Biochem. J. (1981) **200**, 123–132
Printed in Great Britain

On the formation of amino acids deriving from spermidine and spermine

Nikolaus SEILER, Bernd KNÖDGEN and Maurice W. GITTOS
Centre de Recherche Merrell International, 16 rue d'Ankara, 67084 Strasbourg-cedex, France

Wai Yee CHAN, Guy GRIESMANN and Owen M. RENNERT
Department of Pediatrics, Children's Memorial Hospital, The University of Oklahoma Health Services Center, P.O. Box 26901, Oklahoma City, OK 79290, U.S.A.

(*Received 1 May 1981/Accepted 17 June 1981*)

Evidence obtained from experiments with rats and mice is presented suggesting that the naturally occurring amino acids putreanine and N^8-(2-carboxyethyl)spermidine, and most probably also related compounds deriving from the polyamines spermidine and spermine by oxidative metabolism, are formed within two anatomical compartments. In the first step polyamines are converted into aldehydes by serum spermine oxidase in the circulation. A certain portion of these aldehydes can be taken up by liver and other organs and transformed by aldehyde dehydrogenase into the corresponding amino acids. Putreanine is not only derived from spermidine, but can also be formed from N^8-(2-carboxyethyl)spermidine by oxidative deamination, catalysed by serum spermine oxidase, and subsequent spontaneous elimination of acrolein.

In the course of the last decade a number of amino acids have been detected in tissues and urine that are obviously oxidative metabolites of spermidine and spermine. Putreanine [*N*-(4-aminobutyl)-3-aminopropionic acid; N^1-(2-carboxyethyl)-1,4-diaminobutane] was first detected in rat brain (Kakimoto *et al.*, 1969; Shiba & Kaneko, 1969) and was subsequently found in human (Perry *et al.*, 1972; Kremzner, 1973) and monkey brain (Kremzner & Sturman, 1979) and in various organs of rat and rabbit (Nakajima & Matsuoka, 1971; Nakajima, 1973). Furthermore it is formed in mouse neuroblastoma cell cultures (Kremzner, 1973; Kremzner *et al.*, 1975). Putreanine, isoputreanine [*N*-(3-aminopropyl)-4-aminobutyric acid; N^1-(3-carboxypropyl)-1,3-diaminopropane] and the spermine derivatives N^8-(2-carboxyethyl)spermidine [N^1-(3-aminopropyl)-N^4-(2-carboxyethyl)-1,4-diaminobutane] and spermic acid [N^1N^4-bis-(2-carboxyethyl)-1,4-diaminobutane] are constituents of rat urine (Noto *et al.*, 1978; Asatoor, 1979). The latter compound was also found in bovine brain (Imaoka & Matsuoka, 1974).

The intraventricular injection of labelled putrescine or spermidine led to the detection of labelled putreanine in brain; intraperitoneal injections resulted in the occurrence of radioactive putreanine and isoputreanine in liver and urine (Nakajima, 1973; Noto *et al.*, 1978). Intraperitoneal [^{14}C]spermine injections produced the corresponding labelled mono- and di-carboxylic acids, which were found in the urine (Noto *et al.*, 1978).

Although the precursor–product relationships are clear, nothing is known of the enzymes involved in the metabolic transformations of spermidine and spermine, which lead to the formation of their amino acid derivatives. Several authors (Kakimoto *et al.*, 1969; Nakajima, 1973) have speculated that an oxidase analogous to serum spermine oxidase (Blaschko, 1962), which can form a monoaldehyde from spermidine and a mono- or di-aldehyde from spermine, may catalyse the first step. These aldehydes spontaneously eliminate acrolein (Tabor *et al.*, 1964). But the corresponding amino acids could be formed from these aldehydes by the action of an aldehyde dehydrogenase, before acrolein elimination took place (Fig. 1). However, a tissue oxidase having these properties has not been demonstrated (Morgan, 1980).

In the present paper we furnish evidence that the formation of the acidic polyamine derivatives is the result of metabolic events taking place in two different anatomical compartments; the first step, namely oxidative deamination of the polyamines, occurs in the circulation; some of the aldehydes thus formed enter the cellular compartment and are intracellularly transformed into the corresponding acids.

0306-3283/81/100123-10$01.50/1

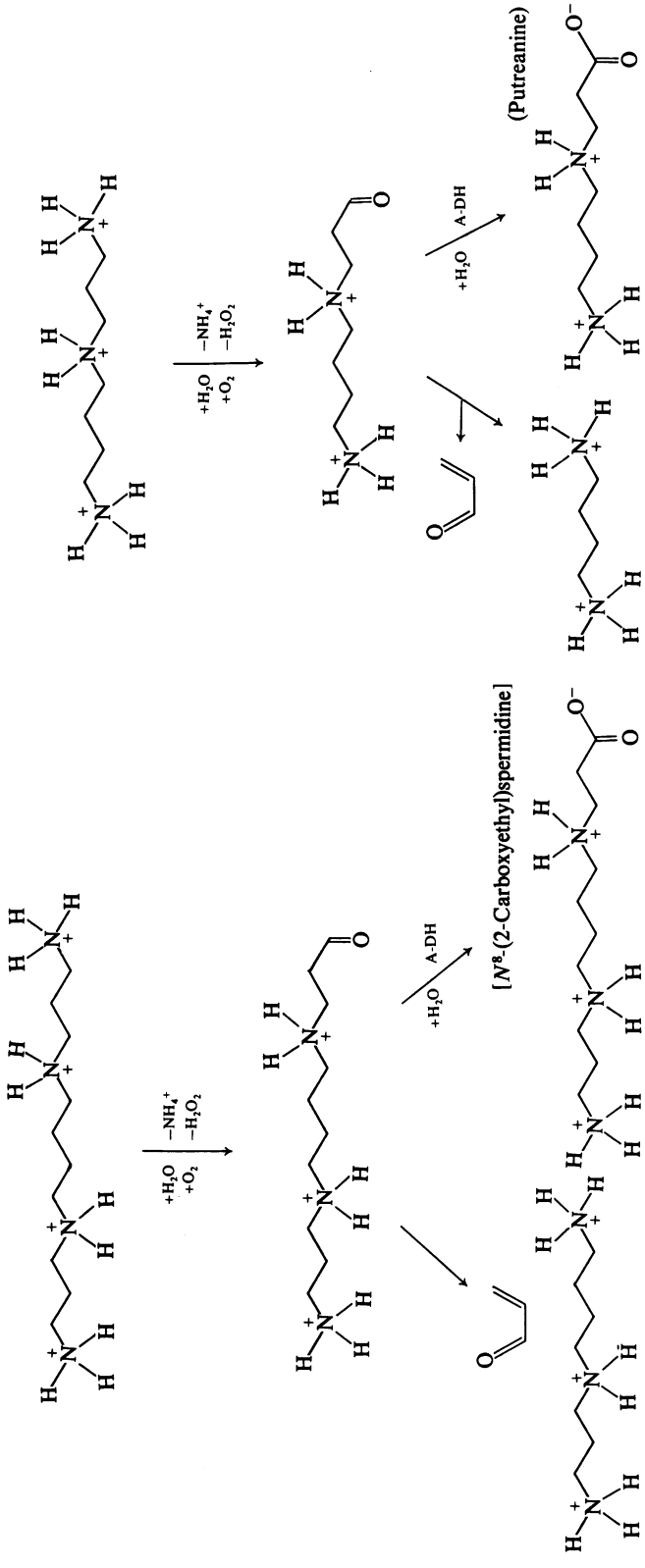

Fig. 1. *Reactions involved in the transformation of spermine into N^8-(2-carboxyethyl)spermidine and spermidine and of spermidine into putreanine and putrescine*

Abbreviation used: A-DH, aldehyde dehydrogenase. The oxidative steps forming the aldehydes are catalysed by serum spermine oxidase; the elimination of acrolein is a spontaneous, non-enzymic reaction (Tabor *et al.*, 1964).

Materials and methods

Determination of polyamines and their acidic metabolites

(*a*) *Liquid chromatography.* For the determination of the polyamines and of N^8-(2-carboxyethyl)spermine a previously published method was used that in essence separates on a reversed-phase column the ion pairs formed between the amines and octane sulphonate (Seiler & Knödgen, 1980). For quantification the reaction of primary amines with *o*-phthalaldehyde and 2-mercaptoethanol (Roth & Hampai, 1973) was utilized with continuous monitoring of fluorescence at 455nm (fluorescence excitation at 345nm).

Putreanine was determined by using [*tetramethylene*-1,4-^{14}C]spermidine as precursor. Samples were separated with a Durrum amino acid analyser by using the elution program designed by Folk *et al.* (1980). Fractions were collected every 1.5min and radioactivity was counted by liquid-scintillation spectrometry. Calculations of amount were made on the assumption that the specific radioactivities of putreanine and of its precursor spermidine were identical.

(*b*) *Dansylation procedure.* Reaction with dansyl (5-dimethylaminonaphthalene-1-sulphonyl) chloride was carried out as previously described (Seiler, 1970, 1975). After excess of reagent had been allowed to react by addition of 50μl of an aq. 40% solution of dimethylamine, the dansyl derivatives were extracted from a reaction mixture, consisting of 0.3ml of aqueous phase and 0.9ml of acetone, by addition of 2ml of ethyl acetate and after thorough mixing with 1ml of toluene. Samples (3.5ml) of the organic layer were applied on small alumina columns. These were prepared by introducing 400mg of alumina (basic, cationotropic; activity grade I; Woelm, Eschwege, Germany) into 1ml pipette tips, which had a cotton wool plug at the constricted end. To elute dansylated polyamines and dansylated acetylpolyamines the columns were washed with 3ml of methanol. Elution of bis-dansylputreanine and trisdansyl-N^8-(2-carboxyethyl)spermidine was achieved with 3ml of methanol/conc. NH_3 (4:1, v/v). The residue of this eluate was dissolved in 0.2ml of ethyl acetate and then 0.1ml of toluene was added. Portions of this solution were applied on silica-gel plates (20cm × 20cm; silica-gel 60; Merck, Darmstadt, Germany). For the determination of N^8-(2-carboxyethyl)spermidine the plates were developed with chloroform/tetrachloromethane/methanol (14:6:1, by vol.) (two runs). For putreanine determinations, development with chloroform/acetic acid/water (10:9:1, by vol.) (one run) was found to be more advantageous. The polyamines and acetylpolyamines can be determined in portions of the combined ethyl acetate and methanol fractions in the usual manner (Seiler & Knödgen, 1979).

Determination of serum spermine oxidase activity

The activity of this enzyme in calf serum was determined by using spermine tetrahydrochloride or spermidine trihydrochloride as substrates, and measurement of peroxide formation by the method of Snyder & Hendley (1968). The incubation mixture consisted of 2000μl of incubation buffer, 50μl of newborn-calf serum, 200μl of substrate solution and 750μl of the appropriate oxidase inhibitor solution. The incubation buffer contained 8mg of homovanillic acid and 2mg of horseradish peroxidase per 100ml of Sörensen phosphate buffer, pH 7.4. The substrate concentration was 0.5mM in the final incubation mixture. Incubations were carried out for 60min at 37°C. After cooling to 0°C fluorescence intensity was directly measured; activation was at 315nm, and maximum fluorescence emission was at 425nm.

Formation of putreanine and N^8-(2-carboxyethyl)-spermidine from spermidine and spermine respectively in vitro

Homogenates were prepared from rat or mouse livers, which were perfused with ice-cold 0.9% NaCl solution before excision. Usually the incubation mixtures consisted of 200μl of homogenate (1:3, w/v) in Sörensen phosphate buffer, pH 7.4, 60μl of serum (freshly prepared mouse serum, or newborn-calf serum), 20μl of substrate solution and 20μl of inhibitor solution (or buffer). The substrate concentration was 1mM, unless otherwise stated. Incubations were carried out at 37°C, usually for 30min. Putreanine determinations were performed using the dansylation procedure; in some experiments [*tetramethylene*-1,4-^{14}C]spermidine (sp. radioactivity 13Ci/mol) was used as substrate, and the above liquid chromatographic procedure was applied. N^8-(2-carboxyethyl)spermidine was determined by reversed-phase liquid chromatography.

Laboratory animals

Male albino CD1 mice (30 ± 2g) and Sprague–Dawley rats (300 ± 50g) were from Charles River, Saint Aubin-les-Elbeuf, France. Male Cox (SW) mice, weighing 34–36g, were purchased from the Laboratory Supply Company, Indianapolis, IN, U.S.A.

All animals had access to standard diet and water *ab libitum* throughout the experiments. The experiments were carried out during the natural 12h light period of the animals.

Chemicals

Usual laboratory chemicals were from Baker Chemicals, Deventer, the Netherlands, or from

Merck, Darmstadt, Germany. Putreanine, H_2SO_4,-$\frac{1}{2}H_2O$ was purchased from CalBiochem, Los Angles, CA, U.S.A.; aminoguanidine sulphate was from Schuchardt, München, Germany; pargyline hydrochloride was from Abbot Laboratories, North Chicago, IL, U.S.A.; marsilid (iproniazid) was from Hoffmann-La Roche, Grenzach, Germany; 3-hydroxybenzyloxyamine phosphate was from Sandev, Harlow, Essex, U.K. Aminoguanidine sulphate is an inhibitor of diamine oxidase (Schuler, 1952) and was shown in the present work also to be an inhibitor of serum spermine oxidase. Marsilid inhibits both monoamine oxidase (Zeller & Barsky, 1952) and serum spermine oxidase (Tabor *et al.*, 1954). The inhibition of the latter enzyme by 3-hydroxybenzyloxyamine was reported by Gaugas (1981). Pargyline is both an inhibitor of monoamine oxidase (Taylor *et al.*, 1960) and of aldehyde dehydrogenase(s) (Shirota *et al.*, 1979; Lebsack & Anderson, 1979). The newborn-calf serum was from Gibco (Europe), Glasgow, Scotland, U.K.; horseradish peroxidase was from Boehringer, Mannheim, Germany. Homovanillic acid, spermine tetrahydrochloride and spermidine trihydrochloride were from Fluka, Buchs, Switzerland. [*Tetramethylene*-1,4-^{14}C]spermidine trihydrochloride (sp. radioactivity 13 Ci/mol) was purchased from New England Nuclear, Boston, MA, U.S.A.

N^8-(2-Carboxyethyl)spermidine

A new synthetic route has been worked out for this compound. To protect the nitrogen atom of the three-carbon unit of spermidine the urea derivative was formed by the method of McManis & Ganem (1980). Stirring of equimolar amounts of the urea derivative and benzaldehyde in dichloromethane gave the corresponding Schiff-base in quantitative yield. This was catalytically reduced (with H_2 and Pd/carbon) to the benzyl derivative. Condensation of the benzyl derivative with methyl acrylate afforded the propionic ester, which was debenzylated (with H_2 and PtO_2) and hydrolysed (with 5 M-HCl at 100°C for 16 h) to give N^8-(2-carboxyethyl)spermidine. Purification of the product was achieved by chromatography on Amberlite CG-50, using the method suggested by Tsuji *et al.* (1975) for the purification of *N*-acetylpolyamines. Subsequently the compound was run through a Dowex 50W (X8; H^+ form) column using an HCl gradient (Tabor *et al.*, 1958) to form the hydrochloride. The compound (colourless crystals) was chromatographically pure in all solvent systems tested.

Results

Formation of putreanine and N^8-(2-carboxyethyl)spermidine in vivo

The separation of the $HClO_4$ extract of the liver of a mouse that received 0.5 mmol of spermine tetrahydrochloride/kg body wt. intraperitoneally 2 h before excision showed a large peak close to the position where N^1-acetylspermidine was expected to be eluted (Fig. 2*b*). Only a small peak was observed at this position in control liver extracts (Fig. 2*a*). In contrast with N^1-acetylspermidine the compound corresponding to this new peak was stable to hydrolysis with 6 M-HCl (105°C and 16 h). Its reaction with a solution of HCl in methanol produced a new compound with a retention time between that of spermidine and spermine. From these findings the identity of the compound as N^8-(2-carboxyethyl)spermidine was assumed. The chromatographic pattern of this newly formed compound after spermine injection *in vivo* showed identity with an authentic sample on reversed-phase chromatography. This was also true for the methyl

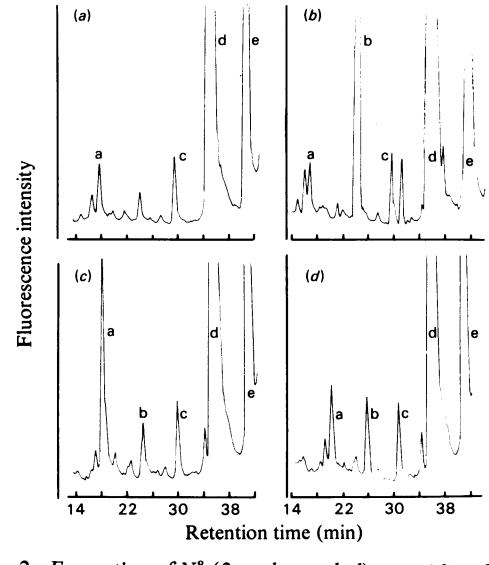

Fig. 2. *Formation of N^8-(2-carboxyethyl)spermidine from spermine in vivo*

High-pressure-liquid-chromatographic separations of liver extracts of mice are shown. (*a*) Control animal; (*b*) mouse treated with 0.5 mmol of spermine tetrahydrochloride/kg body wt. intraperitoneally 2 h before liver excision; (*c*) animal treated as in (*b*), but it received 100 mg of aminoguanidine sulphate/kg body wt. intraperitoneally 3.5 h before spermine administration; (*d*) treatment as in (*b*), but pretreatment with 200 mg of pargyline hydrochloride/kg body wt. 3.5 h before spermidine administration. a = putrescine; b = N^8-(2-carboxyethyl)spermidine; c = 1,7-diaminoheptane (internal standard); d = spermidine; e = spermine. (For details of the chromatographic system see Seiler & Knödgen, 1980.)

esters. Further identification was achieved by chromatographic comparison of the dansyl derivatives, using the procedures described in detail in the Materials and methods section. The thin-layer chromatogram of the dansylated liver extract of a spermine-treated mouse showed a massive spot co-migrating with authentic trisdansyl-N^8-(2-carboxyethyl)spermidine. A peak corresponding with bisdansyl-putreanine could be demonstrated in the liver extract of a spermidine-treated animal. The chromatographic patterns of the liver extracts of control and putrescine-treated animals were identical.

Our high-pressure-liquid-chromatographic procedure (Seiler & Knödgen, 1980) allows the determination of N^8-(2-carboxyethyl)spermidine only in the absence of N^1-acetylspermidine and vice versa; putreanine is eluted in a region that overlaps with normal liver constituents. The dansylation technique described in the Materials and methods section is suitable for the determination of both putreanine and N^8-(2-carboxyethyl)spermidine. Its sensitivity is comparable with that of the high-pressure-liquid-chromatographic method. Both procedures allow the measurement of less than 200pmol of these amino acids.

Time and dose relationships of putreanine and N^8-(2-carboxyethyl)spermidine formation in vivo

Amounts of [^{14}C]spermidine trihydrochloride and spermine tetrahydrochloride ranging from 0.01mmol/kg body wt. to 0.5mmol/kg body wt. (1mmol of spermine tetrahydrochloride/kg body wt. was lethal within 30min) were administered intraperitoneally to mice. At various times after administration animals were killed and the livers and other organs were extracted with 0.2M-$HClO_4$, and the extracts were separated as described in the Materials and methods section. The high-pressure-liquid-chromatographic method (Seiler & Knödgen, 1980) could be used for N^8-(2-carboxyethyl)-spermidine measurement in liver and other tissues, since the absence of significant amounts of N^1-acetylspermidine had already been established. This was achieved by showing the stability of the measured compound against hydrolysis with 6M-HCl. Authentic N^8-(2-carboxyethyl)spermidine was used as standard.

There was a well-defined dose and time relationship between the amount of intraperitoneally administered polyamines and the appearance of putreanine and N^8-(2-carboxyethyl)spermidine in liver respectively, as shown in Table 1. Measurable amounts of these compounds were seen at 30min after a dose of 0.01mmol of spermidine or spermine/kg body wt.; a massive increase of these amino acids was observed with increasing polyamine doses. Even at toxic doses (>0.1mmol/kg body wt.), the transformation process was not saturated. Concentrations of putreanine were usually higher at 30min than at 2h, whereas N^8-(2-carboxyethyl)-spermidine peaked about 2h after spermine administration.

At 24h after intraperitoneal administration of 0.1mmol of the precursor amines/kg body wt., significant amounts of the polyamine-derived amino acids were still observed. However, 2h after an oral dose of 0.5mmol of spermine tetrahydrochloride/kg

Table 1. *Concentration of putreanine and N^8-(2-carboxyethyl)spermidine in liver of mice after intraperitoneal administration of spermidine trihydrochloride and spermine tetrahydrochloride*

Values are means of two experiments. Abbreviation used: nt, not tested.

Polyamine dose (mmol/kg body wt.)	Time after polyamine administration (h)	Putreanine (nmol/g of liver)*	N^8-(2-Carboxyethyl)spermidine (nmol/g of liver)†
0.01	0.5	2.0	nt
	2	0.6	1.9
	6	0.6	nt
	24	0.2	nt
0.1	0.5	17.8	82
	2	11.3	128
	6	8.2	93
	24	1.1	19
0.5	2	nt	454
1.0	0.5	125.5	nt
	2	151.2	nt
	6	74.3	nt
	24	2.0	nt

* Results for male Cox mice (35 ± 1g).

† Results for male CD1 mice (30 ± 2g).

body wt. no significant amounts of N^8-(2-carboxyethyl)spermidine was found in mouse liver.

The distribution of N^8-(2-carboxyethyl)spermidine was estimated in various mouse organs 2 h after an intraperitoneal dose of 0.5 mmol of spermine tetrahydrochloride/kg body wt. Highest concentrations were found in liver (up to 645 nmol/g) and kidney (494 nmol/g). Lower concentrations were found in small intestine (53 nmol/g) and skeletal muscle (20 nmol/g). All other organs and blood contained less than 10 nmol of N^8-(2-carboxyethyl)spermidine/g.

The distribution of putreanine in male Cox mice was estimated in these organs after an injection of 1.0 mmol of spermidine/kg body wt. Highest amounts of putreanine were found in liver (109.4 nmol/g) and kidney (7.6 nmol/g). All other tissues either contained very small amounts of putreanine or the concentration was not detectable with the procedure employed.

Effects of some oxidase inhibitors on the formation of putreanine and N^8-(2-carboxyethyl)spermidine in vivo

Mice were treated with intraperitoneal doses of 100 mg or 200 mg of aminoguanidine sulphate or pargyline hydrochloride/kg body wt. After 3.5 h they received either 1.0 mmol of spermidine trihydrochloride/kg body wt. or 0.5 mmol of spermine tetrahydrochloride/kg body wt. intraperitoneally. After another 2 h the animals wee killed by decapitation, and the liver concentrations of putreanine and N^8-(2-carboxyethyl)spermidine were determined. As can be seen in Fig. 2(*c*) pretreatment with 100 mg of aminoguanidine sulphate/kg body wt. produced a nearly complete block to N^8-(2-carboxyethyl)-spermidine formation, and approximately the same effect was achieved with 200 mg of pargyline/kg body wt. (Fig. 2*d*). With 100 mg of pargyline/kg body wt., inhibition was approx. 50%. Identical results were obtained for the formation of putreanine from spermidine.

Formation of putreanine from N^8-(2-carboxyethyl)-spermidine

The theoretical possibility existed that putreanine was formed not only by oxidative deamination of spermidine, but also from N^8-(2-carboxyethyl)-spermidine.

The reaction sequence (Fig. 3) was presumed to be analogous to that of spermidine formation from spermine (Fig. 1), i.e. oxidative deamination by plasma amine oxidase and spontaneous β-elimination of acrolein.

Incubation of 1 mM-N^8-(2-carboxyethyl)spermidine with newborn-calf serum (60 μl) in Sörensen phosphate buffer generated putreanine. Its formation was demonstrated by t.l.c. of the dansyl derivatives using preseparation on alumina columns as described in the Materials and methods section. The thin-layer chromatograms demonstrated that the amount of putreanine formed increased as a function of incubation time. A second unidentified compound, though present in lower amounts, was also formed under these reaction conditions.

Formation of N^8-(2-carboxyethyl)spermidine from spermine in vitro

Incubation of 1 mM-spermine tetrahydrochloride with 100 μl of Sörensen phosphate buffer and 60 μl of newborn-calf serum for 30 min at 37°C produced a considerable amount of putrescine and spermidine as shown in Fig. 4(*a*). If incubations were performed in the absence of serum with a 1:10 (w/w) homogenate of mouse liver, which had been perfused with 0.9% NaCl solution before homogenization, spermine concentration remained unchanged (results not shown). However, if 60 μl of

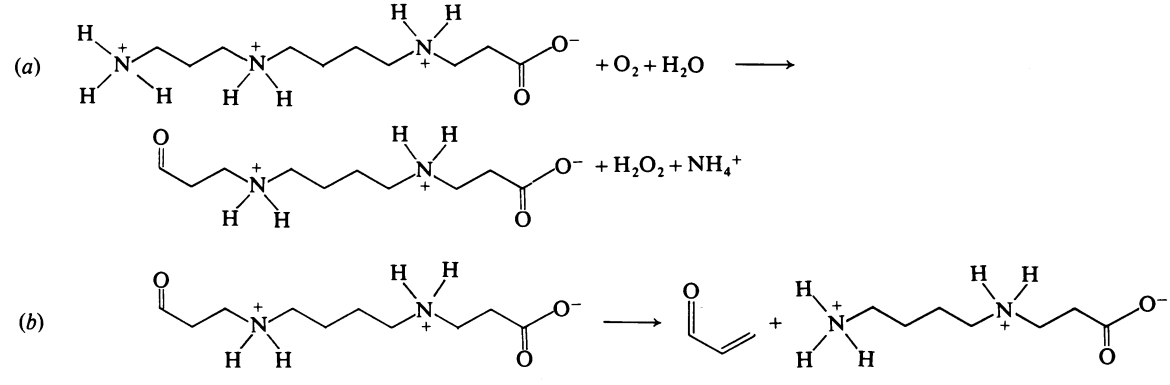

Fig. 3. *Reactions involved in the formation of putreanine from N^8-(2-carboxyethyl)spermidine by serum spermine oxidase*

newborn-calf serum was added to the incubation mixture containing homogenate of mouse liver, a new peak appeared in the chromatograms in addition to those of spermidine and putrescine (Fig. 4*b*). The new peak was identified as N^8-(2-carboxyethyl)spermidine by the same methods that were described for the experiments *in vivo*. (The peak that eluted shortly after spermine is an unknown component of calf serum.)

Under the reaction conditions it appeared that more N^8-(2-carboxyethyl)spermidine than spermidine was formed (Fig. 4*b*). This shows that N^8-(2-carboxyethyl)spermidine can be a major degradation product of spermine under favourable conditions. It could not be established whether spermic acid was formed, since this compound does not give a fluorescent reaction product with *o*-phthalaldehyde, the detector reagent in the high-pressure-liquid-chromatographic method.

Qualitatively the same results were observed if calf serum was substituted with mouse serum. However, the amount of N^8-(2-carboxyethyl)spermidine formed under these conditions was only about 0.4% of that formed with newborn-calf serum.

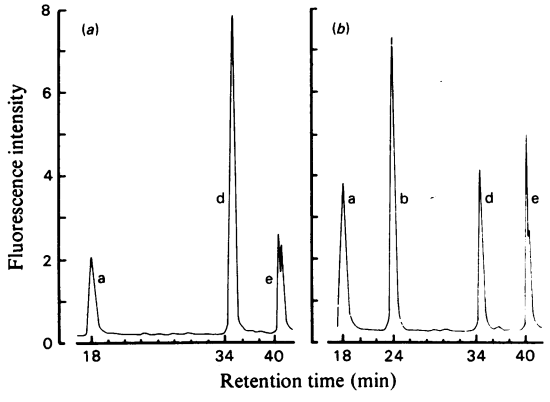

Fig. 4. *Formation of spermidine in vitro from spermine by newborn-calf serum* (*a*) *and of* N^8-(*2-carboxyethyl*)-*spermidine by the combined action of rat liver homogenate and newborn-calf serum* (*b*)

Reversed-phase liquid-chromatographic separation of the components of the incubation mixture was carried out. The chromatographic procedure is the same as that in Fig. 2 (for details see Seiler & Knödgen, 1980). Incubation conditions: 200μl of rat liver homogenate (1:3, w/v) in Sörensen phosphate buffer, pH 7.4, 60μl of newborn-calf serum and 20μl of spermine tetrahydrochloride solution (1 mM in the incubation medium); incubation was for 60 min at 37°C. Peaks are as follows: a, putrescine; b, N^8-(2-carboxyethyl)spermidine; d, spermidine; e, spermine. The peak close to spermine is an unidentified component of the serum.

Formation of putreanine from spermidine in vitro

To test whether intact liver cells were capable of transforming spermidine into putreanine in the presence of calf serum, incubations *in vitro* were performed with identical amounts of tissue from the same rat liver. In one series 0.3 mm thick slices were made from pieces of liver of about 5 mm diameter, using the McIlwain tissue chopper (The Mickle Laboratory Engineering Co., Gomshall, Surrey, U.K.). For the other series, rat liver slices were homogenized with three parts of ice-cold Sörensen phosphate buffer, pH 7.4. The incubation mixtures contained either 900μl of liver homogenate and 100μl of buffer or 225 mg of liver slices suspended in 1000μl of phosphate buffer. In both cases 100μl of spermidine,3HCl solution in phosphate buffer (21.6 mg/5 ml; this corresponds to a final concentration of 1 mM) was used and the reaction was started by addition of 600μl of calf serum. After incubation at 37°C for various intervals with constant shaking the reaction was stopped with 30μl of conc. $HClO_4$. Putreanine formation was determined from a portion of the $HClO_4$ extract, using the dansylation procedure (see the Materials and methods section). The results are summarized in Fig. 5. There was no fundamental difference in the rate of putreanine formation by slices and homogenates. However the onset of the reaction was faster in the

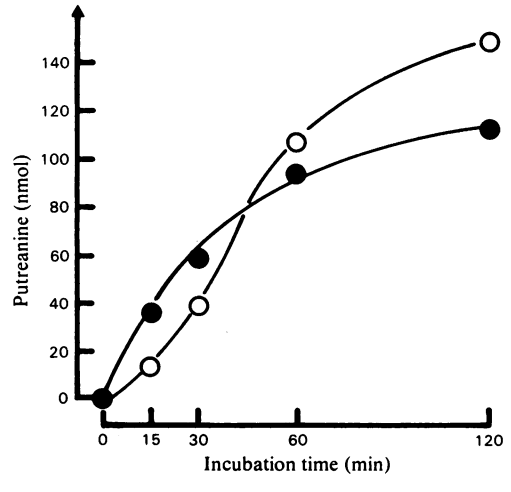

Fig. 5. *Formation of putreanine from spermidine in vitro in liver homogenate* (○) *and slices* (●)

Identical amounts of rat liver slices and homogenized liver were incubated at 37°C with 1 mM-spermidine and newborn-calf serum; putreanine was determined using the dansylation procedure. Each point represents the mean of two experiments for duplicate determinations. For experimental details see the text.

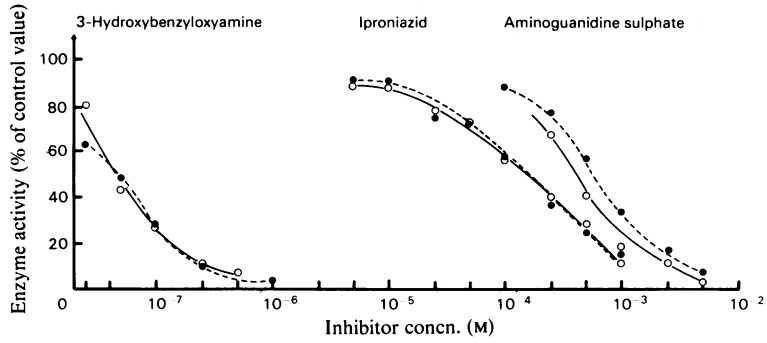

Fig. 6. *Effects of inhibitors of serum spermine oxidase on the rate of spermine deamination by newborn-calf serum* (●) *and putreanine formation from spermidine in incubation mixtures containing rat liver homogenate and newborn-calf serum* (○)

Each point is the mean of two independent experiments. For experimental details see the Materials and methods section.

slices, probably due to a higher initial consumption of O_2 in the homogenate by competing reactions. Maximum rates of putreanine formation were, however, practically the same for both types of incubations, namely 670nmol/g of liver per h for slices and 615nmol/g of liver per h for the homogenates.

Effects of inhibitors on putreanine formation in vitro

If oxidative deamination of spermidine and spermine is the first step in the transformation of the polyamines into their amino acid analogues, inhibitors of serum spermine oxidase would be expected to influence to the same extent the rate of polyamine deamination and of putreanine or N^8-(2-carboxyethyl)spermidine formation. To test this assumption the rate of spermine deamination by newborn-calf serum was determined by measurement of peroxide formation in the presence of inhibitors of serum spermine oxidase. Putreanine formation from spermidine was determined in incubation mixtures that contained both liver homogenate and newborn-calf serum and the same inhibitors. Fig. 6 shows that iproniazid, aminoguanidine sulphate and 3-hydroxybenzyloxyamine affected the two reactions proportionally, although none of these inhibitors is specific for serum spermine oxidase (see the Materials and methods section).

Pargyline had, as expected, no significant influence on spermine or spermidine deamination by calf serum. However, it inhibited putreanine formation by about 40% at 5mM concentration.

Discussion

It has been demonstrated by these experiments that no significant amounts of putreanine and N^8-(2-carboxyethyl)spermidine are formed from spermidine and spermine respectively, after incubation with rat liver homogenates. Addition of mouse serum and especially of calf serum to the incubation mixture markedly increased the formation of these two amino acids. Calf serum is a rich source of spermine oxidase (Blaschko, 1962). However, this enzyme alone is not capable of forming putreanine or N^8-(2-carboxyethyl)-spermidine from spermidine or spermine.

Since serum in conjunction with either liver tissue slices or homogenates was effective in forming putreanine it is concluded that intact cells are capable of performing this reaction. Inhibitors of spermine oxidase decreased the formation of putreanine from spermidine in a manner proportional to the inhibition of spermine oxidation by serum. Pargyline on the other hand did not inhibit spermine oxidation by serum, but decreased the formation of N^8-(2-carboxyethyl)spermidine in the complete system. Pargyline is known to inhibit aldehyde dehydrogenase (Shirota *et al.*, 1979; Lebsack & Anderson, 1979), in addition to being a potent inhibitor of monoamine oxidase.

We conclude that the transformation of spermine into N^8-(2-carboxyethyl)spermidine and of spermidine into putreanine occurs in two steps that take place in two different anatomical compartments; oxidation to the aldehydes is catalysed by spermine oxidase within the circulation; some of the aldehydes are then transferred into the tissues where they are subjected to further oxidation by an aldehyde dehydrogenase to their corresponding amino acids. The reactions are formulated in Fig. 1. Although it has not been experimentally demonstrated, one has to assume that formation of spermic acid follows the

same scheme. In this case, however, two alternative reaction sequences may exist. The dialdehyde may be formed in the circulation, which then is further oxidised to spermic acid within the tissues. As shown in the present work, N^8-(2-carboxyethyl)spermidine is not only formed in excess from spermine, but it is also a substrate of spermine oxidase. When entering the circulation from the tissue, N^8-(2-carboxyethyl)-spermidine could be transformed into the mono-aldehyde. This could either lose an acrolein moiety and thus form putreanine (Fig. 3), or alternatively it could re-enter the tissue and be transformed into spermic acid. It is evident that the formation of putreanine from N^8-(2-carboxyethyl)spermidine is completely analogous to the formation of spermidine from spermine via the monoaldehyde and spontaneous β-elimination of acrolein (Tabor *et al.*, 1964).

It is known that incubation of labelled spermidine with rat brain results in the formation of radioactive putreanine (Nakajima, 1973). This finding is not an argument against the present notion of transformation of the polyamines into their amino acid analogues within two compartments. Since rat serum contains spermine oxidase activity (Blaschko, 1962) the amount of blood remaining in organs that are not perfused before homogenization is sufficiently high so as to catalyse the first step of the reaction sequence. It has been clearly shown in the present work that rat liver, if perfused before homogenization, is not capable of forming significant amounts of putreanine or N^8-(2-carboxyethyl)-spermidine in the absence of plasma.

It must be presumed that the oxidative deamination of the polyamines to the corresponding amino acids is not the only case of a reaction sequence in which subsequent steps take place in two different anatomical compartments. The oxidative deamination of methylamine is almost certainly another example. It was shown (Werner & Seiler, 1963) that inhibitors of both monoamine oxidase and diamine oxidase were effective in decreasing the rate of $^{14}CO_2$ formation from labelled methylamine *in vivo*. Highly potent inhibitors of monoamine oxidase, such as pargyline and tranylcypromine were, however, less effective than the compounds with a hydrazine moiety. *In vitro*, neither mouse nor rat organs were capable of oxidizing methylamine effectively. Beef liver which contains serum spermine oxidase due to residual blood, however, was effective, and with this enzyme source the same inhibitor spectrum was found as in the *in vivo* experiments with mice. These results can be explained without contradiction of the assumption of metabolism in two-compartments.

The observed tissue concentrations of N^8-(2-carboxyethyl)spermidine, i.e. high concentrations in liver and kidney, and low concentrations in most other organs after intraperitoneal administration of spermine is in agreement with a first-pass metabolism in liver and an effective urinary excretion of the spermine and spermidine derived amino acids. A similar distribution pattern was observed with labelled spermidine as a precursor (Nakajima, 1973).

Endogenous putreanine concentrations are higher in brain than in liver (Nakajima & Matsuoka, 1971). Since intraventricular [^{14}C]spermidine injections produced significant amounts of labelled putreanine in the brain one can assume that brain putreanine is formed from spermidine that is directly released from brain cells into the circulation. The effective uptake of the aldehyde by brain cells from the extensive brain capillary system is to be expected. A second possibility is that the polyamines leave the brain via the cerebrospinal fluid. The presence of putrescine, spermidine and also of small amounts of spermine in the cerebrospinal fluid is well established and it has been shown that enhanced polyamine formation in brain tumours is reflected in increased cerebrospinal-fluid polyamine concentrations (Marton *et al.*, 1974). Whether cerebrospinal fluid contains significant spermine oxidase activity to account for the transformation of the polyamines into the corresponding aldehydes is presently not known.

The fact that putreanine was not detected in Rhesus-monkey brain before birth (Kremzner & Sturman, 1979) is probably best explained by the limited capacity of the brain for spermidine and spermine formation due to low *S*-adenosylmethionine decarboxylase activity in early brain development, at a time of high polyamine demand; as a consequence of this phenomenon only a limited amount of polyamine degradation would be predicted along a pathway that causes irreversible losses of polyamines.

The physiological role of the transformation of polyamines into amino acids in a reaction sequence that comprises two different anatomical compartments is speculative at present. Most probably it has no function in the control of intracellular polyamine concentrations. It is more likely that intracellular polyamine concentrations are regulated by the control of the rate of biosynthesis and by a degradation sequence that comprises acetylation and oxidative degradation of N^1-monoacetylspermidine by polyamine oxidase and alternatively excretion of the acetyl derivatives (Seiler, 1981; Bolkenius & Seiler, 1981).

Since an enzyme of the circulation is involved it is suggested that the formation of amino acids from spermidine and spermine is part of the inactivation system that keeps extracellular polyamine concentrations low, thus avoiding toxicity (Tabor & Tabor, 1964). Both exogenous (alimentary) poly-

amines and polyamines of intracellular origin could be physiological substrates of the reaction sequence.

References

Asatoor, A. M. (1979) *Biochim. Biophys. Acta* **586**, 55–62

Blaschko, H. (1962) *Adv. Comp. Physiol. Biochem.* **1**, 67–116

Bolkenius, F. N. & Seiler, N. (1981) *Int. J. Biochem.* **13**, 287–292

Folk, J. E., Park, M. H., II Chung, S., Schrode, J., Lester, E. P. & Cooper, H. L. (1980) *J. Biol. Chem.* **255**, 3695–3700

Gaugas, J. M. (1981) *Adv. Polyamine Res.* **3**, 441–450

Imaoka, N. & Matsuoka, Y. (1974) *J. Neurochem.* **22**, 859–860

Kakimoto, Y., Nakajima, T., Kumon, A., Matsuoka, Y., Imaoka, N., Sano, I. & Kanazawa, A. (1969) *J. Biol. Chem.* **244**, 6003–6007

Kremzner, L. T. (1973) *Polyamines in Normal and Neoplastic Growth* (Russell, D. H., ed.), pp. 27–40, Raven Press, New York

Kremzner, L. T. & Sturman, J. A. (1979) *J. Neurochem.* **33**, 1115–1117

Kremzner, L. T., Hiller, J. M. & Simon, E. J. (1975) *J. Neurochem.* **25**, 889–894

Lebsack, M. E. & Anderson, A. D. (1979) *Res. Commun. Chem. Pathol. Pharmacol.* **26**, 263–275

Marton, L. J., Heby, O., Wilson, C. B. & Lee, P. L. Y. (1974) *FEBS Lett.* **46**, 305–307

McManis, J. S. & Ganem, B. (1980) *J. Org. Chem.* **45**, 2041–2042

Morgan, D. M. L. (1980) *Polyamines in Biomedical Research* (Gaugas, J. M., ed.), pp. 285–302, John Wiley, Chichester, New York, Brisbane and Toronto

Nakajima, T. (1973) *J. Neurochem.* **20**, 735–742

Nakajima, T. & Matsuoka, Y. (1971) *J. Neurochem.* **18**, 2547–2548

Noto, T., Tanaka, T. & Nakajima, T. (1978) *J. Biochem. (Tokyo)* **83**, 543–552

Perry, T. L., Hansen, S. & Kloster, M. (1972) *J. Neurochem.* **19**, 1395–1396

Roth, M. & Hampai, A. (1973) *J. Chromatogr.* **83**, 353–356

Schuler, W. (1952) *Experientia* **8**, 230–232

Seiler, N. (1970) *Methods Biochem. Anal.* **18**, 259–337

Seiler, N. (1975) *Res. Methods Neurochem.* **3**, 409–441

Seiler, N. (1981) in *Polyamines in Biology and Medicine* (Morris, D. R. & Marton, L. J., eds.), pp. 123–145, Marcel Dekker, New York

Seiler, N. & Knödgen, B. (1979) *J. Chromatogr.* **164**, 155–168

Seiler, N. & Knödgen B. (1980) *J. Chromatogr.* **221**, 227–235

Shiba, T. & Kaneko, T. (1969) *J. Biol. Chem.* **244**, 6006–6007

Shirota, F. N., de Master, E. G. & Nagasawa, H. T. (1979) *J. Med. Chem.* **22**, 463–464

Snyder, S. H. & Hendley, E. D. (1968) *J. Pharmacol. Exp. Ther.* **163**, 386–392

Tabor, H. & Tabor, C. W. (1964) *Pharmacol. Rev.* **16**, 245–300

Tabor, H., Rosenthal, S. M. & Tabor C. W. (1958) *J. Biol. Chem.* **233**, 907–914

Tabor, C. W., Tabor, H. & Rosenthal, S. M. (1954) *J. Biol. Chem.* **208**, 645–661

Tabor, C. W., Tabor, H. & Bachrach, U. (1964) *J. Biol. Chem.* **239**, 2194–2203

Taylor, J. D., Wykes, A. A., Gladish, Y. C. & Martin, W. B. (1960) *Nature (London)* **187**, 941–942

Tsuji, M., Nakajima, T. & Sano, I. (1975) *Clin. Chim. Acta* **59**, 161–167

Werner, G. & Seiler, N. (1963) *Biochem. Z.* **337**, 383–396

Zeller, E. A. & Barsky, J. (1952) *Proc. Soc. Exp. Biol. Med.* **81**, 459–461