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N-(3-Aminopropyl)pyrrolidin-2-one, a product of spermidine catabolism *in vivo*

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A high-pressure-liquid-chromatographic method suitable for the separation and sensitive detection of putreanine and isoputreanine is described. This method allowed us to study the formation of the metabolites of the oxidative deamination of spermidine and N^1-acetylspermidine. Administration of spermidine trishydrochloride to mice causes a time-dependent accumulation of putreanine and *N*-(3-aminopropyl)pyrrolidin-2-one in various organs. The latter compound yields isoputreanine by hydrolysis. It can be assumed that the analogous lactam, *N*-(3-acetamidopropyl)pyrrolidin-2-one is formed from N^1-acetylspermidine, since hydrolysis of tissue extracts of N^1-acetylspermidine-treated mice produced isoputreanine. No putreanine is formed under these conditions. Pretreatment of the animals with 25 mg of aminoguanidine sulphate/kg body wt. completely inhibits the formation of putreanine and of the respective isoputreanine precursor from spermidine and N^1-acetylspermidine. This suggests a role for a diamine oxidase-like enzyme in the oxidative deamination of spermidine and N^1-acetylspermidine.

In a recent study we demonstrated that intraperitoneal administration of spermidine to mice causes the accumulation of significant amounts of putreanine [N^1-(2-carboxyethyl)-1,4-diaminobutane] in liver and other organs in a time- and dose-dependent manner. Similarly, spermine was transformed into the corresponding monocarboxylic acid, N^8-(2-carboxyethyl)spermidine (Seiler *et al.*, 1981).

A new method suitable for the separation and sensitive determination of putreanine and isoputreanine [N^1-(3-carboxypropyl)-1,3-diaminopropane] suggested to us the investigation of the metabolism of exogenous N^1-acetylspermidine and the re-investigation of spermidine metabolism with emphasis on isoputreanine formation. This method is presented here together with results that demonstrate the formation of *N*-(3-aminopropyl)pyrrolidin-2-one from injected spermidine.

Materials and methods

Laboratory animals

Male albino CD1 mice (weighing 43 ± 2 g) and female hairless mice (HRS/J strain) (weighing 24 ± 3 g) were used. The albino mice were from Charles River, Saint Aubin-les-Elboeuf, France; the hairless mice were bred in our laboratory.

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

Determination of putreanine and isoputreanine by high-pressure liquid chromatography

For the separation of putreanine and isoputreanine from tissue constituents ion-exchange chromatography using a high-performance column (Whatman; Partisil-10/25 SCXD; 250 mm × 4.6 mm) was used. The main column was protected by a pre-column (100 mm × 3 mm) filled with a pellicular core with C_{18}-brushes (Waters, Paris, France). The chromatographic system consisted of a Varian high-pressure liquid chromatograph (model 8500), to which a loop injector (Valco valve CV-6 UHPa-N60; Valco Instruments, Houston, TX, U.S.A.) (loop volume 250 μl) was attached. Post-column derivative formation with *o*-phthalaldehyde/2-mercaptoethanol reagent (Roth & Hampai, 1973) was used. The reagent was prepared by dissolving 50 g of boric acid, 44 g of KOH and 3 ml of a 30% Brij-35 solution per litre of water. To this solution 2 ml of 2-mercaptoethanol and 400 mg of *o*-phthalaldehyde dissolved in 5 ml of methanol were added before use.

Column effluent and reagent were mixed in a 1:1

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ratio. Fluorescence was continuously monitored at 455nm (activation at 345nm) with a Perkin–Elmer fluorescence spectrometer model 204A equipped with a 10μl flow cell and a 150W xenon arc lamp. Column, mixing T-piece and the mixing coil (2m × 0.5mm int. diameter) were kept at 35 ± 0.5°C.

The elution system consisted of a linear gradient that was prepared by mixing 0.2M-acetic acid (buffer A) and 0.2M-sodium acetate adjusted with acetic acid to pH4.50 (buffer B). Buffer flow rate was 1ml·min^{-1}.

The Partisil SCX column tends to change its chromatographic properties with prolonged use. Therefore, it is not possible to give definitive elution programmes. These have to be adapted to the status of the column. This is, however, usually achieved by adjusting the initial portion of buffer B. With a new column this may be as high as 65%, whereas most of the separations described in this work were performed by equilibration of the column for 14min with a mixture of 15% buffer B and 85% buffer A. After sample application a linear gradient is started; the increment of buffer B may vary between 1%·min^{-1} and 0.5%·min^{-1}, depending on the status of the column. The gradient is run until putrescine has been eluted (usually 20min), then the pumps are switched to the initial conditions. After equilibration, the next sample can be applied. It is thus possible to run samples about every 34min. The column is regularly washed during the night with 250ml of buffer B in order to elute polyamines and other strongly basic compounds.

Sample preparations

Extracts of brain with 0.2M-$HClO_4$ can be separated immediately after dilution with 2–3vol. of 0.2M-acetic acid. Chromatographic separation of tissue extracts is, however, improved by using the following preliminary cleaning procedure. Portions (0.3ml) of tissue extracts prepared by homogenization with 10vol. of 0.2M-$HClO_4$ are run through 0.3ml columns of Dowex 50W (X8; 200–400 mesh, H^+ form) (Serva, Heidelberg, Germany). The columns are washed with 1.5ml of 2M-HCl. Then putreanine, isoputreanine and compounds of related polarity (including putrescine and some of the polyamines) are eluted with 1.5ml of 4M-HCl. This fraction is evaporated to dryness and the residue is dissolved in 0.3ml of 0.2M-$HClO_4$. The samples are kept at −20°C until they are analysed. Before separation, they are diluted appropriately with 0.2M-acetic acid.

Recovery of putreanine and isoputreanine is practically quantitative, as shown by running samples with known amounts of the amino acids through the procedure. Nevertheless, standards were always handled in the same way as tissue extracts and were compared with directly analysed standard solutions.

Preparation of whole-body extracts

Hairless mice were used in these experiments. They were killed by cervical dislocation and immediately immersed in liquid N_2. Using a steel mortar, the frozen carcasses were powdered, while cooled with liquid N_2. Further homogenization was achieved with 10vol. of water by using a Waring Blendor with a stainless-steel beaker. After 1min, 1.7ml of 70% $HClO_4$ was added per 100ml of water, to give a final $HClO_4$ concentration of about 0.2M and homogenization was completed.

After being left for 2h at 3°C the homogenates were centrifuged and the extracts were stored at 20°C until they were analysed.

Portions of the extracts were hydrolysed with 6M-HCl and run through Dowex columns, as described above. However, in this case, 5ml portions of the extracts were 'cleaned up' by using columns of 5ml bed volume. For washing and elution, 25ml portions of 2M-HCl and 4M-HCl were used respectively.

Identification of N-(3-aminopropyl)pyrrolidin-2-one, putreanine and isoputreanine as their dansyl derivatives

$HClO_4$ (0.4ml; 0.2M) tissue extracts were allowed to react with dansyl chloride (5-dimethylaminonapthalene-1-sulphonyl chloride) as previously described (Seiler, 1970). In the case of *N*-(3-aminopropyl)pyrrolidin-2-one determinations, the excess reagent was reacted by addition of proline. In the case of putreanine and isoputreanine, 50μl of an aqueous solution of diethylamine was added.

Extraction of the derivatives from the reaction mixture was in both cases achieved with ethyl acetate/toluene (1:1, v/v).

Separation of monodansyl-*N*-(3-aminopropyl)-pyrrolidin-2-one from all major components of the liver of mice could be achieved by chromatography of portions of the complete mixture of all dansyl derivatives on silica-gel plates (silica gel 60; Merck, Darmstadt, Germany) by two runs in the same direction with ethyl acetate as solvent. Separation was improved by a third run in the same direction, with chloroform/carbon tetrachloride/methanol (14:6:1, by vol.).

For the identification of putreanine and isoputreanine essentially the same procedure as described previously (Seiler *et al.*, 1981) was used, including chromatography on Al_2O_3 columns. The eluate with methanol/conc. NH_3 (4:1, v/v) was evaporated to dryness and dissolved in 0.2ml of a saturated solution of HCl in methanol. After being left overnight at room temperature the methanol/HCl was removed in a vacuum dessicator containing KOH as dessicant. The dry residue containing dansylated amino acid methyl esters was agitated with 0.5ml of a saturated solution of

$NaHCO_3$ in water, and the dansyl derivatives were extracted with 5 ml of toluene. The residue of the toluene extract was redissolved in a small defined volume of toluene, and portions were separated chromatographically on silica-gel plates (silica-gel 60; Merck, Darmstadt, Germany) with chloroform/carbon tetrachloride/triethylamine (5:5:1, by vol.) as solvent. The methyl esters of bis-dansyl-putreanine and -isoputreanine are completely separated by this procedure.

Mass spectrometry

Mass spectra were obtained from chromatographically uniform dansyl-derivatives by direct evaporation from glass capillaries (temperature programme, 60°C/min). The instrument was a quadrupole mass spectrometer (Ribermag R 10-10) having a mass range of 1500 atomic mass units. Ionization was accomplished by chemical ionization with NH_3 or methane as reagent gas.

Chemicals

Usual laboratory chemicals were A.R. grade and were purchased either from E. Merck, Darmstadt, Germany or from Baker Chemicals, Deventer, The Netherlands. Putreanine, $H_2SO_4,\frac{1}{2}H_2O$ was purchased from CalBiochem, Los Angeles, CA, U.S.A. Aminoguanidine sulphate was from Schuchardt, München, Germany. Isoputreanine bishydrochloride was prepared from *N*-(3-aminopropyl)pyrrolidin-2-one (Aldrich, Beerse, Belgium) by hydrolysis with conc. H_2SO_4. It was purified by chromatography on Dowex 50 W (X8; 200–400 mesh; H^+ form) by using an HCl gradient (between 0 and 3 M-HCl). The elemental analysis corresponded to the expected composition. N^1-Acetylspermidine was prepared by acetylation of spermidine by the method of Tabor *et al.* (1971). Spermidine trishydrochloride was from Fluka, Buchs, Switzerland.

Results

Determination of putreanine and isoputreanine in tissue extracts

The method is based on the chromatographic separation of $HClO_4$ tissue extracts using a Whatman Partisil-10 SCX column (250 mm × 4.6 mm) and a linear gradient produced from 0.2 M-acetic acid and 0.2 M-sodium acetate (adjusted to pH 4.50 with acetic acid). Detection is achieved by reaction with *o*-phthalaldehyde/2-mercaptoethanol and continuous monitoring of fluorescence at 455 nm (activation at 345 nm) by the method of Roth & Hampai (1973).

Partisil-10 SCX is a strong cation exchanger. The sulphonic acid functional groups are bound to 10 μm silica-gel particles through siloxane bonds.

One advantage of the method is that it allows the separation of $HClO_4$ tissue extracts after appropriate dilution with 0.2 M-acetic acid.

However, if putreanine has to be determined in tissues containing less than 10 nmol·g^{-1}, it is advisable to use the 'clean-up' step described in the Materials and methods section.

$HClO_4$ extracts of some tissues contain an interfering compound, presumably a peptide, because it can be removed by hydrolysis with 6 M-HCl and subsequent 'clean-up' by chromatography on Dowex 50W (X8). There are no significant losses of the putreanines by this procedure.

Since a well established detection method is used, the sensitivity is of the same order as for other methods of amino acid analysis using the *o*-phthalaldehyde/2-mercaptoethanol reagent in conjunction with a high-pressure-liquid-chromatographic column. Solutions of 50 nM-putreanine and -isoputreanine can be analysed routinely with a precision of about ±5%, if a 250 μl loop injector is used.

Linearity between putreanine and isoputreanine concentrations and peak height was established in the range of 50 nM to 50 μM.

Fig. 1 shows a putreanine standard with 50 pmol

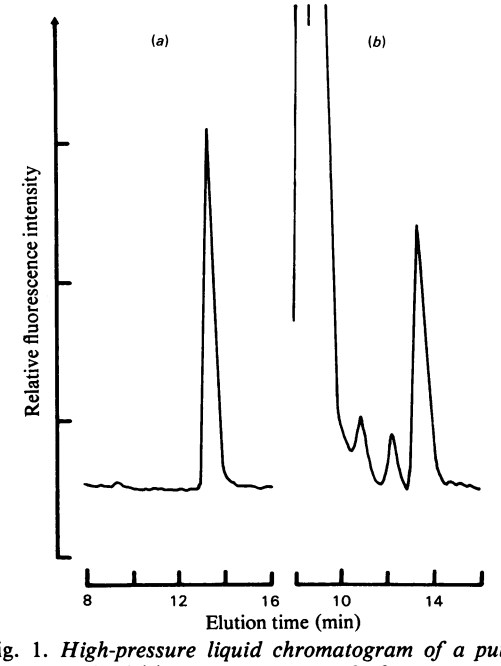

Fig. 1. *High-pressure liquid chromatogram of a putreanine standard (a) containing 50 pmol of putreanine per 0.1 ml and (b) separation of a 0.2 M-$HClO_4$ extract of mouse brain [1:10 (w/v) homogenate] after dilution with 4 vol. of 0.2 M-acetic acid*

For details of the chromatographic conditions see the Materials and methods section.

of putreanine per 0.1 ml and the separation of an $HClO_4$ extract of mouse brain. The analyses of 11 brains of male albino mice weighing between 35 and 45 g gave the following mean value (±S.D.): 16.0 ± 1.4 nmol·g^{-1}. This value is somewhat lower than that published for rat brain by Nakajima & Matsuoka (1971). Putreanine could not be identified unambiguously in either liver or kidney.

Formation of isoputreanine from N^1-acetylspermidine

Groups of three mice received intraperitoneal injections of 1 mmol of N^1-acetylspermidine bishydrochloride/kg body wt. After 10, 20 or 30 min the animals were decapitated and small intestine, kidneys and liver were homogenized with 10 vol. of 0.2 M-$HClO_4$.

Separation of these extracts using the above described high-pressure-liquid-chromatographic system gave chromatograms identical with those of non-treated control animals. However, hydrolysis of the $HClO_4$ extracts with 6 M-HCl formed isoputreanine, but no putreanine was detectable.

The results are summarized in Fig. 2. The precursor of isoputreanine was most rapidly accumulated in small intestine. In contrast, liver and kidney accumulated much less of this compound, and showed less of it at 30 min than at 20 min, indicating a rapid clearance from these organs.

From the mean value at 30 min in Fig. 2 one can calculate an average accumulation rate of 7.3 nmol·g^{-1}·min^{-1} of the precursor of isoputreanine in small intestine under the experimental conditions.

Pretreatment of the animals with 0.2 mmol of aminoguanidine/kg body wt. 4 h before the administration of N^1-acetylspermidine resulted in a complete blockade of isoputreanine accumulation in small intestine and other tissues.

Formation of putreanine and isoputreanine in mice from exogenous spermidine

Groups of five mice (hairless HRS/J strain), weighing 24 ± 3 g, received 1 mmol of spermidine trihydrochloride/kg body wt. intraperitoneally. A non-treated group served as control. At 10, 20 and 30 min after spermidine administration the animals were killed by cervical dislocation and immediately frozen in liquid N_2. Whole-body extracts were prepared with 0.2 M-$HClO_4$. Portions of the extracts were hydrolysed with 6 M-HCl and the hydrolysates were run through Dowex 50 W (X8) columns as described in the Materials and methods section. It was necessary to include the hydrolysis step because of an interfering compound in the non-hydrolysed samples.

Although there was considerable scatter, there was a linear relationship between the amount of putreanine formed and the time after spermidine administration, as shown in Fig. 3(*a*). From the regression line, the average rate of putreanine formation was calculated to be about 0.27 nmol·g^{-1}·min^{-1} under the experimental conditions, i.e. about 0.3% of the injected spermidine was transformed per min and per g body wt. into putreanine.

A time-dependent formation of isoputreanine could also be demonstrated in the hydrolysed whole-body extracts, as shown in Fig. 3(*b*). No further increase of this compound was, however, noticeable after 20 min. Since the whole-body extracts contain the extracts of urine and faeces as well, excretion cannot be responsible for the non-linearity of the relationship.

In a second series, albino male mice were administered 1 mmol of spermidine/kg body wt. intraperitoneally. The $HClO_4$ extracts of liver, kidney and small intestine were separated by high-pressure liquid chromatography, both before and after hydrolysis with 6 M-HCl. As shown in Fig. 4 the peak corresponding to putreanine was not

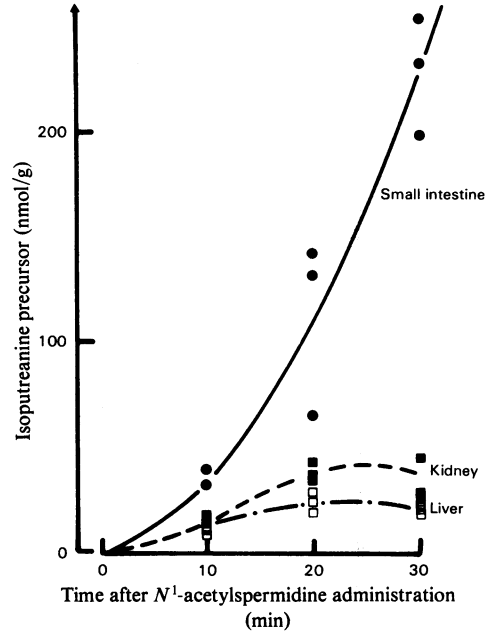

Fig. 2. *Accumulation of the isoputreanine precursor [presumably N-(3-acetamidopropyl)pyrrolidin-2-one] in liver, kidney and small intestine of CD1 albino mice after a single intraperitoneal dose of 1 mmol of N^1-acetylspermidine bishydrochloride/kg body wt.*
The samples were hydrolysed and isoputreanine was determined by high-pressure liquid chromatography. Each point represents the results obtained from a separate organ.

influenced by treatment with HCl, whereas isoputreanine appeared in the chromatograms only after the hydrolytic cleavage, showing that a precursor of isoputreanine had been formed, which can be transformed into isoputreanine by acid hydrolysis.

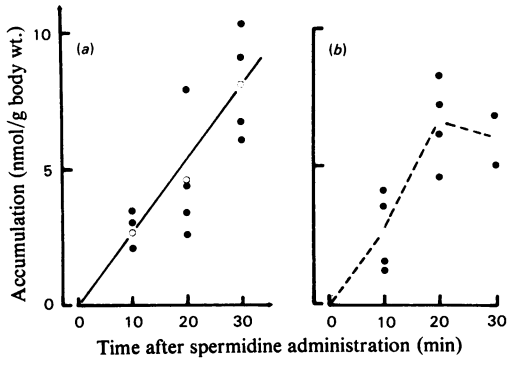

Fig. 3. *Total accumulation of putreanine (a) and of the isoputreanine precursor (b) in hairless mice after a single intraperitoneal dose of 1 mmol of spermidine trishydrochloride/kg body wt.*

Whole-body extracts were prepared with 0.2 M-$HClO_4$. These were hydrolysed and the hydrolysates were subsequently separated by high-pressure liquid chromatography. Filled circles represent results obtained from separate animals. Open circles represent mean values.

Putreanine and the precursor of isoputreanine accumulated in a time-dependent manner in liver and kidney. However, the putreanine content of small intestine was negligibly small. From the data summarized in Fig. 5 one can calculate that putreanine accumulates in the liver of mice at a rate of about 2.1 nmol·g^{-1}·min^{-1}. Taking the average liver weight of these animals of 1.72 g and their body weight into account, one can calculate an accumulation rate in liver per g body wt. of 0.08 nmol·g^{-1}·min^{-1}. This is about 30% of the total putreanine formed by mice under the experimental conditions.

As shown in Fig. 5 a significant amount of putreanine is also found in the kidneys; the rate of accumulation in these organs is not significantly different from that in liver.

The situation is markedly different from isoputreanine formation from N^1-acetylspermidine: in kidney and small intestine a roughly linear increase of the isoputreanine precursor was observed during the first 30 min after spermidine administration, with a rate of 2–2.5 nmol·g^{-1}·min^{-1}. In liver, however, this rate was about 6–7 times higher during the first 10 min, with little further accumulation at later times.

Taking the mean values of putreanine and isoputreanine determinations at 30 min as a basis, one can calculate that the amount of putreanine found in liver and kidneys at this time (142 nmol) corresponds to 0.33% of the injected spermidine.

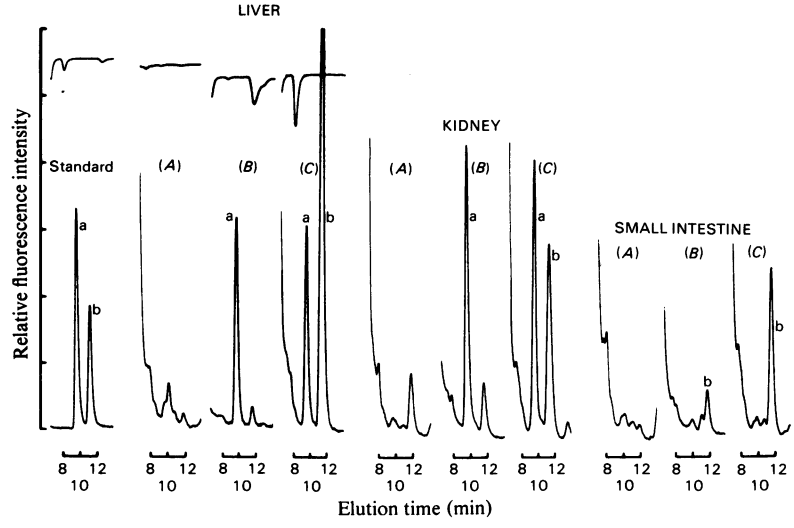

Fig. 4. *High-pressure liquid chromatograms of 0.2 M-$HClO_4$ extracts of liver, kidney and small intestine of albino CD1 mice 30 min after the intraperitoneal administration of 1 mmol of spermidine trishydrochloride/kg body wt.*

(*A*) shows extracts of tissues from non-treated control animals after hydrolysis with 6 M-HCl. The chromatograms of non-hydrolysed extracts from control animals were not significantly different in the part of interest from those of hydrolysed extracts, and are therefore not shown. (*B*) shows 0.2 M-$HClO_4$ extracts of spermidine-treated mice. (*C*) shows separations of the same extracts as in (*B*), but after hydrolysis with 6 M-HCl. (a) Putreanine; (b) isoputreanine.

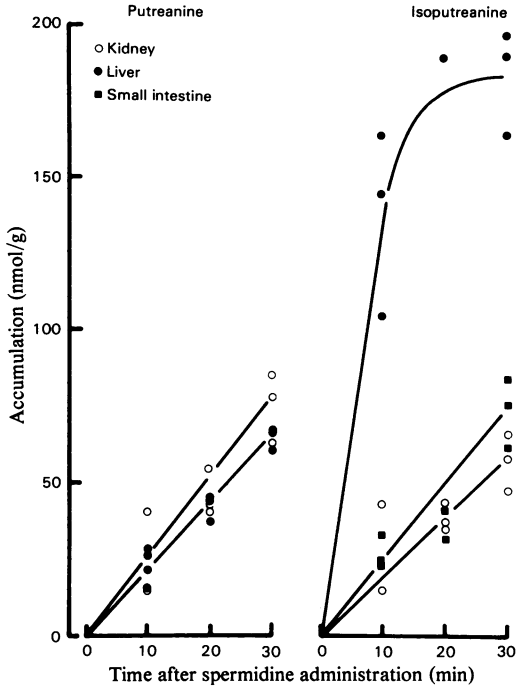

Fig. 5. *Accumulation of putreanine and the precursor of isoputreanine in CD1 mouse organs after the intraperitoneal administration of 1 mmol of spermidine trishydrochloride/kg body wt.*
The experiments include the samples whose separations are shown in Fig. 4. Each point represents the results obtained from a separate tissue sample.

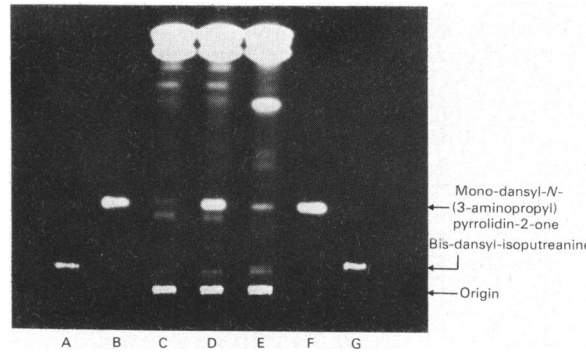

Fig. 6. *T.l.c. separations of the dansyl derivatives from liver extracts*
(A), Bis-dansyl-isoputreanine; (B), mono-dansyl-*N*-(3-aminopropyl)pyrrolidin-2-one; (C), dansyl derivatives of the perchloric acid extract of the liver of a non-treated control animal; (D), dansyl derivatives of a mouse liver extract prepared 30 min after the administration of 1 mmol of spermidine trishydrochloride/kg body wt. (extract identical with that whose separation is shown in Fig. 4); (E), same extract as in (D), but hydrolysed with 6 M-HCl before the reaction with dansyl chloride (identical with the hydrolysed liver extract in Fig. 4); (F), mono-dansyl-*N*-(3-aminopropyl)pyrrolidin-2-one; (G), bis-dansyl-isoputreanine.

The value found for the putreanine content in whole-body extracts is 0.8%.

For the isoputreanine precursor the corresponding values are 0.8% for kidney plus liver of the albino mice and 0.6% for the whole-body extracts.

The comparison between the results obtained from whole-animal extracts and the amino acid determinations in extracts of individual organs is hampered by the fact that they were obtained from different strains of mice. Moreover, the measured rates are most probably not reflecting the maximum capacity of mice to transform spermidine into putreanine. Because of unacceptable toxicity it could not be established whether the rate-limiting step in the sequence of events participating in the transformation processes was saturated with its substrate under the experimental conditions. It nevertheless shows that mice have a considerable capacity to form putreanine and a derivative of isoputreanine from exogenous spermidine, and demonstrates that liver and kidney (and in the case of the isoputreanine derivative, also the small intestine) are the organs most significantly involved in the accumulation of these amino acids.

Intraperitoneal administration of 0.2 mmol of aminoguanidine/kg body wt. 4 h before spermidine administration completely inhibits the accumulation of putreanine and of the isoputreanine derivative in liver, kidney and small intestine, indicating that some type of Cu^{2+}-containing diamine oxidase-like enzyme is involved in the process.

Identification of N-(3-aminopropyl)pyrrolidin-2-one

$HClO_4$ liver extracts of mice pretreated with 1 mmol of spermidine trishydrochloride/kg body wt. were reacted with dansyl chloride and the derivatives separated on silica-gel thin-layer plates. Development of the plates with ethyl acetate (two runs in the same direction) or chloroform/carbon tetrachloride/methanol (14:6:1, by vol.) or a combination of the two solvents revealed a fluorescent spot on the chromatograms, which co-migrated with authentic mono-dansyl-*N*-(3-aminopropyl)pyrrolidin-2-one (Fig. 6). In liver extracts of non-treated control animals only a very minor fluorescent spot migrated in this position. Likewise, the spot was hardly detectable in chromatograms of dansylated liver extract of N^1-acetylspermidine-treated mice. If the $HClO_4$ extract was hydrolysed before reaction with dansyl chloride, a spot became visible on the

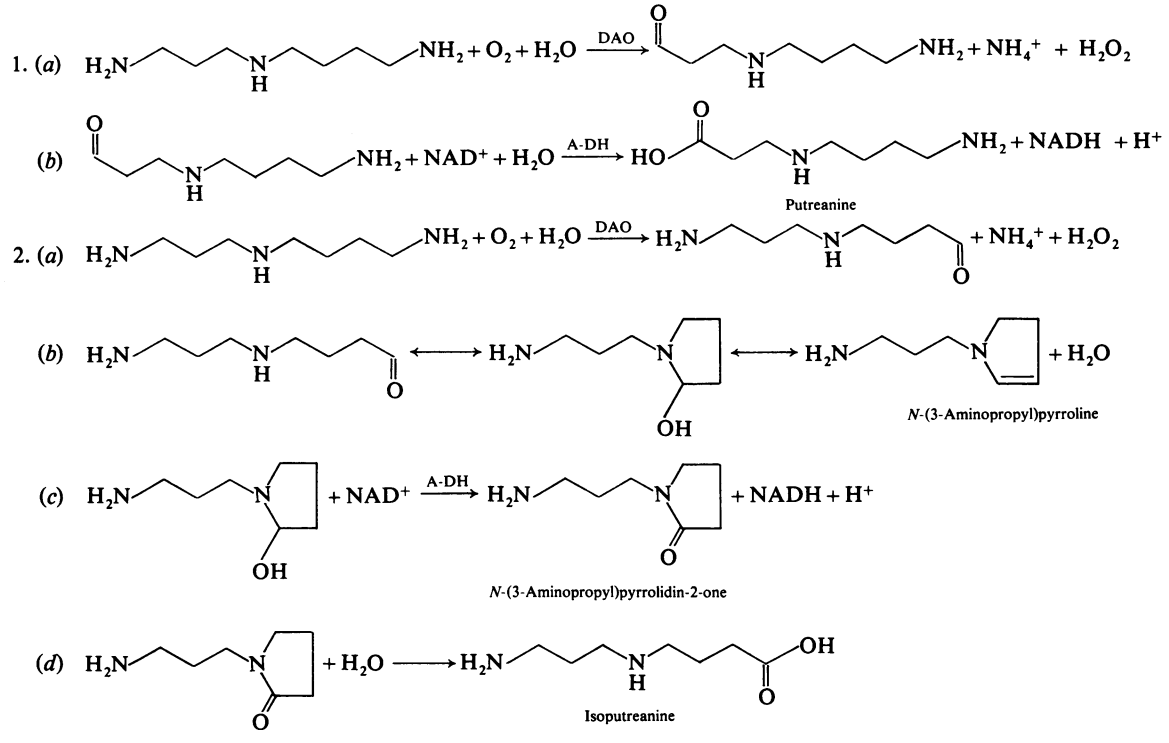

Fig. 7. *Reactions involved in putreanine and isoputreanine formation from spermidine*
Abbreviations: DAO, aminoguanidine-sensitive oxidase (diamine oxidase); A-DH, aldehyde dehydrogenase.

chromatograms, which co-migrated with bis-dansyl-isoputreanine.

The identity of the above compound with mono-dansyl-*N*-(3-aminopropyl)pyrrolidin-2-one was unambiguously established by comparison of mass spectra from spots of the unknown and the reference compound. By using methane as the reagent gas, they showed the expected pseudo molecular ions at *m/z* 376 ($M^+ + H$), 404 ($M^+ + C_2H_5$) and 416 ($M^+ + C_3H_5$) besides ions typical for dansyl derivatives (Seiler *et al.*, 1970).

Isoputreanine was identified in hydrolysed extracts of the liver of spermidine-treated mice essentially by the same methodology. However, in order to improve the specificity of the chromatographic system, the dansyl derivatives were first separated on a small Al_2O_3 column, as described previously (Seiler *et al.*, 1981). The amino acid fraction, which elutes with methanol/conc. NH_3 (4:1, v/v), was evaporated to dryness and the residue was esterified with methanol/HCl. The methyl esters were subsequently separated on silica-gel thin-layer plates, with chloroform/carbon tetrachloride/triethylamine (5:5:1, v/v) as solvent, and mass spectra were determined of the compound isolated from liver and of an authentic sample of bis-dansyl-isoputreanine. By using NH_3 as reagent gas for chemical ionization, the spectra were identical, with the molecular ion $M + H^+$ at *m/z* 641 as the base peak, and typical fragment ions at *m/z* 406, 376, 351 and 172.

Discussion

The high-pressure-liquid-chromatographic method reported in this paper allowed us to demonstrate putreanine and isoputreanine formation from exogenous spermidine *in vivo*. Putreanine is the end-product of the oxidative deamination of the 3-aminopropyl moiety of spermidine. Oxidative deamination of the aminobutyl moiety generates the corresponding lactam, which can be hydrolysed to isoputreanine and determined as such in the various tissues. However, by using the dansylation method, the demonstration of *N*-(3-aminopropyl)pyrrolidin-2-one in the tissues of spermidine-treated animals was possible.

Fig. 7 summarizes the reaction sequence most likely to be involved in the formation of putreanine

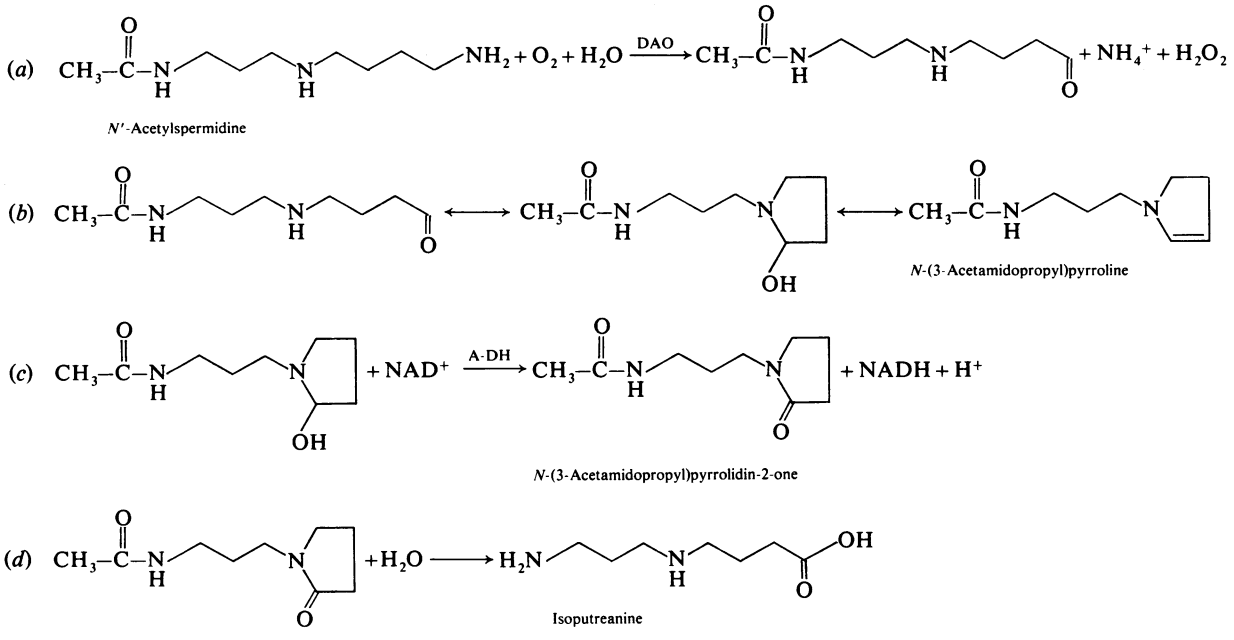

Fig. 8. *Reactions involved in isoputreanine formation from N^1-acetylspermidine*
Abbreviations: DAO, aminoguanidine-sensitive oxidase (diamine oxidase); A-DH, aldehyde dehydrogenase.

and isoputreanine from spermidine. The oxidative deamination of the aminobutyl moiety resembles 1-pyrroline formation from putrescine (Tabor, 1951) and spermidine and of *N*-(3-aminopropyl)-2-pyrroline formation from spermine by diamine oxidases (Hasse & Schürer, 1962; Smith, 1972). The latter reactions, which involve fission of the polyamine chain with formation of 1,3-diaminopropane, are characteristic of plant enzymes (Bachrach, 1973).

Generation of the lactam, instead of the amino acid, in the case of the oxidative deamination of the 4-aminobutyl moiety is energetically advantageous since a stable five-membered ring is formed from the corresponding 4-aminoaldehyde. The analogous formation of a four-membered ring is unfavourable. Putreanine is therefore the product of the oxidative deamination of the 3-aminopropyl moiety of spermidine.

Formation of a γ-lactam has previously been reported: incubations of putrescine with rat liver slices produce pyrrolidin-2-one among other species (Lundgren & Hankins, 1978). This reaction and the formation *in vivo* of *N*-(3-aminopropyl)pyrrolidin-2-one involve most probably the same reaction steps.

Whether *N*-(3-aminopropyl)-2-pyrroline or, as formulated in Fig. 7, *N*-(3-aminopropyl)-2-hydroxypyrrolidine is the actual substrate of the aldehyde dehydrogenase is presently a matter of speculation. In the case of pyrrolidin-2-one formation, Lundgren *et al.* (1980) argue in favour of 1-pyrroline as the intermediary metabolite. 2-Hydroxypyrrolidine should, however, be an intermediate in the formation of 1-pyrroline from 4-aminobutyraldehyde, and it may also be formed by addition of water to 1-pyrroline and thus be present in the solution in equilibrium with 1-pyrroline. From the above considerations and from the fact that no hydrolase is known in the mammalian organism that splits N^1-acetylspermidine into acetate and spermidine, it is clear why only isoputreanine was found in the hydrolysed $HClO_4$ extracts of the tissues of mice that received N^1-acetylspermidine.

In Fig. 8 the formation of *N*-(3-acetamidopropyl)pyrrolidin-2-one was formulated, by analogy with the formation of *N*-(3-aminopropyl)pyrrolidin-2-one formation from spermidine. Even if substantial amounts of *N*-acetylisoputreanine were formed, they would not have been detected by the high-pressure-liquid-chromatographic method without hydrolytic cleavage because acetylation of the primary amino group prohibits reaction with the *o*-phthalaldehyde reagent.

Two groups reported the determination of isoputreanine in urine samples (Nakajima *et al.*, 1980; Noto *et al.*, 1978; Asatoor, 1979). Since acid hydrolysis was essential to reveal the presence of

isoputreanine in urine, it is suggested that *N*-(3-aminopropyl)pyrrolidin-2-one and *N*-(3-acetamido)pyrrolidin-2-one are the natural excretory products.

As demonstrated by the results summarized in Fig. 2, the accumulation of the isoputreanine precursor is most prominent in small intestine, if N^1-acetylspermidine was administered. In liver and kidney, maximal values were already found at 20 min, indicating the rapid elimination of the isoputreanine derivative from these organs, or its metabolism.

In surprising contrast, *N*-(3-aminopropyl)pyrrolidin-2-one, which was formed from spermidine, was more rapidly accumulated in liver than in small intestine and kidney, in liver even more rapidly than putreanine (Fig. 6) and there was no putreanine accumulation observed in small intestine.

It is not possible to explain these findings in detail at present because they reflect the end result of a number of processes that are either unknown or unknown with respect to their quantitative role.

Different rates of absorption of spermidine and N^1-acetylspermidine from the peritoneum is of major importance only for exogenous amines, but the following aspects are of importance and need further consideration.

(*a*) Distribution by the circulation and retention by various organs plays a role in the metabolism of both exogenous and endogenous amines.

(*b*) Spermidine and N^1-acetylspermidine have different substrate characteristics with regard to diamine oxidases (Suzuki *et al.*, 1981; Gahl & Pitot, 1981) but they may be even preferentially oxidized by two different enzymes.

From the fact that 0.2 mmol of aminoguanidine/kg body wt. doses are capable of producing a nearly complete inhibition of putreanine and isoputreanine formation, it is reasonable to assume the role of diamine oxidase-like enzymes in this reaction *in vivo*. Serum spermine oxidase is known to attack exclusively the aminopropyl moiety of spermidine (Tabor *et al.*, 1964). In agreement with this is the fact that N^1-acetylspermidine, in contrast with N^8-acetylspermidine, is not a substrate of this enzyme (Mamont *et al.*, 1981; Gahl & Pitot, 1982). Hence one can conclude that serum spermine oxidase does not participate in isoputreanine formation.

(*c*) The substrate characteristics for aldehyde dehydrogenases of *N*-(3-aminopropyl)-2-hydroxypyrrolidine, *N*-(3-acetamidopropyl)-2-hydroxypyrrolidine and *N*-(4-aminobutyl)-3-aminopropanal, the presumed reaction products of the oxidase reaction (Figs. 7 and 8), may differ considerably, owing to the absence of a positive charge in the N^1-acetylspermidine derivative and the presence of one and two basic nitrogens respectively in the spermidine metabolites.

(*d*) The urinary elimination rate of the acetylated and non-acetylated lactam may differ considerably, and their rate of elimination may also differ considerably from that of putreanine.

It is conceivable that the formation of *N*-(3-aminopropyl)pyrrolidin-2-one or its conjugate reflects excessive spermidine formation, its excessive liberation into the circulation from tissues or an unusually high diamine oxidase activity in certain tissues. Thus it may prove to be a useful marker for certain pathological states.

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