chromatography

TLC is used for preliminary identification and semiquantitation of polyamines. It may not be as specific and precise as other methods, but as a simple and rapid method with no need for sophisticated equipment, it is still helpful in the quantitation of polyamines.

Polyamine determination by TLC requires, like other chromatographic methods, at least two steps: (1) separation from all other compounds containing amino groups, usually by extraction with perchloric acid; (2) derivative formation. Since polyamines do not have native fluorescence and adsorb in the same wavelength in which interfering compounds are detectable, their direct detection remains very difficult. Therefore, the primary and secondary amino groups are generally derivatized with 5-dimethylaminonaphthalene-1-sulfonyl chloride (dansyl chloride) to form fluorescent compounds suitable for thin-layer chromatographic determination [67–72]. When added in excess, it reacts with putrescine, spermidine and spermine to give intensely fluorescent (activation maximum near 365 nm, emission maximum at about 510 nm) di-, tri- and tetra-"dansyl" derivatives, respectively. For the separation of derivatized polyamines, alumina and kieselguhr plates have been used in times past, but silica plates (20×20 cm, 100 or 200 μm in thickness) are preferred for their higher performance [182]. Samples are applied on thin-layer plates using precision pipettes in preference to graduated pipettes or microsyringes and separation is carried out by ascending chromatography in filter-paper-lined chromatographic tanks with different developing solvents (Fig. 1).

Quantitative estimation is obtained by either in situ scanning of fluorescence or, after elution, by conventional fluorimetry, with a sensitivity ranging from 10 to 100 pmol. Since dansyl derivatives are light-sensitive and can be irreversibly destroyed by light, it is essential to keep the dry plates in the dark until they are evaluated.

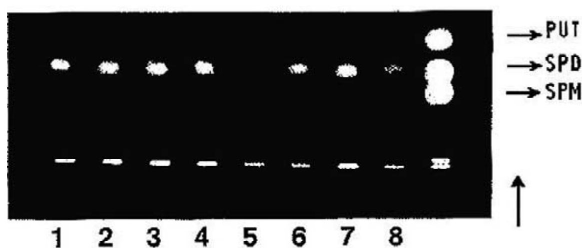


Fig. 1. Separation pattern of dansylated putrescine, spermidine and spermine on a precoated silica gel plate using ethylacetate/cyclohexane (2:3) as the solvent system. The lane at the extreme right shows a separation pattern of standard polyamines. Arrows indicate: putrescine (PUT), spermidine (SPD) and spermine (SPM) bands. Lanes 1–4 show polyamine separation in the strain UR-6. Lanes 5–8 show polyamine separation in the strain AG-83. UR-6 and AG-83 are strains of *Leishmania donovani*. Reproduced from Madhubala [72] with permission.

Radioactive polyamines, determined for metabolic studies, can be dansylated and identified as spots by autoradiography. The spots are then scraped into vials containing liquid scintillation fluid and counted in a scintillation counter [183].

Table 2 gives a comparison of three TLC methods for polyamine assay published from 1980 to 1998.

3.2. Gas chromatography

Methods using GC were introduced to polyamine analysis in 1969 [73]. However, despite quite considerable efforts, GC of free amines has not gained much importance, either because of its poor sensitivity or because of difficulties in obtaining derivatizable salt-free extracts from biological fluids.

GC with flame ionization detection (FID) was the first system employed. Since its sensitivity is modest, its application has been limited to the analysis of urine, which usually contains much higher levels of polyamines than other physiological fluids such as blood and cerebrospinal fluid [76,83,92]. Improvements in FID sensitivity gained using trifluoroacetylacetone (FAA) as derivatizing reagent have recently allowed us to quantify putrescine and cadaverine in the serum of cancer patients (Fig. 2) [94].

The introduction of electron-capture detection

Table 2
Determination of polyamines using TLC

Year	Sample	Extraction	Derivatization	Solvent system	Refs.
1980	Cultured human lymphocytes	Cells were washed in 0.9% (w/v) NaCl and extracted with 3% (v/v) perchloric acid	Dansyl chloride	Benzene/methanol (19:1, v/v) cyclohexane/ethyl acetate (3:2, v/v) chloroform/propan-2-ol (10:1, v/v)	[183]
1983	Urine	Urine was hydrolysed with hydrochloric acid at 110 °C for 16 h. Following hydrolysis the test-tubes were placed in a refrigerator (4 °C) for 12 h. The resulting precipitate was filtered and the sample was evaporated to dryness at 70 °C. The resulting residue was taken up into 2% (v/v) perchloric acid	Dansyl chloride	Chloroform/triethylamine (85:17, v/v) chloroform/toluene/triethylamine (60:28:12, v/v/v)	[70]
1998	Tissue or cultured cells	Specimens were washed with phosphate-buffered saline (PBS) and homogenized at 4 °C with 2% (v/v) perchloric acid	Dansyl chloride	Cyclohexane/ethylacetate (2:3, v/v)	[72]

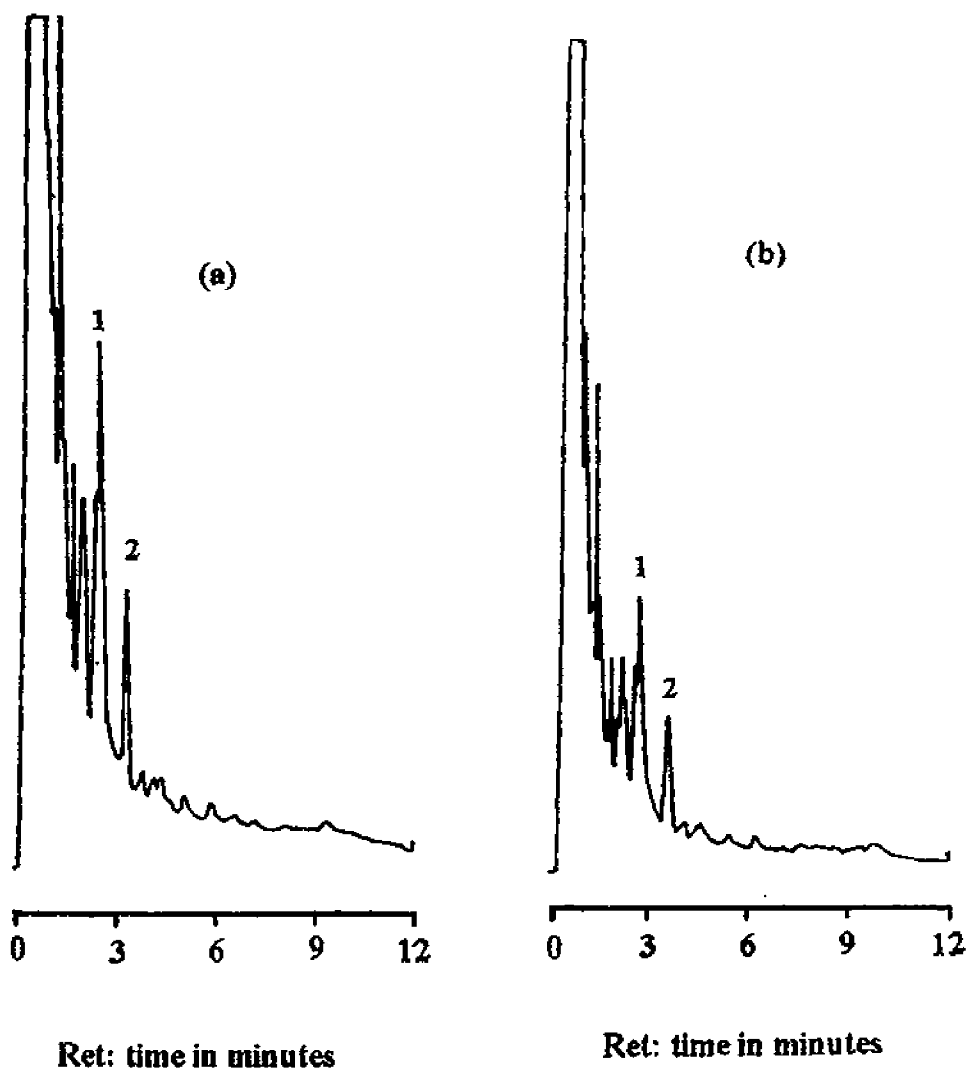


Fig. 2. Gas chromatographic determination of: (1) putrescine and (2) cadaverine from serum of a cancer patient with carcinoma of the lung before (a) and after (b) radiotherapy. Polyamines were derivatized with trifluoroacetylacetone (FAA). Conditions: Column BP1 (12 m×0.22 mm I.D.) with layer thickness 0.25 μm; initial temperature 200 °C with a rise of 2 °C/min up to 220 °C for 2 min, injection port 280 °C and detector 285 °C. Nitrogen flow-rate 3.5 ml/min split ratio 1:10 and flame ionization detection (FID). Reproduced from Khuhawar et al. [94].

(ECD) in combination with suitable amine derivatives allowed the detection limits to decrease markedly. Makita et al. first applied this detection system to the quantitative assay of putrescine and spermidine, using pentafluorobenzyl chloride for derivatization, resulting in the unsatisfactory determination of spermine [78]. Four years later, Rattenbury et al. [81] developed an ECD method for measuring urinary polyamines as their pentafluoropropionyl derivatives. But, in spite of its excellent sensitivity, which enabled the measurement of polyamines at pmol levels, this method remained restricted to the analysis of urinary polyamines because of a cross-reactivity occurring between amino acids and the acylating reagent, pentafluoropropionic anhydride. Only in 1983 did Fujihara et al. [85] devise a simple ECD-GC method for polyamine determination even in a sample, such as human blood, containing large

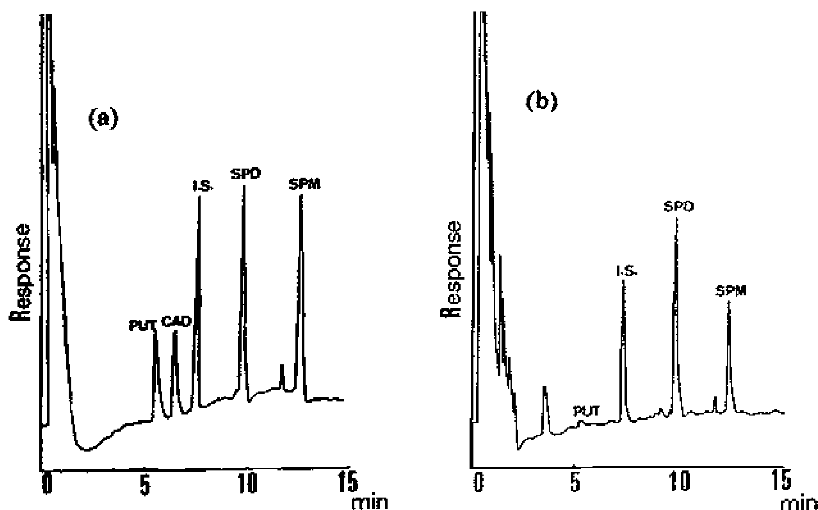


Fig. 3. Gas chromatogram of polyamines from a standard mixture (a) and human erythrocytes (b). Polyamines were derivatized with heptafluorobutyric anhydride (HFBA). Column: 3% OV-17 on 80–100 mesh Chromosorb W HP, 150 cm×3 mm I.D. glass. Nitrogen flow-rate: 60 ml/min. Instrumental conditions: initial temperature 120 °C, delay 2.5 min, 15 °C/min, and final temperature 280 °C. Detector: ^{63}Ni electron-capture detector, 300 °C at a pulse interval of 50 μs. PUT, putrescine; SPD, spermidine; SPM, spermine; I.S., 1,5-diamino-3-azapentane. Reproduced from Fujihara et al. [85] with permission.

quantities of amino compounds (Fig. 3). They used an activated Permutit for the clean-up of samples and heptafluorobutyric anhydride (HFBA) as an excellent acylating reagent, more selective than pentafluoropropionic anhydride. This technique allowed the measurement of 0.02 to 0.1 pmol amounts.

Nitrogen–phosphorus (N-P) detectors also provided very low detection limits in several organic samples (Fig. 4) and, therefore, they have been widely used [82,86,87,90,91,93].

The use of capillary instead of packed columns increased further the sensitivity of GC systems. The advantages of using capillary columns were a higher operating temperature in the stationary phase, greater resistance to low pH samples, less bleed, extreme robustness (strength) and excellent reproducibility. Moreover, thanks to their very small diameter and considerable length, the capillaries allowed enormous resolution capacity and high efficiency. Polyamines of interest could, therefore, be measured in small amounts and almost without disturbance of interfering compounds [86,87,89,92–94]. Moreover, capillary GC was able to analyse, rapidly and simultaneously, polyamines and acetylpolyamines, minimizing the problems of adsorption and reducing the time required for measurements [93].

Further developments in GC analyses comprised both pre-purification and derivatization procedure improvements. The former employed extraction with alkaline butanol [74,80], trichloroacetic acid (TCA) [85,86,94], diisopropyl ether [89], cation-exchange chromatography [75,77,81] or adsorption with silica gel [79,83,87,92,93]. The latter led mostly to the formation of isobutyloxycarbonyl [82], pentafluoropropionyl [81], ethyloxycarbonyl [83,88], trifluoroacetyl [92,94] or heptafluorobutyryl derivatives [85,87,91,93].

The progress made over the years in detection and column systems, isolation procedures and derivatization techniques allowed the introduction of GC to routine laboratory usage.

Some of the GC techniques for polyamine determination reported in the last two decades are summarised in Table 3.

3.3. High-performance liquid chromatography

In 1976, Samejima et al. [95] reported the first HPLC method for polyamines. Since that time, a large number of papers have been published about ion-exchange [99,100,102,103,106,118,133] or reversed-phase [96–98,101,105,107–114,117,119–123,

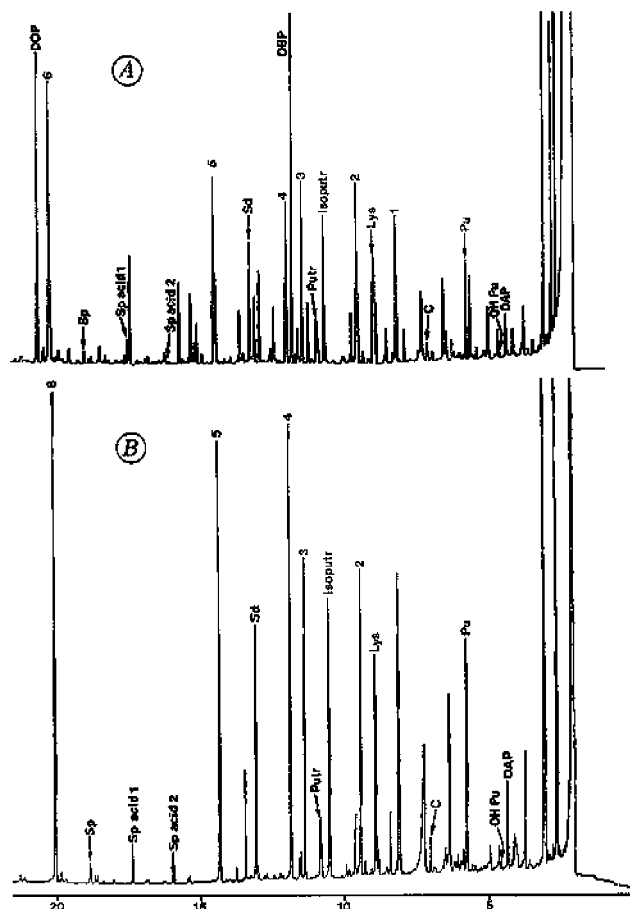


Fig. 4. Comparison between typical chromatograms obtained with flame-ionization (a) and nitrogen–phosphorus (b) detectors after capillary gas chromatography of methyl-heptafluorobutyryl-derivatized extracts of acid-hydrolyzed urine from a normal man. Column: 35 m×0.2 mm (I.D.) fused-silica capillary, coated with cross-linked methyl silicone (film thickness 0.11 μm), and siloxane deactivated. Helium flow-rate: 0.5 ml/min, split ratio 1:12. Detector and injector temperature: 300 °C. Oven temperature program: start at 120 °C, increase by 7 °C/min to 260 °C, then hold for 20 min at 260 °C. Abbreviations used: DAP, 1,3-diaminopropane; OH Pu, 2-hydroxyputrescine; Pu, putrescine; C, cadaverine; 1, 1,6-diaminohexane; Lys, lysine; 2, 1,7-diaminoheptane; Isoputr, isoputreanine; Putr, putreanine; 3, N^1-methylisoputreanine; DBP, dibutylphthalate; 4, bis(3-aminopropyl)amine; Sd, spermidine; 5, *N*-(3-aminopropyl)-1,5-diaminopentane; Sp acid 2, *N*,*N*-bis(2-carboxyethyl)-1,4-diaminobutane; Sp acid 1, *N*-(3-aminopropyl)-N^1-(2-carboxyethyl)-1,4-diaminobutane; Sp, spermine; 6, *N*,*N*-bis(3-aminopropyl)-1,5-diaminopentane; DOP, dioctylphthalate. 1 through 6 are added internal standards. Reproduced from Muskiet et al. [87] with permission.

125–132,134,136] chromatographic methods. Over the years, the HPLC system has been improved to make the determination faster and more sensitive and to obtain better resolution. Now, HPLC remains the technique of choice for polyamine assay because of its high reproducibility and sensitivity as well as its suitability to full automation.

Polyamine derivatives may be obtained either by pre- or post-column mode and detected by fluorimetry or spectrophotometry. The pre-column derivatization has the advantage of producing reaction products soluble in organic solvents that can be directly extracted and pre-concentrated from body fluids [120]. Reagents such as dansyl chloride [96,97,105,109,128,130,132,134], fluorescamine [98,129] and *o*-phthaldialdehyde (OPA) [99–101,103,108,111,113,117,121,126,136] have been proposed for fluorimetric detection. On the other

Table 3
Determination of polyamines using GC

Year	Sample	Extraction and derivatization	Conditions	Detection limits	Refs.
1982	Urine	The monoacetyl polyamines were isolated and concentrated from urine using a silica gel column. Derivation was made by adding ethyl chloroformate. The derivatives were extracted with chloroform. The combined extracts were dried over anhydrous sodium sulphate and evaporated to dryness at 65 °C in a gentle stream of nitrogen. The residues were dissolved in ethyl acetate	Uniport HP column (1 m×3 mm I.D.) impregnated with 0.5% SP-1000, T= 150–280 °C, nitrogen flow-rate=40 ml/min and flame ionization detection (FID)	10 nmol/ml for monoacetyl-cadaverine N^1-acetyl-spermidine, N^8-acetyl-spermidine and 20 nmol/ml for monoacetyl-putrescine	[83]
1983	Erythrocytes	Extraction was carried out with trichloroacetic acid (TCA) 10% (w/v). Derivatization was made by adding heptafluorobutyric anhydride (HFBA). The mixture was evaporated to dryness under a stream of nitrogen and the residue was redissolved in diethyl ether	Column of Pyrex glass (150 cm×3 mm I.D.) packed with 3% silicon OV-17 on 80–100 mesh Chromosorb W HP, T=120–280 °C, nitrogen flow-rate=60 ml/min and ^{63}Ni Electron Capture (EC) detection (pulse interval=50 μs)	0.1 pmol/μl for putrescine and cadaverine, 0.02 pmol/μl for spermidine and spermine	[85]
1983	Plasma	Samples were deproteinized with TCA 10% (w/v). Derivatization was made by adding isobutyl chloroformate. The derivatives were extracted with diethyl ether	Column of borosilicate glass (7 m×0.2 mm I.D.), T=190–280 °C, helium flow-rate=6 ml/min and N-P detection	0.05 nmol/ml for spermidine	[86]
1984	Urine	After acid hydrolysis, the compounds were isolated by adsorption onto silica gel and converted into their methyl-heptafluorobutyryl derivatives. The derivative-containing solution was evaporated at room temperature under a stream of air. The residue was dissolved in phosphate buffer and the derivatives were extracted into dichloromethane. After evaporation, the residue was redissolved in ethyl acetate	Fused silica capillary column (35 m×0.2 mm I.D.), T=120–260 °C, helium flow-rate 0.5 ml/min and N-P detection	No data were reported, but levels from both healthy people and patients with metastatic melanoma were detectable	[87]
1984	Urine	Urine samples were directly treated with ethyl chloroformate in an alkaline medium. After extraction of the resulting *N*-ethoxycarbonyl derivatives with chloroform, the combined extract was applied to a silica gel minicolumn to remove interfering substances	Column of silanized glass (1 m×3 mm I.D.), T=150-250 °C, helium flow-rate=40 ml/min	The minimum detectable quantities were in the range of 0.1–0.3 pmol of acetylpolyamines	[88]
1990	Urine	The polyamines were isolated and concentrated from urine using a silica gel column. Derivation was made by adding trifluoroacetic anhydride (TFAA). The derivatives were evaporated to dryness at room temperature under a gentle stream of nitrogen. The residue was dissolved in acetone	SE-54 capillary column (27 m×0.25 mm I.D.), T=100–250 °C, nitrogen flow-rate= 1.07 ml/min and FID	2 ng/μl for putrescine, 5 ng/μl for spermidine and 25 μg/ml for spermine	[92]
1997	Murine L1210 lymphocytic leukaemia cells	Prepurification comprises deproteinization with 12% sulfosalycilic acid, isolation with Sep-Pak silica at pH 9, conversion to heptafluorobutyryl derivatives and post-derivatization organic fluid extraction	Fused-silica capillary column (37.5 m×0.2 mm I.D.), T=120–280 °C, helium flow-rate=0.6 ml/min and nitrogen–phosphorus (N-P) detection	0.7 pmol/μl for putrescine, 0.4 pmol/μl for spermidine and 0.5 pmol/μl for spermine	[93]
1999	Serum	Extraction was made by TCA 10% (w/v). Derivation was carried out with trifluoroacetylacetone (FAA) (3% v/v in methanol) and sodium acetate (pH 6.75). The derivatives were extracted with chloroform	Column BP1 (12 m×0.22 mm I.D.), T=200–220 °C, nitrogen flow-rate=3.5 ml/min and FID Column BP5 (50 m×0.22 mm I.D.), T=240–260 °C, nitrogen flow-rate=4.5 ml/min and FID	0.6 μg/ml for putrescine and 0.5 μg/ml for cadaverine	[94]

hand, UV–Vis spectrophotometry requires derivatization reactions with quinoline-8-sulfonyl chloride (benzoyl chloride) [112,119,120,122,123,125,131, 135], 4-dimethylaminoazobenzene-4′-sulfonyl chloride (dabsyl chloride) [104,110] or *p*-toluenesulfonic chloride (tosyl chloride) [184,185]. Owing to their high molar extinction coefficients, dansyl derivatives can also be monitored in column eluate by UV absorption [182]. The detection limits reached with both fluorescamine and OPA are generally better than those obtained with all the spectrophotometric reagents [134]. The fluorogenic agents react only with primary amino groups, making them more sensitive than acid chlorides, which form derivatives with both primary and secondary amino groups, imidazole nitrogen and phenolic hydroxyls and even with some alcohols [123,182]. Moreover, fluorescamine and OPA require a very short time for derivatization as well as simpler or faster elution procedures [181,186]. On the other hand, acid chlorides have the advantage of providing reaction products more stable than fluorescamine or OPA-derivatives [134]. However, despite the progress achieved, the derivatization procedures often suffer from various drawbacks, such as tedious sample preparation, interference from by-products, long analysis times and the risk of indeterminate errors. Furthermore, some post-column derivatization methods may form unstable derivatives and give rise to broad and derivatization-reagent-diluted peaks [133]. Recently, Molins-Legua et al. [134] reported a method able to solve many of these problems. They described a simple and quick off-line HPLC procedure employing solid-phase extraction cartridges for both clean-up and derivatization of polyamines from urine. The use of solid-phase supports enabled purification of the sample and, at the same time, concentrating and derivatizing the analytes, leading to a marked decrease in sample handing as well as a shorter analysis time relative to conventional methods (Fig. 5).

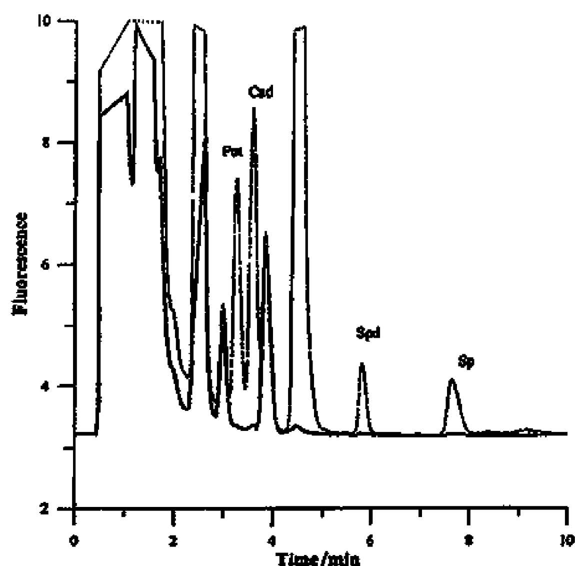


Fig. 5. Chromatograms obtained from blank urine (−) and spiked with putrescine (Put) 0.83 μg/ml, cadaverine (Cad) 0.88 μg/ml, spermidine (Spd) 0.88 μg/ml and spermine (Sp) 0.93 μg/ml (−). Polyamines were derivatized with dansyl chloride. Column: C_{18} Lichrospher (125×4 mm I.D.). An acetonitrile-imidazol solution (1 m*M*, pH 7.0) (70+30 v/v) mixture in gradient elution mode was used as the eluent at a flow-rate of 1 ml min^{-1}. The gradient used was 70% of acetonitrile at zero time, 90% at 5 min, and 70% at 9 min. After 9 min, the percentage of acetonitrile was kept constant. A fluorescence detector was used for data acquisition (excitation at 252 nm and emission at 500 nm). Reproduced from Molins-Legua et al. [134] with permission.

Alternative detection strategies have also provided increased sensitivity without requiring any preliminary derivatization procedures. In 1989, Maruta et al. [115] described a sensitive (sub-pmol) and selective analytical method for quantifying polyamines in tissue and acid-hydrolysed urine. They employed an isocratic ion-pairing reversed-phase chromatography and a post-column reactor immobilizing PAO. The generation of hydrogen peroxide was monitored by ECD. Since these researchers used PAO derived from soybean seedlings that were not commercially available, other experiments have been carried out using other oxidases. The enzyme from *Aspergillus terreus* was found to be very suitable for the task [187].

A few years later, a system based on a similar principle involving immobilized acetylpolyamine amidohydrolase (DAL) allowed better separation and simultaneous determination of free as well as acetylated polyamines (Fig. 6) [124]. Recently, detection with an integrated pulsed amperometer (IPAD), integrated voltameter (IVD), integrated square wave (ISWD) and condensation nucleation light scattering (CNLSD) has been developed [133].

Detection through the chemiluminescence measurement of hydrogen peroxide produced by on-

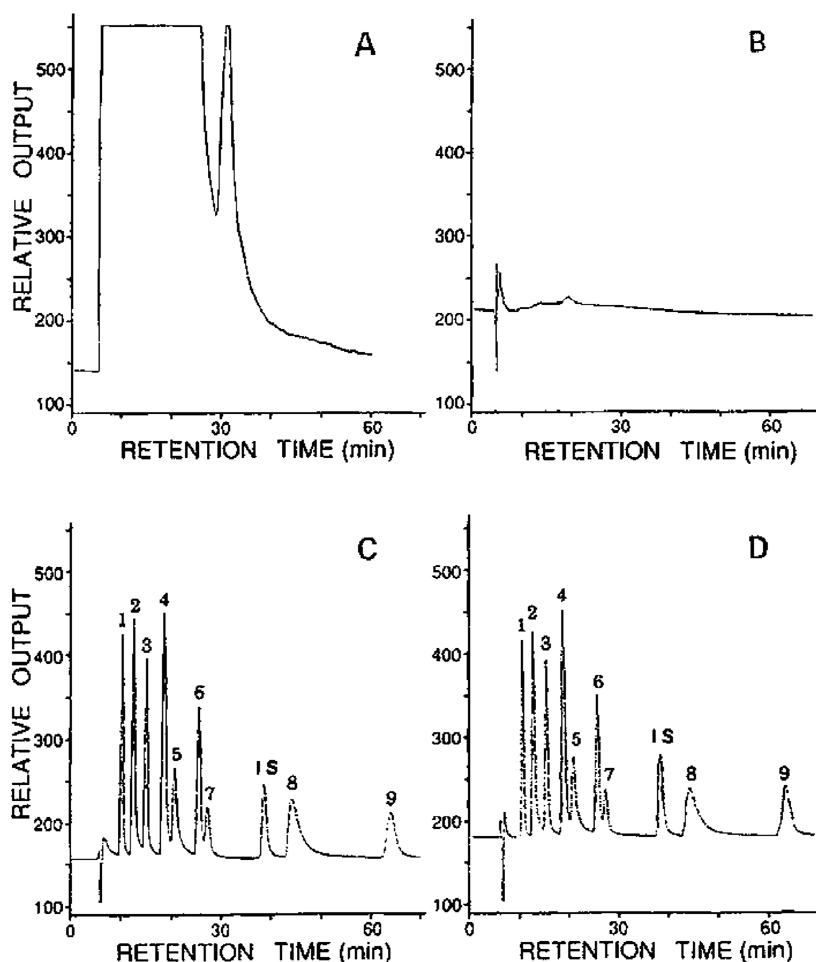


Fig. 6. Detection of polyamines with the improved HPLC system. An urine sample without pre-treatment (a) or after pre-treatment (b), and a mixture of nine polyamines containing an internal standard without pre-treatment (c) or after pre-treatment (d) were analysed with the improved HPLC system with (a, b, and d) or without (b) an enzyme reactor. 1, Putrescine; 2, *N*-acetylputrescine; 3, cadaverine; 4, spermidine; 5, *N*-acetylcadaverine; 6, N^1-acetylspermidine; 7, N^8-acetylspermidine; 8, spermine; 9, *N*-acetylspermine; I.S., internal standard. Reproduced from Hiramatsu et al. [124] with permission.

column immobilized putrescine oxidase (*Micrococcus flavidus*) and PAO (*A. terreus*) was specific and highly sensitive, as shown by Kamei et al. [116]. Nevertheless, to our knowledge, this detection system has not been widely accepted, since it has not been reported by others.

Some of the HPLC methods for polyamine assay reported in the last two decades are summarised in Table 4.

4. Hyphenation procedures

Hyphenation procedures, including LC–MS and GC–MS, are emerging techniques able to combine the separation power of HPLC or GC with the sensitivity and specificity of MS. Their application for the quantitative determination of polyamines has spread widely in the last decade.

GC–MS was first used by Walle for the quantita-

Table 4
Determination of polyamines using HPLC

Year	Compounds	Sample	Derivatization	Column	Elution system	Detection mode	Detection limits	Refs.
1982	Acetylpolyamines	Urine	OPA	Bio-Rad A-9	The column was equilibrated for 15 min with the Buffer A 0.2 *M* sodium citrate in double-distilled water (DDW). After sample injection, the Buffer B, 0.2 *M* sodium hydroxide and sodium chloride in DDW, was started and run for 38.5 min. The Buffer C, 0.44 *M* sodium hydroxide and sodium chloride in DDW, was then started and run for 20 min after which the column was washed with Buffer D, sodium hydroxide in DDW, for 15 min. Buffer flow-rate: 30 ml/h	Fluorescence	Not given	[103]
1983	Free and total polyamines	Urine and serum	Dabsyl chloride	Radialpak C_8	A linear gradient elution was realized between solutions A (0.25 N triethylammonium phosphate/methanol, 50:50) and B (0.25 N triethylammonium phosphate/methanol, 20:80) going from 0 to 100% of B in 10 min. Flow-rate: 1.5 ml/min	Fluorescence	10 pmol for spermine and 5 pmol for the other polyamines	[105]
1984	Polyamines	Human serum, children duodenal biopsy and mouse brain	Dabsyl chloride	Ultrasphere ODS (150×4.6 mm I.D.)	Part I (12-min analytical run): the solvent composition is changed after 1.5 min (70 to 100% B in 1 min) and 5 min (100 to 70% B in 0.5 min) Part II (25-min analytical run): the solvent composition is changed after 9 min (57 to 100% B in 7 min) and 20 min (100 to 37% B in 1 min). Solvent A: 20 m*M* heptanesulphonate and 20 m*M* acetic acid in water. Solvent B: acetonitrile. Flow-rate: 2 ml/min	Fluorescence	0.6 pmol for putrescine; 0.3 pmol for spermine and spermidine	[107]
1984	Putrescine	Cerebrospinal fluid	OPA	C_{18} radial-compression (11.5 cm×8 mm I.D.)	Isocratic elution with the mixture of methanol/de-ionized water/phosphoric acid/*N*,*N*-dimethylcyclohexylamine (70:27:1.5:1.5 by vol.)	Fluorescence	50 pmol/ml of putrescine	[108]
1986	Polyamines and acetylpolyamines	Urine, cerebrospinal fluid and tissues	Dabsyl chloride	Ultrasphere ODS (250×4.6 mm I.D.)	Gradient I: solvent A, acetonitrile; solvent B, 10 m*M* phosphate buffer, pH 4,4. Equilibration time, 6 min; total time, 26 min Gradient II: solvent A, acetonitrile–methanol (185:15) solvent B, 10 m*M* phosphate buffer, pH 4.4. Equilibration time, 6 min; total time, 46 min	Fluorescence	0.12 pmol for putrescine, 0.08 pmol for spermidine, and 0.06 pmol for spermine	[109]
1987	Polyamines and acetylpolyamines	Cultured bacteria	Dabsyl chloride	Spherisorb S5 ODS2 (250×4.6 mm I.D.)	Linear gradient from 40% sodium acetate buffer/60% acetonitrile to 100% acetonitrile in 20 min, whereafter the elution was continued for 5 min with 100% acetonitrile. Flow-rate: 1.5 ml/min	UV	0.6 pmol for putrescine; 0.3 pmol for spermine and spermidine	[110]
1988	Polyamines and acetylpolyamines	Urine, serum and tissues	OPA	NovaPak C_{18} (150×4.6 mm I.D.)	Gradient I: solvent A, 0.1 *M* sodium acetate; solvent B, 0.2 *M* sodium acetate/acetonitrile (10:3, v/v); equilibration time, 8 min; total time, 25 min Gradient II: solvent A, 0.1 *M* sodium acetate; solvent B, 0.2 *M* sodium acetate/acetonitrile (10:3, v/v), equilibration time, 10 min; total time, 56 min	Fluorescence	0.5–1.0 pmol for each analyte	[111]

Table 4. Continued

Year	Compounds	Sample	Derivatization	Column	Elution system	Detection mode	Detection limits	Refs.
1989	Polyamines	Rat dorsal root ganglia	9-Fluorenylmethyl chloroformate	NovaPak C_{18}	Binary solvent delivery system	Fluorescence	Not given	[114]
1990	Polyamines	Incised skin wounds of rats	OPA	Cation-exchanger column Shim-pack ISC-05/S0504 P (50×4 mm I.D.)	The column was first developed with solvent A (0.2 N sodium citrate and 0.7 N sodium chloride) for 5 min, and then with a linear gradient of 0–100% solvent B (2.5 N sodium chloride and 0.2 N sodium citrate) for 10 min. Finally, 100% solvent B was run for 25 min. Flow-rate: 0.6 ml/min	Fluorescence	Not given	[118]
1991	Polyamines	Urine	OPA	Ultrasphere ODS (250×4.6 mm I.D.)	Linear gradient elution of a solution of acetonitrile in water (70:30 by vol) to 100% over 10 min. Flow-rate: 2 ml/min	Fluorescence	15 pmol for spermidine and spermine. The content of putrescine was negligible	[121]
1991	Polyamines	Erythrocytes	Dabsyl chloride	Separon C_{18} (15×0.32 cm I.D.)	a) Isocratic system: 83% methanol, 17% water b) Gradient system: 0 min 75% methanol in water; 6 min 95% methanol in water; 15 min 95% methanol in water; 20 min 75% methanol in water	Fluorescence and UV	40 pmol for putrescine, 25 pmol for spermidine and 20 pmol for spermine	[120]
1992	Polyamines	Human seminal plasma	Benzoyl chloride	Bio-Sil ODS-5S (250×4.0 mm I.D.)	Isocratic elution with a solution of methanol/water (60:40, v/v)	UV	57 pmol for putrescine, 117 pmol for spermidine and 124 pmol for spermine	[122]
1996	Polyamines	Human urine, pig plasma and pig intestine biopsies	OPA	Inertisil (250×3.2 mm I.D.)	Two mobile phases were used: (A) sodium citrate buffer (125 m*M*), pH 6.5, containing 50 ml/l of tetrahydrofuran and (B) consisting of a solution of citrate buffer, acetonitrile and tetrahydrofuran	Fluorescence	0.1 pmol for all tested	[126]
1998	Polyamines and acetylpolyamines	Biological specimens		Two columns in series were used: ODS C_{15} (75×4.6 mm I.D.) and LC_{18} (150×4.6 mm I.D.)	Mobile phase for elution was a gradient between A: 0.02 *M* 1-heptane–sulphonic acid (pH 3.4), acetonitrile, methanol (5:3:2 by vol), and B: acetonitrile, methanol (3:2, v/v)	Fluorescence	33.6, 8.33, 2.72, 2.67, 4.76, 4.27 and 2.27 fmol/µl for N^1-acetylspermidine, N^8-acetylspermidine, putrescine, 1,6-hexanediamine, 1,7-diaminoheptane, spermine and spermidine, respectively	[132]

1998	Polyamines	Cultured cells	Benzoyl chloride	Spherisorb ODS2 (250×4.6 mm I.D.)	Mobile phase: methanol in water (60:40, by vol). Flow-rate: 0.4 ml/min	UV	Not given	[131]
1998	Polyamines	Cultured cells	Florescamine	ODS reversed-phase (250×4.6 mm I.D.)	Two mobile phases were used: (A) 0.25% (w/v) lithium camphorsulfonate, pH 2.65 and (B) 0.25% (w/v) lithium camphorsulfonate, pH 2.65, in 25% (v/v) 1-propanol	Fluorescence	50 pmol/injection for putrescine, 400 pmol/injection for spermidine and spermine	[129]
1998	Polyamines	Cultured cells	Dabsyl chloride	ODS reversed-phase (300×4.6 mm I.D.)	Two mobile phases were used: (A) 10 m*M* sodium dihydrogen phosphate and (B) acetonitrile. Elution gradient on injection: 45% B to 80% B over 14 min; at 14 min: 80% B to 90% B over 1 min; at 15 min: hold at 90% B for 7 min; at 22 min: 90% B to 45% B over 1 min; at 29 min: next sample injected	Fluorescence	10 pmol for putrescine 5 pmol for spermine and spermidine	[130]
1998	Polyamines	Human prostate	Dabsyl chloride	µBondapak C_{18} (250×4.6 mm I.D.)	Samples were separated at a flow-rate of 1.0 ml/min with a one-step linear gradient from 80 to 100% methanol in 11 min. The oven temperature was 50 °C	Fluorescence	0.05 nmol/ml for putrescine, 0.08 nmol/ml for spermidine and 0.06 nmol/ml for spermine	[128]
1999	Polyamines	Urine	Dabsyl chloride	C_{18} Lichrospher (125×4.0 mm I.D.)	An acetonitrile–imidazol solution (1 m*M*, pH 7.0) (70+30 v/v) mixture in gradient elution mode was used as the eluent at a flow-rate of 1 ml/min. The gradient used was 70% acetonitrile at zero time, 90% at 5 min and 70% at 9 min. After 9 min the percentage of acetonitrile was kept constant	Fluorescence	10 ng/ml for each analyte	[133]
2000	Polyamines and acetylpolyamines	Chick embryo retina	Benzoyl chloride	Spherisorb C_{18} S3 ODS2 (150×4.6 mm I.D.)	Two mobile phases were used: (A) water and (B) methanol. Gradient elution: from 61% B (0 to 3 min) to 67% of B (4.5 to 14 min). The gradient was then immediately returned to 61% of solvent B (14 to 15 min) and the initial conditions restored in 5 min. The flow-rate was 0.8 ml/min	UV	Not given	[135]
2001	Polyamines	Human saliva	OPA	Nucleosil ODS (250×4.6 mm I.D.)	The gradient elution was carried out with two mobile phases: (A) water and (B) methanol at a flow-rate of 0.8 ml/min	Fluorescence	0.04 nmol/ml for spermine, 0.05 nmol/ml for spermidine and 0.06 nmol/ml for putrescine	[136]

tion of urinary polyamines using trifluoroacetylation for derivative formation [137]. Since then, many papers about polyamine detection by high-resolution capillary GC in combination with MS have been published [138–146,150,181].

Various derivatization methods have been developed, such as perfluoroacylation [138,139, 143,144,147,149], alkylsilylation [188] and alkoxycarbonylation [142,148,150]. While perfluoroacylation and alkylsilylation require multiple sample-purification steps that are normally very tedious and time consuming, alkoxycarbonylation allows derivatization and extraction of amines in aqueous solutions to be done simultaneously. Recently, Choi et al. [150] developed a GC–MS method able to combine alkoxycarbonylation with subsequent perfluoroacylation to block the remaining active hydrogen atoms. Under these conditions, polyamine separation as *N*-ethyloxycarbonyl-*N*-perfluoroacyl (N-EOC-N-PFP) derivatives was achieved with excellent peak shapes, higher responses and shorter analysis times compared to single N-EOC derivatives for the same amount. Moreover, the faster elution (1–2 min) due to the increase in volatility of N-EOC-N-PFP derivatives completed the separation within 20 min and the enhancement of responses by 7- to 19-fold reduced the detection limits at the same time (Fig. 7).

In order to avoid a rapid deterioration of the GC–MS instrumentation, a sample clean-up step is recommended before amine derivatization. Adsorption on silica [141] and cationic resin [139,138,173] or cation-exchange chromatography [143,144,174] are the most suitable procedures.

MS ionization is carried out by both chemical-ionisation and electron-impact modes. The first employs methane, while the latter ionising energy at 70 eV, ionising current at 60–300 μA, accelerating voltage at 3–3.5 kV and an ion source temperature at 200–250 °C.

In the methane chemical ionization spectrum, the quasi-molecular ion $[M+H]^+$ is the base peak [138,141], owing to the low signals corresponding to other fragmentations (Fig. 8). Therefore, it is the preferred technique when the molecular mass is to be determined with high sensitivity.

In the electron-impact spectrum of the polyamines, the base peaks are different depending on the derivatization reagent used. With trifluoroacetylation $[M\text{-}CF_3]^+$ is the highest fragment of practical use (Fig. 9) [141]; the characteristic ions of the N-EOC

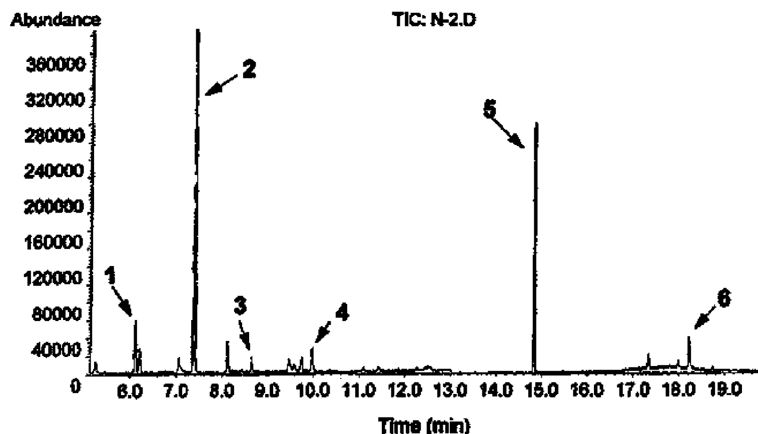


Fig. 7. Selected-ion current chromatogram of hair polyamines as their *N*-ethoxycarbonyl-*N*-pentafluoropropionyl (N-EOC-N-PFP) derivatives from a male subject obtained in the selected ion monitoring (SIM) acquisition mode by monitoring three ions for each amine. The start time was programmed from 5.0 for the first group of 12 ions and then from 12.0 min for the second group of six ions with a dwell time of 80 ms for the first group and 120 ms for the second group. Column: DB-5 (30×0.25 mm I.D., 0.25 μm film thickness) fused-silica capillary column. The oven temperature was programmed: from 140 to 210 °C (2 min) at 8 °C/min and then to 320 °C (3.75 min) at 20 °C/min. Peaks: 1, 1,3-diaminopropane; 2, putrescine; 3, cadaverine; 4, 1,6-diaminohexane (I.S.); 5, spermidine; 6, spermine. Reproduced from Choi et al. [150] with permission.

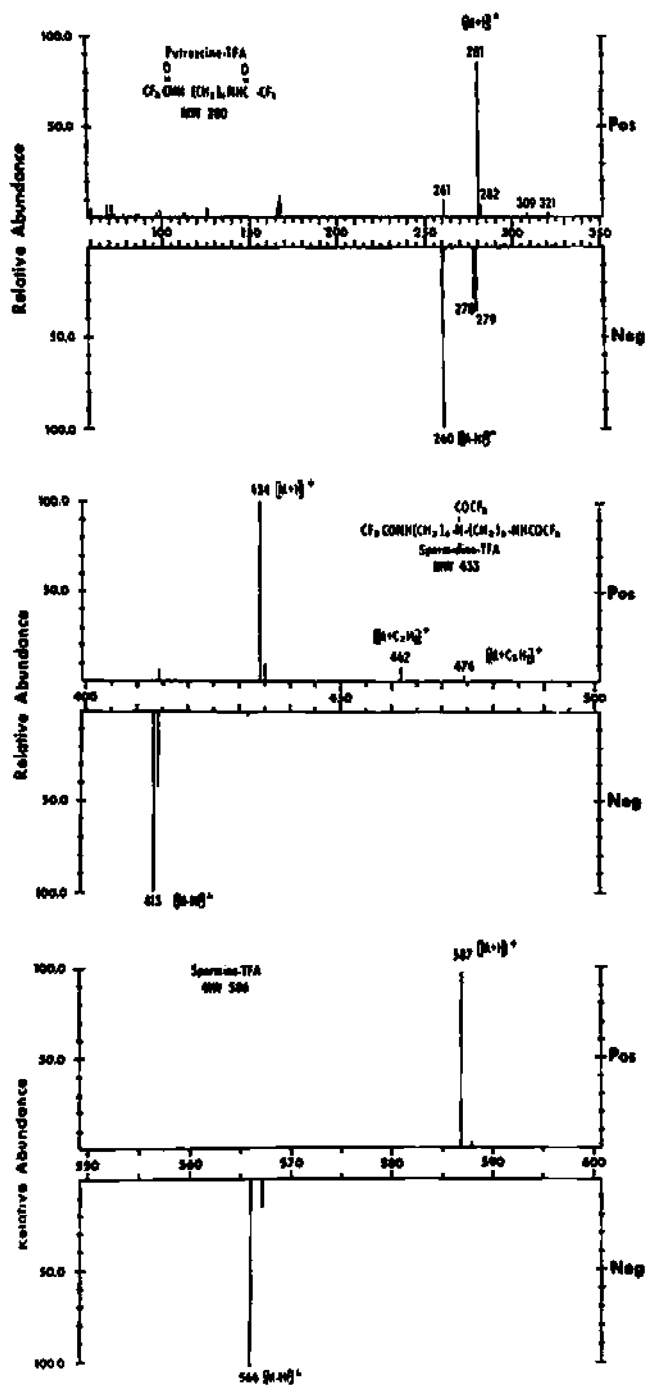


Fig. 8. Pulsed positive–negative chemical-ionization spectra on the trifluoroacetyl (TFA) derivatives of: (a) putrescine, (b) spermidine and (c) spermine. Reproduced from Shipe et al. [138] with permission.

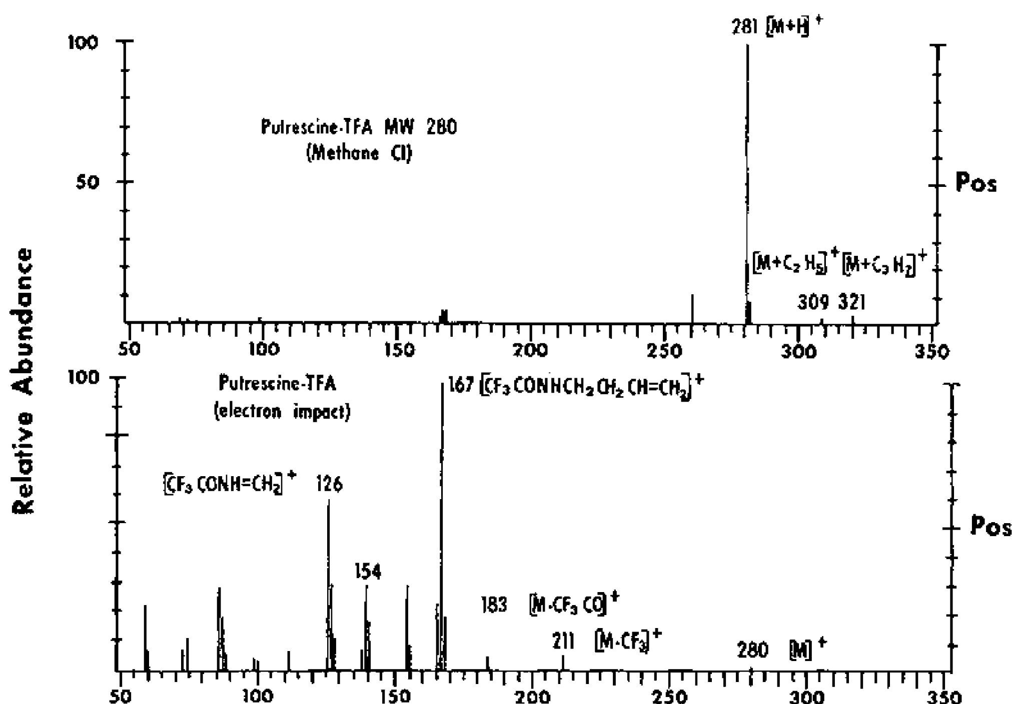


Fig. 9. Comparison of methane chemical ionisation (upper) and 70 eV electron impact spectra of TFA-putrescine. Reproduced from Shipe et al. [138] with permission.

derivatives are $[M\text{-}45]^+$, $[M\text{-}46]^+$, $[M\text{-}73]^+$ and $[M\text{-}119]^+$ for putrescine, spermidine and cadaverine, and $[M\text{-}(73+46X2)]^+$, $[M\text{-}(130+46)]^+$, $[M\text{-}(116+46x2)]^+$, $[M\text{-}(259+46)]^+$, $[M\text{-}(245+46X2)]^+$ for spermine [142]; with N-EOC-N-PFP derivatives, the most intense ions correspond to $[M\text{-}119]^+$ for 1,3-diaminopropane, putrescine and 1,6-diaminohexane, $[M\text{-}308]^+$ for cadaverine and $[M\text{-}73]^+$ for spermidine and spermine (Fig. 10) [150].

Detection of ions may be carried out by either full-scan or selected ion monitoring (SIM) mode. The former shows all peaks derived from molecular fragmentations and rearrangements of each polyamine derivative. All mass/charge (m/z) values are recorded, usually ranging from 50 to 800. In the latter method, the ion current is continuously monitored at preselected m/z ratios for the detection and quantitation of a few characteristic ions. Its sensitivity and selectivity may be improved using deuterium-labeled analogs, whose physical and chemical properties are quite similar to those of the unlabeled polyamines [141].

5. Electromigration methods

5.1. Paper and high-voltage electrophoresis

Despite their notable simplicity, both paper and high-voltage electrophoresis for polyamine assay have been abandoned in recent years, in favour of more sensitive and specific advanced electrophoretic techniques. Already in 1977, Seiler [151] in his review of assay procedures for polyamines in urine, serum and cerebrospinal fluid, concluded that none of the methods based on paper chromatography, thin-layer chromatography, paper electrophoresis and high-voltage paper electrophoresis were appropriate for routine assay of polyamines. He stated that the spermine values, as measured after staining of the electrophoretograms with ninhydrin according to Russell [152], were considerably higher than those found with other methods owing to an unidentified urinary constituent which co-migrated with spermine under the conditions of the electrophoretic separation. As was also pointed out by Marton et al.

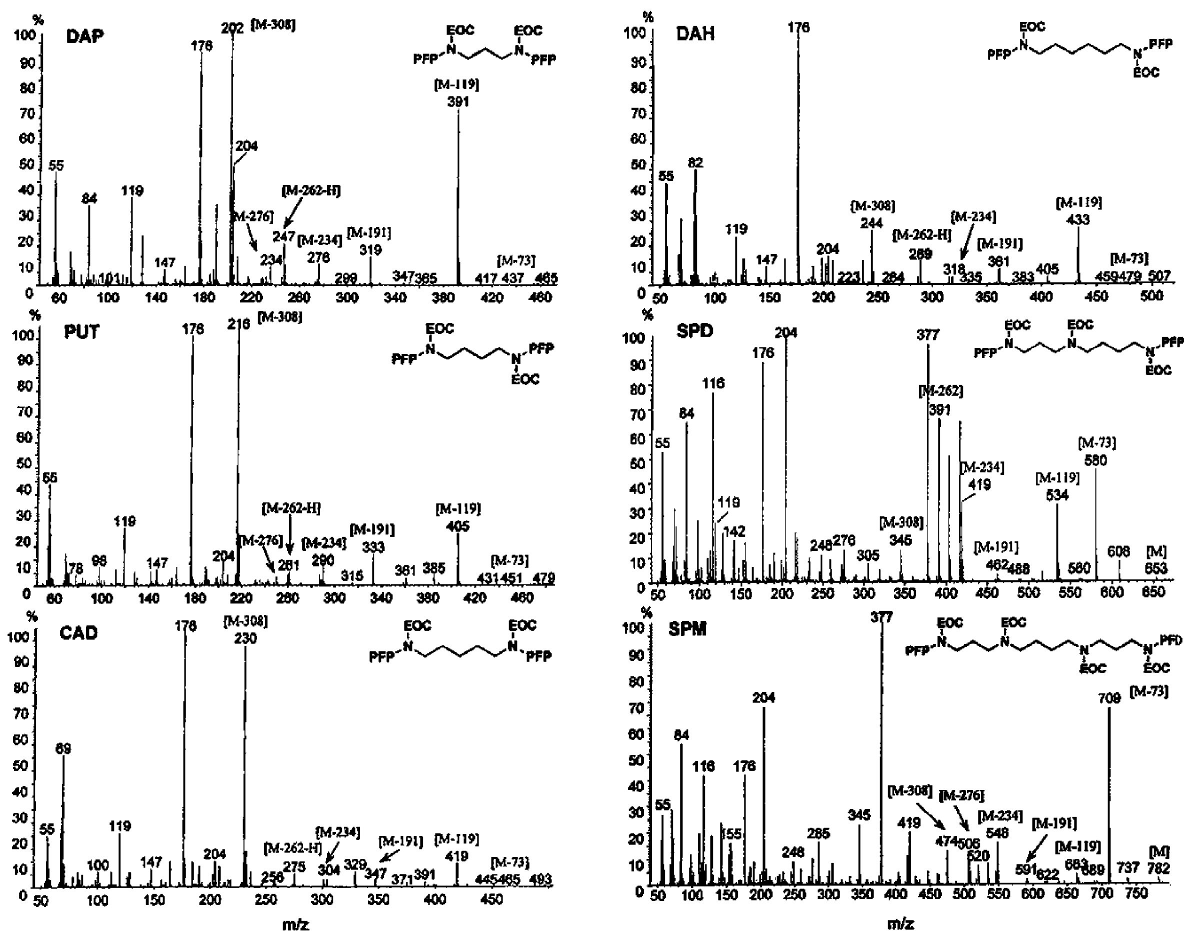


Fig. 10. Electron-impact mass spectra of six polyamines as their N-EOC-N-PFP derivatives obtained in the scanning mode at a rate of 0.42 scan/s with a mass range of m/z 50–800. Peaks: DAP, 1,3-diaminopropane; PUT, putrescine; CAD, cadaverine; DAH, 1,6-diaminohexane; SPD, spermidine; SPM, spermine. Reproduced from Choi et al. [150] with permission.

[153] and by Russell and Russell [154], Russell's initial method based on high-voltage paper electrophoresis was time-consuming and neither very sensitive nor specific when applied to physiological fluids. Therefore, it was modified in 1980 by Fujita et al. [155] who introduced several conditions to improve the sensitivity and reproducibility. Their method required: (a) collection of urine samples at room temperature under toluene instead of refrigeration; (b) a hydrolysis time of 3 h rather than 12–16 h; (c) isolation of polyamines by cation-exchange column chromatography rather than butanol extraction; (d) direct densitometry by dual-wavelength photometry by the zigzag scanning method instead of colorimetric measurement of the spots. However, despite these useful modifications, the method was still limited because of its poor specificity and sensitivity. In 1989, Kanda et al. [156] reported a simple and rapid assay method for polyamines in urine and tissues using electrophoresis on Titan III cellulose acetate. In this procedure, polyamines were first extracted from a hydrolysate of urine or from supernatants of tissue homogenates by use of a Bio-Rex 70 minicolumn. After electrophoretic separation, polyamines were fluorimetrically detected by the reaction with OPA-2-mercaptoethanol. The proposed method

required only 11 min for electrophoresis and subsequent detection, and six samples and two external standards could be assayed on one strip. Furthermore, linear scanning was sufficient for the measurement of separated polyamines. The sensitivity of this method was comparable to that of the HPLC method.

5.2. Capillary electrophoresis (CE)

In the last 10 years, CE has become an important analytical technique, particularly in the analysis and separation of biological specimens [157]. For its versatility coupled with an analyte resolution similar to that assured by chromatographic techniques, it was widely applied in many laboratories. CE differentiates charged species on the basis of electrophoretic mobility under the influence of an applied electric field. The value of the electrophoretic mobility (μ) of a species is directly related to its net charge and inversely related to its hydrodynamic mass. CE requires only small quantities of material, is applicable to water-soluble, non-volatile, high-molecular-mass species, is readily automated, and can be manipulated by altering pH, ionic strength and electrolyte composition, or by incorporating electrolyte additives [158]. Nevertheless, CE methods, despite some attractive merits, are less sensitive than GC and HPLC because of the short detector cell pathlength through the capillary. In order to increase their sensitivity, several derivatization procedures have been adopted. But, unfortunately, chemical modifications are time-consuming and result in the dilution of the sample, affecting the separation process, and are difficult to implement with very small sample volumes. To solve this problem, an indirect detection technique, involving neither pre- nor post-capillary derivatization procedures, can be employed [159–162]. By using a run buffer containing a background electrolyte (BGE) in which a chromophore or fluorophore is present, the native analytes can be indirectly detected in the capillary with a high degree of sensitivity. Another way, named ‘‘in-capillary’’ or ‘‘on-column’’ derivatization, offering the same advantages as indirect detection, has also been investigated for the same purpose. Separations and derivatizations of analytes are carried out simultaneously during the electromigration of native analytes in a separation capillary tube filled with a run buffer containing derivatization reagent.

CE separations include capillary zone electrophoresis (CZE), micellar electrokinetic capillary electrophoresis (MEKC), capillary electrochromatography (CEC), capillary isoelectric focusing (CIEF), capillary gel electrophoresis (CGE) and capillary isotachophoresis (CITP). Very recently, there has also been interest in microchip electrophoresis, which combines the possibility offered by gels of performing multiple, parallel analysis with the automation assured by capillary electrophoresis. Micro-fabrication allows the possibility of miniaturizing traditional analytical instrumentation, with the advantages of speed, automation, and volumetric reduction of samples, reagents and wastes. The applications of electromigration techniques to polyamine detection were recently well reviewed by Ogury [163]. On the basis of the reported data, he concluded that at present CZE is the most recommended electromigration method for the quantitative determination of free amines and polyamines and that the indirect detection system is to be preferred.

6. Immunoassays

An alternative approach to the above-described chemical methods is represented by RIA [164–166] and ELISA [167–170]. Both techniques provide high sensitivity, require small tissue samples and allow multiple analyses in parallel. They also offer the considerable advantages of convenience and rapidity, avoiding the requirement for sample derivatization. Nevertheless, they are not completely satisfactory either for their modest specificity and for the inability to obtain antibodies against putrescine. Moreover, RIA may be hazardous because of its radioactivity.

In 1975, Bartos et al. [164] described a RIA for spermine and spermidine in human serum. However, the usefulness of the method was limited by the 20% cross-reactivity of the antispermine antibody with spermidine. Later, they produced polyclonal antibodies that were specific for spermine [166] or spermidine [165]. In 1983, Fujiwara et al. [189] developed an enzyme immunoassay (EIA) employing two antibodies against spermine and spermidine.

But cross-reaction problems were present, thus preventing discrimination between the two polyamines. In 1989, Garthwaite et al. [190] raised monoclonal antibodies to spermine and spermidine that they used in both RIA and ELISA. However, the immunocytochemical applicability and specificity of their antibodies were not described. Four years later, the same authors [167] prepared a monoclonal antibody, IAG-1, with a high affinity for free spermine, which showed only 10% cross-reactivity with spermidine and negligible cross-reactivity with a range of other polyamine analogues. This monoclonal antibody, used in ELISA, was able to quantify free spermine, with a detection limit in the order of 1 pmol. Subsequently, they described the production and characterization of a novel monoclonal antibody, JAC-1, specific for free spermidine [170]. It allowed measurement of free spermidine with high sensitivity (lower limit of detection 10 pmol), and furthermore, showed a lower cross-reactivity (4% with spermine) relative to previous immunoassays [165,166,168].

Recently, several ELISA systems have been developed to quantify acetylpolyamines also. Fujiwara et al. [191] first described an ELISA for N^1,N^{12}-diacetylspermine using the monoclonal antibody ASPM-2, raised against spermine conjugated with human serum albumin via *N*-(γ-maleimidobutyryloxy)succinimide (GMBS) [192]. The assay was highly sensitive, but poorly specific, for N^1,N^{12}-diacetylspermine, because of high cross-reactivity with acetylspermine and N^1-acetylspermidine.

The cross-reaction with acetylspermine, but not with N^1-acetylspermidine, may be overridden in testing human urine, because of the low content of the former and the higher levels of the latter relative to urinary N^1,N^{12}-diacetylspermine concentrations [193,194]. In 1998, this procedure was further improved by the same authors when they developed three monoclonal antibodies, ACSPM-1 to -3, against N^1,N^{12}-diacetylspermine, of which ACSPM-2 was the most specific, exhibiting only 0.85% cross-reaction with N^1-acetylspermidine [195]. In the same year, Hiramatsu et al. [196] succeeded in obtaining an antibody highly specific to N^1,N^{12}-diacetylspermine with only 0.03% cross-reactivity with N^1-acetylspermidine.

N^1,N^8-diacetylspermidine was determined with high sensitivity and selectivity only in 1997, when an ELISA system using a highly specific antibody was reported [197].

7. Critical evaluation of polyamine biological relevance

7.1. Cell differentiation and proliferation

The role of polyamines in cell differentiation and proliferation has been established mainly through experiments using polyamine synthesis inhibitors. Although the biosynthesis is accomplished by a coordinated involvement of four different enzymes [198], the inhibitors used most have been those specific for ODC, such as α-difluoromethylornithine (DFMO)—a potent enzyme-activated irreversible agent [199]—and for SAMDC, such as the spermidine analogue [200] methylglycoxal-bis guanylhydrazone (MGBG) [201]. Generally, to clarify the specific role of polyamines, experiments have been carried out by inhibiting the synthesis and reverting the observed inhibitory effects by adding all or single polyamines.

Other sets of experiments not involving the inhibition of synthesis can evaluate the effects of intracellular concentrations of polyamines on proliferative and differentiative processes. In fact, the cellular polyamine content is regulated, apart from the endogenous synthesis, by specific uptake into and release out of the cell [202–206].

While the role of polyamines in cellular growth and proliferation has been well assessed and established as similar and ubiquitous in all cell types, involvement in differentiation is dependent on the type of tissue and differentiating agent.

Similarly, different and controversial are the mechanisms by which polyamines interfere with the differentiative cell apparatus. For these reasons and because of the availability of reviews about the biological role of polyamines, only the most recent mammalian research is reported here.

In several cell types, among which are keratinocytes and condrocytes, polyamines are involved in the differentiative process by acting as amine donors and as a substrate of transglutaminase (TG), which establishes covalent cross-links between cytoskeletal proteins [207–213].

The involvement of polyamines on the glycosylation and secretory machinery of the cell has been well demonstrated [214]. In polyamine-deprived cells, the total glycosylation capacity was not affected, while the synthesis of a high-molecular-mass proteoglycan containing chondroitin and keratan sulfate was completely inhibited [215]. Since treatment with DFMO and another ODC inhibitor 1-aminooxy-3-amino-propane (APA) caused swelling of endoplasmic reticulum (ER) and medial and trans-Golgi cisternae and involution of rough ER, it was hypothesized that rough ER and cytoskeleton may be the target of the proliferative and differentiative effects of polyamines.

A positive role for polyamines in intestinal maturation has recently been elucidated. Dietary polyamines display direct as well as indirect trophic effects on rat's immature intestine as factors of luminal growth and maturation [216]. The mechanism by which the ingestion of spermine induces precocious maturation of rat small intestine, and particularly the increase in maltase- and sucrase-specific activities, has been hypothesized to be mediated by a T-cell/IL-2-dependent immune response [217].

If in many cell systems the differentiative process is positively regulated by polyamines, in many others it appears to be inhibited, such as in several tumoral cells. Of interest is the role of polyamines in the differentiation of tumoral cell lines, such as B16 melanoma-, colon carcinoma-, murine embryonal teratocarcinoma F9- and human leukaemia-cells. B16 melanoma cells treated with DFMO and the polyanionic drug suramin, which affects polyamine metabolism and transport, are induced to differentiate and to stop proliferation [218]. In HT-29 human colon carcinoma cells, the differentiation is regulated by polyamines in a more complex and diversified manner. While uptake of putrescine, spermidine and spermine was not modified in differentiating cells, putrescine synthesis and release were abolished [204]. The former phenomenon was due to a dramatic decrease in ODC activity and a marked retroinhibition of enzymic activity by endogenous polyamines. The latter has been hypothesized to be a compensatory mechanism occurring when endogenous synthesis of putrescine is reduced. Caco-2 cells offer a good model for studying the significance of polyamine cellular content on differentiation, since, in contrast to most tumor cell lines, they modify polyamine metabolism during differentiation [219]. An increase in spermine content may be toxic for the cells and may impair differentiation [219], while on the other hand, polyamine deficiency has been shown to inhibit both proliferation and differentiation [220]. Recently, it was found that during the differentiation of murine embryonal teratocarcinoma F9 stem cells induced by irreversible inhibition of the ODC activity, a c-Myc protein accumulation occurred [221]. In transfection experiments, using ODC promoter–reporter gene fusion constructs, the accumulation of c-Myc protein, resulting from polyamine depletion, led to increased reporter ODC gene expression, accounting for a previously unknown role for polyamines as negative regulators of c-Myc. Similarly, in the human leukemia cell line HL60, monodansylcadaverine, which binds to the active site of TG, annulled the positive effect of retinoic acid on differentiation [222], indicating that the polyamine interaction with TG in transformed and normal cells has different outcomes. Striking evidence for the polyamine association with cell growth has been well established by numerous findings in several physiological as well as pathological cell types [223–225]. Endothelial cell (EC) proliferation has been shown to be regulated by arginase I or arginase II activities through the production of ornithine for polyamine biosynthesis in bovine coronary venular EC transfection experiments. Addition of DFMO to the culture medium abolished the differences in putrescine concentrations and reduced those in spermidine content in EC transfected with rat arginase I cDNA (AI-EC), EC transfected with mouse arginase II cDNA (AII-EC) and EC control transfected with lacZ gene (lacZ-EC) [226], and therefore prevented the increase in AI-EC and AII-EC proliferation compared with lacZ-EC. Also, smooth muscle cell proliferation in rat aorta was increased by high levels of arginase I expression, as demonstrated by transfection experiments with two selective inhibitors of arginase, *N*(G)-hydroxy-L-arginine and *S*-(2-boronoethyl)-L-cysteine [227].

The mechanism by which polyamines regulate the growth of other cell lines is quite different. The untransformed intestinal crypt cell line (IEC-6) is arrested in the presence of DFMO in the G(1) phase

of the cell cycle as a consequence of the accumulation of p21, the product of Waf1/Cip1 gene, and p53, as well as the inhibition of cyclin-dependent kinase (CdK) 2 [228]. Transfection of human prostatic epithelial cells PNT1A and PNT2 with antisense ODC RNA or ODC antizyme (OAZ) cDNA decreased ODC activity and putrescine concentration, whereas transfection with SSAT cDNA induced a more marked inhibition of spermidine and spermine content. PNT1A and PNT2 cells transfected with all the three constructs are blocked in the S phase and in the G0/G1 or G2/M phases, respectively. Transfection of both cell lines with antisense ODC and/or OAZ cDNA resulted in cell accumulation in the S phase [229]. Bettuzzi et al. [230] showed an increase in polyamines from the G0 phase that reached a peak in the late G1/early S phase, and a slight decrease in spermidine and spermine and a drastic drop in putrescine in the mid S phase, allowing a better definition of the role played by polyamines in cell cycle progression, with respect to other studies [231–239].

In view of the essential role of polyamines in the proliferation of mammalian cells, understanding of the regulatory mechanisms of the metabolism and the rate-limiting enzymes of the polyamine pathway may be an important tool for the prevention of carcinogenesis. In a recent review, Shantz and Pegg [240] reported that ODC and SAMDC are both regulated at the translational level, but in a different manner. ODC regulation is dependent on the availability of the cap-binding subunit of the e IF-4F complex which mediates translation initiation, while SAMDC is regulated by the internal open reading frames (ORF) which block ribosomes and decrease the efficiency by which the downstream ORF encoding SAMDC protein is translated.

The significance of the polyamine content in cell growth prompted many studies to use their concentrations as a marker of neoplasia. But, surprisingly, despite the necessity of polyamines for cell proliferation and the activation of apoptosis by inhibitors of their synthesis [241–248], recent reports have indicated that they can activate apoptosis when they are present in excess [249,250] and depending on cell system signals [251–253]. An excess of spermine and spermidine, but not putrescine, activated the processing of pro-caspase-3 and the exit of cytochrome *c* from mitochondria in a cell-free model, suggesting that the activation of programmed cell death may be induced by polyamine toxicity [249]. Similar conclusions have been drawn by the same authors exposing human promyelocytic-leukemia cells to excessive intracellular levels of spermine [250]. Human promyelocytic leukemia cells undergoing 5,6-benzylidene-L-ascorbate-induced apoptosis displayed a decline in putrescine concentrations and unchanged levels of spermidine and spermine [251]. The behaviour of the human T-cell line induced to activate programmed cell death by dimethyl sulfoxide appears slightly different. They showed a decrease in ODC activity and polyamine content paralleled by a reduction in c-myc expression. The lack of apoptosis and the changes in c-myc expression, despite growth inhibition in DFMO-treated cells, suggested that the programmed cell death induced by dimethyl sulfoxide is mediated by a c-Myc-dependent decrease in ODC activity and polyamine concentrations [252]. During B-cell clonal deletion [253], the apoptosis induced by B-cell antigen receptor cross-linking was coupled with down-regulation of the genes involved in polyamine biosynthesis and up-regulation of the genes responsible for polyamine catabolism, leading to reduced levels of all three polyamines. The addition of spermine could repress the apoptosis by attenuating the potential loss of mitochondrial membrane and the activation of caspase-7-induced by B-cell receptor signaling.

The main catabolic enzyme of polyamines, PAO, has been shown to exert an opposite role in apoptosis. Although its activity decreased as the size and histological grade of human breast cancer increased, and the hydrogen peroxides produced by PAO were involved in inducing apoptosis [254,255], *N*,*N*′-bis (2,3-butadienyl)-1,4-butanediamine, an inhibitor of PAO, is able to induce apoptosis through the down-regulation of the antiapoptotic protein Bcl-X(L) [256].

All results obtained about the role of polyamines on the proliferation of normal and cancer cell lines have indicated that they are necessary for cell growth and that polyamine analogues and polyamine synthesis inhibitors may be effective agents in blocking tumor growth [257–265].

Therefore, the use of polyamine analogues to-

gether with the inhibitors of ODC activity and polyamine levels represents a novel therapeutic strategy in the in vivo treatment of tumors.

7.2. Polyamines as second messengers

It has been established for a long time that polyamines are messengers that regulate Ca^{2+} signals and mediate different Ca^{2+}-sensitive responses to hormones, neurotransmitters and extracellular stimuli. Their significance in mediating insulin, androgenic and thyroid hormones as well as β-adrenergic agonist effects was extensively surveyed by Koenig et al. [266]. As second messengers of extracellular stimuli they mediate calcium-related events at the cell membrane (putrescine) and at the mitochondria (spermine) of neuronal cells [267] and the effects of inotropic agents, namely ouabain, noradrenaline and calcium, on rat ventricle strips [268]. This latter role is significant, even though the basal isometric tension after treatment with polyamine synthesis inhibitors is similar to that in controls. Therefore, an efficient ODC/polyamine system has been postulated to be involved in calcium ion movements or in Ca^{2+} sensitivity. Experiments with murine splenocytes [269] highlighted an opposite effect of polyamines, especially putrescine, which inhibited the mitogen-stimulated Ca^{2+} influx in T cells. The more sensitive T-cell set to the putrescine effect appeared to be the CD4+, since cytoplasmatic free calcium in CD8+ lymphocytes was not affected by putrescine. Therefore, the effects of polyamines on calcium influx was not only agent-dependent, but also cell-type-dependent. This statement was confirmed by other studies that showed a putrescine-induced and protein kinase C-mediated enhancement of the L-type calcium channel activity in mouse neuroblastoma cells [270].

An inhibitory effect of spermidine on the increase in passive Ca^{2+} efflux promoted by trifluoroperazine has been observed in sarcoplasmic reticulum [271], but not in platelet vesicles [272], likely to be in relation to the different amino acid sequences of the two Ca^{2+} ATPases involved.

The effects of spermine on Ca^{2+} accumulation in mitochondria has been well known for many years [273–276], but only recently it has been demonstrated that spermine increases mitochondrial Ca^{2+} uptake via a new mechanism called RaM, responsible for sequestering Ca^{2+} from physiological transients or pulses [277]. Spermine has been shown to interfere with the phosphoinositide/Ca^{2+} signalling pathway, since it competes with calcium cations, present in the intracellular medium, in binding to negatively charged molecules like phosphatidylinositol 4,5-diphosphate [278], phosphatidylinositol 1,4,5-trisphosphate [279] and phosphatidylinositol 1,2,6-trisphosphate [280]. Since spermine interacts preferentially with phosphatidylinositol 4,5-diphosphate in comparison to phosphatidate and phosphatidylserine, the phosphorylation of phosphatidyl inositol modulates the membrane fusion activated by the spermine/acidic phospholipid interaction [278]. The formation of complexes between spermine and D-myo-inositol 1,4,5-trisphosphate probably leads to the inhibition of the promotion of Ca^{2+} release [279], whereas the interactions of D-myo-inositol 1,2,6-trisphosphate with spermine and zinc cations have been considered relevant in the regulation of biological processes [280]. But spermine plays a pivotal role in modulating the pathways controlled by inositolphosphatases also through mechanisms not involving complex formation. The inhibitory effect of spermine on inositol 1,4,5-trisphosphate 5′-phosphatase has been implicated in the inhibition of the thrombin-induced increase in cytoskeletal actin, mediated by inositol 1,4-bisphosphate [281].

The interference of spermine with the phosphoinositol/Ca^{2+} pathway in several systems stimulated investigations about the effect of spermine on the passive permeability of native alveolar epithelium and alveolar liquid absorption [282]. Both were increased, probably through an indirect mechanism involving a mediator responsible for the change, and inhibited by putrescine and spermidine. Of particular interest was the apparent concomitant increase in transepithelial Na^{+} transport and paracellular permeability, never seen with other agents, like hormones, cytokines, growth factors, and toxins acting on the alveolar epithelium. The well-known effect of spermine on the increase in *N*-methyl-D-aspartate subtype of glutamate receptors [282] has been excluded, because of the absence of edema produced in isolated lung by the stimulation of these receptors [283]. It has been postulated that spermine may act by decreasing the Ca^{2+}-release from in-

tracellular stores, since it progressively decreased the inositol 1,4,5-triphosphate concentration and its effects on permeability and alveolar liquid absorption were increased by a thiol agent, like mersalyl, that blocks inositol triphosphate-gated Ca^{2+} channels.

7.3. Tissue regeneration

Polyamines play a fundamental regulatory role in all the processes involving tissue regeneration, such as healing of stress-related ulcers [284–286], post-hepatectomy regeneration [287–292], compensatory renal growth [293,294] and compensatory lung growth after partial pneumonectomy [295–298].

The physiological biosynthesis of polyamines increased significantly in rats during the healing of stress-induced gastric mucosa erosions [286], and TG activity, whose polyamines are the substrates (as seen in Section 7.1), concomitantly markedly increased as a result [284]. Treatment with DFMO (500 mg/kg), completely inhibiting the significant increases in both ODC activity and polyamine synthesis, led to the blockage of mucosa repair [285], while the oral administration of spermidine (100 mg/kg) immediately after stress prevented the inhibition of mucosa healing [284]. Stressed mucosa also exhibited increased expression of the proto-oncogenes c-fos and c-myc, which decreased after DFMO administration. These findings account for the involvement of c-fos and c-myc in the mechanism of polyamine-stimulated healing of gastric mucosal stress ulcers [285], apart from the role played by these proto-oncogenes in the regulation of proliferation and apoptosis [221,252].

The significance of ODC expression and polyamine concentrations on rat liver regeneration has been discussed for years without definitive data being reached. Some reports indicated that both ODC activity and polyamine levels (primarily putrescine and spermidine) increased substantially and proportion-dependently [289,292] during liver regeneration after partial hepatectomy in the rat [287,288]. More recent data by Beyer et al. [290] show that, although during liver regeneration ODC activity, putrescine and spermidine increased significantly, treatment with DFMO did not modify the integrity of the regeneration process. Another study on the mechanisms by which 1-year-old rats had lower levels of hepatic regeneration showed that, although ODC mRNA content, enzyme activity and polyamine levels were lower than in 6-week-old rats, the supplementation of putrescine to older rats did not change the rate of regeneration [290]. A report from a Japanese surgery group partly contradicted the above mentioned results, since it pointed out a novel role for putrescine in the regenerative ability of the rat liver after ischemia and hepatectomy. The increase in lipid peroxide levels in DFMO-treated rats which underwent induced ischemia and hepatectomy and the corresponding decrease in [^{3}H]thymidine incorporation together with a reverse response induced by the administration of putrescine suggest that putrescine suppressed the production of lipid peroxides and promoted DNA synthesis [291]. Data from other laboratories have confirmed the essential role of putrescine in liver regeneration, since they showed that exogenous putrescine was able to stimulate liver regeneration following liver failure and alcohol-induced injury [299,300], but not cirrhosis [301]. Moreover, putrescine administration to rats was able to ameliorate the diminished hepatocyte regenerative capacity induced by cadmium treatment by shifting the first burst of DNA synthesis earlier [302]. The thymidine-kinase activity inhibited by cadmium administration was improved, together with the rate of hepatic DNA synthesis, by intraperitoneal injection of putrescine during the initial phase after partial hepatectomy.

Also, the inhibitory effect on DNA biosynthesis induced by IFN-α was paralleled by the suppression of ODC activity and the reduction of putrescine levels in regenerating mouse liver after partial hepatectomy and was reversed by putrescine administration [303]. But putrescine administered in partially hepatectomized rats treated simultaneously with IFN-α, even though it significantly increased thymidine-kinase activity, did not affect the rate of DNA synthesis [304]. These data and the lack of correlation between the alterations of thymidine-kinase activity in IFN-α-treated rats and the liver regenerative status showed that in this system hepatic regeneration is affected by IFN-α independently of its action on liver thymidine-kinase activity and putrescine levels. Putrescine has also been implicated as a mediator of the human and recombinant human hepatocyte growth factor effects on the stimulation

of DNA synthesis after 70% hepatectomy in rats [305].

A direct role of ODC in renal hypertrophy [306,307] has been proposed, but contrasting results have been obtained on the significance of polyamine concentrations in regulating the compensatory kidney response [293,307–313]. In fact, even though ODC activity [314] and the renal levels of putrescine increased after unilateral nephrectomy at the same time as the increase in the weight of the remaining kidney, the treatment of rats with DFMO did not impair compensatory renal hypertrophy. The inhibition of both baseline renal ODC activity and putrescine levels as well as the unilateral nephrectomy-induced increases had no effect on the hypertrophic renal response [293]. Other models of renal hypertrophy in mice have shown that testosterone-induced kidney enlargement [294] and kidney growth in pubertal mice [315] were not inhibited by DFMO, although a dramatic increase in renal ODC was observed as an effect of the testosterone treatment [294]. Similar results have been obtained in renal hypertrophy in mice with thyroxine, fluorocortisone and potassium depletion [294]. Data obtained on compensatory renal growth in mice, showing a lack of ODC activity increase despite the increase in contralateral kidney weight [294], did not agree with those obtained in rats [306,314]. The testosterone-induced ODC increase in mouse kidney without an impairment of hypertrophy after DFMO treatment has been speculated to be involved in biological processes other than renal growth [294], and the discrepancies between data from rats and mice have been explained in the light of differences in the mechanism of the ODC response [316].

Polyamine involvement in the repair processes of the lung, either following lobectomy or after exposure to toxic drugs, has been well evaluated by Bardocz [317], and since 1989, to our knowledge, few more recent findings have been published in this regard.

For a long time, it has been known that the rapid compensatory growth of the remaining lung after unilateral pneumonectomy and the post-operative increases in lung mass are preceded by enhanced uptake of exogenous spermidine and by the increase in cAMP-dependent protein kinase activity [296,297]. Moreover, 7 days after left pneumonectomy, and during the rapid compensatory growth of the right lung, type II pneumocytes incorporated thymidine at a rate 224% greater than cells isolated from control rats and increased the uptake of exogenous spermidine by 50% through enhancement of the maximal velocity of transport [295]. The uptake of spermidine and the exogenous polyamine substrate analogue MGBG increased by partial pneumonectomy were doubled in rats adrenalectomized 5 days before left partial pneumonectomy, in parallel with an increased rate of lung restoration [298].

7.4. Neoplastic growth

The involvement of polyamines and ODC in tumor promotion has been widely accepted. Data from several sources are available on polyamine metabolism in human neoplastic lesions, such as carcinoma of the breast [11,318], esophageus [319], stomach [13,320], colon [13,321], and liver [322,323], and a correlation between ODC activity and growth rates of several hepatoma cell lines has been known for a long time [324]. The mechanisms by which polyamines exert the tumorigenic effect may, however, be quite different. Transformation of the epithelial cells in skin of ODC Ras double transgenic mice is characterized by an exceptionally high histone acetyltransferase activity with specificity for lysine-12 in the tail domain of histone H4 involved in gene transcription. Treatment with DFMO induced tumor regression and reversed the histone acetyltransferase effects, indicating that polyamine biosynthesis is implicated in the regulation of histone acetylation, even indirectly, since polyamines do not directly stimulate the enzymatic activity of either p300 or p300/CREB-binding protein (CBP)-associated classes of histone acetyltransferase [25]. On the basis of these results, multiple effects of polyamines on the chromatin structure, the gene expression and the promotion of the neoplastic process have been postulated. A different aspect of polyamine involvement in neoplasia has been elucidated for adenocarcinoma of the prostate gland. Spermine and spermidine were the major cations found in the eosinophilic prostatic secretory granules (PSG) that are lost in neoplasia. In untreated carcinoma, the decrease in spermine/spermidine was directly related to loss of PSG, but after chemical

castration, surviving malignant cells were positive for spermine/spermidine, despite the significant reduction or absence of PSG. Therefore, the physiological arrest of polyamine production induced by androgen blockade is ineffective in androgen-resistant tumor clones that continue to synthesize spermine and spermidine, uncoupled from hormone regulation [24]. In addition to the different mechanisms by which polyamines affect tumorigenesis, the regional distribution of ODC and the entity of polyamine concentrations have been related to the degree of malignancy. In experimental gliomas of cats, ODC activity increased within the tumor in parallel with a high number of mitoses, while putrescine levels increased in the whole tumor and the peritumoral edema. These results have stressed the relevance of ODC as a biochemical marker of proliferation in brain tumors [23]. A prospective study about the states of DNA, RNA and protein in human uterine, breast and rectal cancers has shown an increase in the levels of all the parameters in carcinomatous tissues in comparison to their respective adjacent normal tissue, implicating the increased synthesis of putrescine in neoplasia [22]. The polyamine accumulation in cancer cells and the overproduction of ODC have been shown to elicit the acquisition of the transformed phenotype, and that polyamines can trigger the transformation and are not only secondary by-products has been recently confirmed [325]. Stimulation of c-myc and c-fos proto-oncogene expression was preferentially operated by spermidine and putrescine, respectively. ODC overexpression alone was sufficient in activating hair follicle keratinocytes to expand clonally to form epidermal tumors. In fact, in transgenic mice it induced a greater sensitivity to initiation with a single low dose of carcinogen in respect to littermate controls and they did not require further treatment with tumor promoters for tumors to develop [326].

The usefulness of polyamines as markers of malignancy in human carcinoma has been reported for some time. The spermidine/spermine ratio in colo-rectal cancer correlated with the histologic grade of the tumor [327]; elevated spermine concentrations correlated with the histology of renal cell carcinoma [328], and more recently, ODC mRNA expression correlated with malignancy of breast cancer [318]. A direct relationship of polyamine metabolism to the degree of malignancy of human hepatocellular carcinoma (tested by the tumor volume doubling time, the differentiation of the tumor, the prevalence of portal invasion and the intrahepatic metastasis) has been well established [18]. ODC activity was significantly higher in moderately and poorly differentiated human hepatocellular carcinoma and in patients with portal invasion or intrahepatic metastasis. In these patients, the spermidine/spermine ratio was also significantly higher. ODC activity and the spermidine/spermine ratio were well correlated too with tumor volume doubling time, indicating that they may be clinically useful indices of tumor malignancy. Polyamine—especially cadaverine and spermidine—levels in ascites have been shown to be one of the cancer-associated markers, since their increase in malignant ascites was significantly higher than that in cirrhosis and tuberculosis [17]. And in breast cancer staging the ratio of N1-acetyl spermine/spermidine was a useful marker together with the ratio of 16alpha-OHE1/2-OHE1, so it was suggested that the alteration in polyamine oxidase activity may play an important role in the development of breast cancer [329]. Deacetylated spermidine and spermine in human urine increased significantly in patients with urogenital malignancies and decreased during treatment and partial remission [27].

Normal or near-normal values after treatment were considered an index of a good prognosis whereas an increase during the period of follow-up indicated the recurrence of a cancer or the onset of a second one. The close correlation of polyamine synthesis with pre-malignant and malignant stages of cancer development was confirmed by the administration of a single oral dose of ornithine in rats treated with the carcinogen 1,2-dimethylhydrazine. Putrescine, spermidine and spermine blood concentrations significantly increased with respect to control rats, indicating the enhancement of ODC activity and the validity of blood polyamine levels as predictive markers of cancer development [330].

Reports about successful cancer therapies and prevention with suppressors of polyamine biosynthesis [20,21,331] and/or polyamine analogues able to induce SSAT activity, and therefore the catabolic polyamine pathway, are numerous. DFMO in combination with tritiated radioemitters or cytotoxic

agents, such as MGBG, was shown to be an effective antitumor agent, especially when the exogenous polyamines were eliminated, even though brain tumor growth recurred upon termination of treatment [332]. In contrast, competitive polyamine analogues continued to inhibit the growth of brain tumors for several weeks after cessation of treatment. The enforcement of DFMO efficacy in blocking tumor growth by the depletion of the polyamine source has been proved also in MCF-7 human breast cancer cells in mice, since the blockade of the gastrointestinal supply of polyamines enhanced the intracellular polyamine depletion induced by DFMO [19]. Complete polyamine deprivation by DFMO combined with inhibition of the bacterial production of gastrointestinal polyamine and a polyamine-free regimen was effective against tumor growth, both for a standard MCF-7 tumor and a variant MCF-7 tumor with a tamoxifen-acquired resistance [333]. Therefore, it has been hypothesized that the ability of polyamine deprivation to inhibit the growth also of those tumors becoming tamoxifen-resistant—a common outcome of the hormonal therapy for advanced breast cancer—could offer a therapeutic advantage. Several kinds of polyamine analogues have been shown to effectively inhibit solid tumor growth and stages in breast cancer, in addition to preclinical studies aimed at examining combinations of conventional cytotoxics, phase II trials to determine the efficacy of polyamine analogues are in progress [334]. N^1,N^{11}-Bis(ethyl)norspermine is a potent inducer of a high expression of SSAT in human primary lung cancer, and its detection in clinical specimens has been proposed as a prognostic indicator of drug response [335].

Moreover, SSAT induction has been shown to prolong inhibition of tumor growth and to induce long-term tumor regression of different human solid tumor xenografts [336]. Another polyamine analogue, BE-4-4-4-4, was shown to inhibit ODC activity and to deplete all three polyamines, indicating that it is more effective as an antiproliferative and cytotoxic agent than DFMO against human carcinoid tumors [337].

An indirect demonstration that aberrations in polyamine biosynthesis are closely related to the development of tumors has been provided also by the usefulness of vitamin E as a suppressor of the development of lung tumors through the modulation of polyamine biosynthesis and cell proliferation during the initiation and promotion phases of urethane-induced lung carcinogenesis in mice [338].

7.5. Polyamine significance in other physiological and pathological conditions

Several studies have been carried out on the correlation of physiological life processes (such as circadian rhythm, age and sex, as well as the menstrual cycle and reproductive system) with polyamine metabolism.

Significant fluctuations in cellular polyamine levels were found throughout the day in male rats fed ad libitum and maintained in a 12-h light, 12-h dark cycle (lights on at 07:00 h). A peak of putrescine levels was found in liver at 24:00 h and in testicular seminiferous tubules late in the dark phase. In the Harderian glands or in pineal glands, spermidine and spermine contents reached a maximum at 06:00 h (late in the dark phase), while in the anterior pituitary gland the peak was observed at 18:00 h (late in the light phase) [339]. A circadian rhythmicity in the urinary levels of total polyamines and N^1- and N^8-acetylspermidine from healthy individuals was described by Poyhonen et al. [340]. The analysis of 24-h excretion, split into four consecutive periods, demonstrated that the excretion rates of these polyamines were highest in the morning. Most authors have determined the total urinary polyamine excretion excluding cadaverine, since it has been shown that the large physiological changes occurring in urinary polyamine excretion were related to cadaverine level variations [341]. Recent studies have examined the concentration and secretion of polyamines in colostrum and milk of dairy goats during 90 days of lactation and have shown that the spermidine content in milk sampled in the evening was usually higher than in that from morning milking [342].

Contrasting opinions have been reported about the effect of aging and sex on the polyamine biosynthesis pathway. In 1980, Beninati et al. reported that age was able to influence the excretion of spermine in male subjects and that sex significantly affected the urinary content of polyamines, resulting in higher excretion of putrescine and spermine in female

subjects than in males [343]. A recent work [340] did not show any correlation between polyamine excretion and age, but did show marked sex-dependent variations, since excretion of spermidine, N^8-acetylspermidine and spermine were higher in men and excretion of putrescine was higher in women. Examination of the polyamine distribution in neurologically normal autopsied human brain showed that spermidine levels in the occipital cortex increased markedly from birth, reaching maximal levels at 40 years of age [344] and that SAMDC activity increased by 600% from age 6 months to near maximal levels at age 10 years [345], accounting for a selective role of spermidine in the functions of developing and mature brain. In contrast to these observations, other data showed a consistent negative correlation between both spermine and spermidine content in different brain areas and age that was maintained in many degenerative movement disorders. Only in Huntington's disease was a decrease in the concentration of spermine in the putamen observed, suggesting an imbalance of polyamine content only in the presence of severe atrophy. Age and sex had a great influence also on the effects of testosterone on mouse renal PAO and DAO activities that at the age of 20 days were higher in males than in females, while during the maturational processes of kidneys increased mainly in females. Therefore, testosterone was able not only to stimulate biosynthesis of polyamines, but also to modulate their catabolism [346].

The relationship between the fluctuations in both ODC activity and polyamine levels and the different phases of the menstrual cycle and pregnancy has been extensively studied.

Since 1978, it has been known that the excretion of all the polyamines is higher during the menstrual period in parallel with the necrosis of endometrial cells [347]. A subsequent finding [348] reported that the urinary excretion of putrescine and spermidine appeared more marked during the follicular phase, while spermine excretion levels were higher during the luteal phase. According to Molinka and Gunpide [349], ODC activity was significantly higher in human proliferative endometrium during the oestrogen-dominated follicular phase than in secretory endometrium after the formation of the progesterone-secreting corpus luteum. Opposite results have been obtained more recently by Poyhonem et al. [340], who did not find any correlation between polyamine excretion and the menstrual cycle.

The data about the role of ODC and polyamines in pregnancy are indeed unanimous, in that ODC activity is essential in the early stages of mammalian embryogenesis [350,351] and DFMO has a marked contragestational effect when administered during pregnancy [350,352,353]. Polyamine concentrations increased in the plasma and urine of pregnant women as a function of gestational age [354], and their accumulation in human placenta was related to the extensive protein synthesis required for growth and the production of hormones occurring in the early events of placental development and in the later part of the pregnancy [354]. The regulatory role of polyamines in pregnancy is also supported by the fact that the contragestational effect induced in rats by moderate hyperthermia during a critical period of gestation was completely prevented by exogenous administration of polyamines [355], even though hyperthermia may affect many other cellular targets [356,357].

Recently, Mehrotra et al. [358] demonstrated that during the peri- and immediate post-implantation stages of pregnancy, SAMDC inhibitor failed to arrest the pregnancy in the hamster, while PAO inhibitor was 100% effective [358]. It has been postulated that this enzyme affects the regulatory processes of maternal immune reactions, preventing rejection of the fetus on the basis that PAO suppresses mitogen-stimulated lymphocyte proliferation [359,360] and that very low PAO activity in the serum of human subjects was associated with spontaneous abortion [361].

Moreover, it was shown that in mice spermine increased on day 9 and spermidine on day 8 of pregnancy [362] together with morphogenesis of the ectoplacental cone [363].

The relevance of polyamines in pregnancy was also demonstrated by the effects of DL111-IT, a new hormonal contragestational agent, on ODC activity and polyamine levels in hamsters and rats. Both decreased significantly, leading to degeneration and dissolution of decidual cells [364] and to the early arrest of pregnancy [365].

Alterations in intracellular and extracellular polyamines in non-tumoral pathological conditions, such

as psoriasis, muscular dystrophy, systemic lupus erythematosus, cystic fibrosis, uremia, liver cirrhosis and periodontal diseases have been extensively demonstrated.

Psoriasis, a disease characterized by benign but unrestricted epidermal proliferation, shows increased local ODC activity [35], elevated systemic polyamine levels and increased urinary polyamine excretion [33]. Epidermal shave biopsies showed that putrescine levels were higher in involved psoriasis than in uninvolved and normal skin, whereas spermidine and spermine were increased in both psoriasis and uninvolved skin areas compared with normals.

Moreover, the spermidine/spermine ratio was greater in involved skin than uninvolved and normal epidermal sites [32]. Cultured human keratinocytes obtained by suction blisters from normal skin and uninvolved skin of psoriatic patients showed a marked increase in spermidine and spermine during the entire period of culture and a transient rise in putrescine only at the beginning of the active growth phase. Treatment with DMFO decreased the concentrations of putrescine and spermidine in both normal and uninvolved psoriatic keratinocytes, but had no effect on either DNA or protein synthesis.

Although no significant differences have been observed between the cells derived from normal and uninvolved psoriatic epidermis, the psoriatic keratinocytes appeared to be more sensitive to the action of polyamine antimetabolites [34]. In fact, differently from normal keratinocytes, cellular macromolecule synthesis was profoundly inhibited by the synergistic effect of DFMO and the antimetabolite MGB.

Abnormalities of polyamine metabolism have been found in the hereditary muscular dystrophies. Increased urinary excretion of spermine, spermidine and putrescine as well as elevated erythrocyte spermine levels in patients affected by Duchenne Muscular Dystrophy (DMD) were reported many years ago [62,63]. Also, analysis of DMD muscle samples revealed elevated concentrations of putrescine and spermidine and normal spermine content [61]. In a more recent investigation, polyamine concentrations were significantly more elevated in polymyopathic (dystrophic) hamster tissue relative to their age-matched controls and in dystrophic hamster myoblast and fibroblast primary cultures compared to cultures from normal hamsters. In contrast, no significant dystrophy-related abnormalities in ODC or SAMDC activities were noticed [64]. These data suggest that the elevated polyamine contents of dystrophic hamster tissue and primary cultures may be due to a deficiency in polyamine catabolism or transport.

Altered levels of polyamines in the blood and urine as well as in culture medium of cells from patients affected by cystic fibrosis were reported many years ago [50–54] and to our knowledge more recent studies in this regard are not available.

Recent in vitro studies have demonstrated that, although free polyamines did not have any toxic effect on the organotype cultures, they inhibited VERO (fibroblast-like cells) proliferation in the presence of dialysates from uremic patients containing toxic stimuli, even at low concentrations [42]. A toxic effect of polyamine-protein conjugates (PPC_s), synthesized in vitro from the plasma of hemodialyzed patients, on the proliferation of the erythroid colony-forming units (CFU-E) has been shown [43].

In non-malignant liver diseases, contrasting results are available about polyamine concentrations in plasma, urine and ascitic fluid. The increased putrescine plasma levels in about 60% of patients affected by cirrhosis found by Desser [45] have not been confirmed by others. Some reported no modifications in polyamine levels [46] and others only increases in spermine concentrations [47]. More recent studies have shown a significant increase, not related to the severity of liver disease, in the urinary excretion of all free and monoacetylated polyamines in cirrhotic patients, with or without diabetes or impaired glucose tolerance [48]. Other data showed that the percentages of N_1-acetylspermidine and putrescine were elevated, whereas spermine levels were decreased in the hepatic tissues of patients with fulminant hepatitis and liver cirrhosis [49].

Polyamine metabolism is altered in some autoimmune disorders, especially systemic lupus erythematosus, in which the increased levels of polyamines are responsible for the disruption of chromatin structure, hampering repair of damaged DNA sites and apoptosis, by interacting with nucleus and membrane [39]. Earlier studies of the effects of DFMO on lupus nephritis revealed a post-transcriptional modification of ODC which might be responsible for the increased ODC activity and polyamine

concentrations found in the kidney [37,38] and in the spleen [40] of MRL-lpr/lpr (lpr) mice, which spontaneously develop a lupus-like syndrome, with respect to other strains. The increased levels of polyamines in splenic T cells contributed to the defective signal-transduction pathways and the pathogenesis of symptoms [40], while DFMO treatment resulted in an increase in the expression of Fas mRNA in the thymus and the beneficial effects on these mice [41].

Few, but interesting, data are available on the relationship between polyamine metabolism and periodontal diseases. In ligature-induced periodontitis of dogs, both putrescine levels and ODC activity increased significantly, while no noticeable increment in spermine and spermidine concentrations occurred. When the ligature was removed, a sharp rise in spermine and spermidine content was observed [366]. Moreover, putrescine was the only polyamine detectable in gingival crevicular fluid of patients with gingivitis or periodontitis, demonstrating a significant role of this polyamine in the periodontium [367]. Studies by us, aimed at defining the real in vivo biocompatibility of Ni–Ti alloy for long periods of time, showed that spermine and spermidine salivary levels increased significantly only during the pubertal period, in parallel with the maximum levels of fertility hormones, while putrescine levels did not display any variation during the entire period of 1 year of orthodontic treatment [368]. Our further investigations showed that these increases in spermine and spermidine content were significant long before the onset of a gingivopathy revealed by a high gingival index, indicating that the salivary amounts of spermine and spermidine may be considered as an earlier indicator of gingival hyperplasia than the gingival index (submitted paper). We also showed that in human gingival tissue, spermine and spermidine concentrations were significantly higher in patients suffering from gingivitis hyperplastica, while putrescine significantly increased only in the course of periodontitis.

A selective role for spermine has been described in the metabolism of glucose. In diabetic rats, the observed decrease in pancreatic putrescine levels was restored with L-arginine, indicating that this polyamine may be used in the regenerating processes or for recovering the endocrine pancreatic function [57]. In vitro studies by Welsh and Sjoholm [55] showed that only spermine was able to stimulate (pro)insulin biosynthesis and to increase insulin m-RNA content, while depletion in the putrescine and spermidine concentrations of islets did not affect insulin release in response to glucose. Spermine involvement in modulating insulin binding [56] and in proliferation and insulin production by normal and tumoral pancreatic β-cells has also been well established [58–60].

Recent unpublished studies by us, corroborating the exclusive role for spermine in the metabolism of glucose and diabetes mellitus, showed significant variations of spermine in the vitreous fluids of patients affected by proliferative diabetic retinopathy (submitted paper).

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