Biochimica et Biophysica Acta, 615 (1980) 480—488

BBA 69102

POLYAMINE OXIDASE IN RAT TISSUES

N. SEILER, F.N. BOLKENIUS, B. KNÖDGEN and P. MAMONT

Centre de Recherche Merrell International, 16, rue d'Ankara, 67084 Strasbourg Cedex (France)

(Received March 19th, 1980)

Key words: Acetylpolyamine; Polyamine oxidase assay; (Rat)

Summary

An assay procedure for polyamine oxidase in tissue homogenates was devised. The method is based on the degradation of N^1,N^{12}-diacetylspermine to N^1-acetylspermidine and the determination using TLC of the latter.

Polyamine oxidase activity is high in most tissues. Its activity is comparable to that of spermidine and spermine synthase. The independence of this enzyme from cellular proliferation rates and its relatively long biological half-life are indicative of a passive role of polyamine oxidase in the regulation of cellular polyamine levels.

Introduction

Polyamine oxidase from rat liver is a flavin enzyme. It is capable of transforming spermine into spermidine, and spermidine into putrescine [1]. However, it was shown recently [2,3] that N^1-acetylspermidine *, N^1-acetylspermine and even N^1,N^{12}-diacetylspermine are much better substrates than the non-conjugated polyamines, and it was presumed that the acetylated polyamines are natural substrates of this enzyme.

Polyamine oxidase splits N^1,N^{12}-diacetylspermine first into N^1-acetylspermidine and 3-acetamidopropanal. N^1-acetylspermidine can react further to form putrescine and a second molecule of 3-acetamidopropanal. The reaction sequence is formulated in Fig. 1.

Recently, we published a sensitive method for the determination of acetyl-derivatives of polyamines [5]. The suitability of this method together with the fact that tissue levels of acetylpolyamines are usually very low (Seiler, N.,

* The nomenclature follows that of Tabor et al. [4].

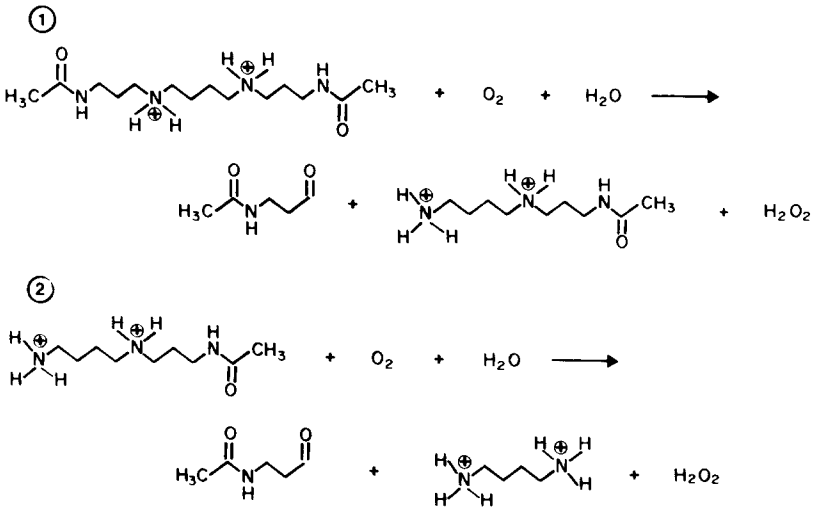

Fig. 1. Scheme of degradation of N^1,N^{12}-diacetylspermine by polyamine oxidase.

unpublished data), prompted us to establish optimum conditions for the formation of N^1-acetylspermidine from N^1,N^{12}-diacetylspermine by tissue homogenates, and to apply it to the study of some aspects of polyamine oxidase.

Materials and Methods

Chemicals. Usual laboratory chemicals and solvents were from Baker Chemicals (Deventer, The Netherlands), or from E. Merck (Darmstadt, F.R.G.). D,L-dithiothreitol, cycloheximide and bovine serum albumin were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Yeast RNA and deoxyribose from Serva (Heidelberg, F.R.G.). Pargyline was a gift from Abbot (France). N^1,N^{12}-diacetylspermine [2], N^1-acetylspermidine [4], 5-dimethylaminonaphthalene-1-sulfonyl chloride [6] and D,L-α-difluoromethyl ornithine (RMI 71782) [7] were synthesized by published procedures. [*methyl*-^{3}H]Thymidine (specific radioactivity 20 Ci/mmol and L-[4,5-^{3}H]leucine (specific radioactivity 52 Ci/mmol) were supplied by New England Nuclear Co. (Boston, MA, U.S.A.). The oil-in-water emulsion used as a vehicle for topical applications of cycloheximide was a gift from Vick International (Slough, U.K.).

Laboratory Animals. Male Sprague-Dawley rats weighing 200—250 g (Charles River, France) and male hairless mice weighing 22 ± 2 g (HRS/J-strain bred in our animal quarters) were used. They were kept under a standarized 12 h light-dark cycle and had access to standard diet and tap water ad libitum.

Preparation of tissue homogenates. Rats were decapitated and the various organs removed within 1 min. They were homogenized with 10 parts of ice-cold borate buffer pH 9 (15.5 g boric acid, 50 g sodium tetraborate, 60 ml 1 N NaOH per 5 l; EDTA and dithiothreitol were added at a final concentration of 1 mM and 0.02 mM, respectively).

Partial hepatectomy. This was done as described by Higgins and Anderson [8]. At different times after surgery the rats were killed by decapitation. Sham-

operated animals were used as controls for each time point.

Preparation of skin slices. Hairless mice were killed by inhalation of diethyl ether. About 1 × 3 cm-pieces of dorsal skin were prepared, cleaned and then fixed on a glass plate by freezing on solid CO_2. While still frozen, the skin samples were chopped, using a McIlwain tissue chopper (The Mickle Laboratory Engineering Co., Cromshall, Surrey, U.K.) into 0.1 × 10 mm slices.

Enzyme assay. The incubation mixture contained in a final volume of 180 μl homogenate or slices corresponding to about 10 mg of tissue, 0.56 mM N^1,N^{12}-diacetylspermine, 0.56 mM aminoguanidine sulfate and 0.036 mM pargyline. All solutions were prepared in the above mentioned borate buffer, pH 9.0. The samples were incubated in glass tubes of 9 mm inner diameter at constant-shaking frequency for 30 min at 37°C (air at atmosphere). The reaction was stopped by addition of a solution of 20 mg dansyl chloride (5-dimethylamino-naphthalene-1-sulfonyl chloride) in 300 μl acetone. After saturation with sodium carbonate, the reaction mixture was left overnight at room temperature to ensure completion of the reaction [6]. After reaction of the excessive dansyl chloride with proline the dansyl derivatives were extracted with 2 ml toluene.

In most cases it was adequate to apply 20 μl aliquots of the toluene solution to silica gel plates (silica gel 60, E. Merck, Darmstadt) and to develop the plates first with ethyl acetate, and subsequently with chloroform/tetrachloro-methane/methanol (70 : 30 : 5) (two runs). For stabilization the plates were dipped in a 10% solution in cyclohexane of Rhodorsil oil SI 710 (Prolabo, Paris, France) or paraffin oil.

The air-dried plates were quantitatively evaluated by direct scanning of fluorescence. The Camag (Muttenz, Switzerland) thin-layer scanner was used for this purpose. Fluorescence was activated at 360 nm.

If a spot interfering with bisdansyl-N^1-acetylspermidine was observed on the plate, the toluene solution was evaporated in a stream of air. The residue was heated for 30 min at 50°C with 150 μl of 5 M KOH in methanol, in order to remove hydrolyzable dansyl derivatives. Addition of 1.5 ml of a solution KH_2PO_4 + Na_2HPO_4 (100 mg each per 1.5 ml) and extraction with 2 ml toluene recovers the dansyl derivatives. (For more details of the method of acetylpoly-amine determination see Ref. 5.)

Determination of protein, RNA and DNA. Proteins were estimated using the method of Lowry et al. [25] modified by Hartree [9]. Isolation and separation of RNA and DNA was accomplished using the method described by Shibko et al. [10]. RNA was estimated by ultraviolet spectrophotometry, DNA was measured using the method of Burton [26] as modified by Giles, K.W. and Myers, A. [11]. Bovine serum albumin, yeast RNA and 2-deoxyribose were used as standards.

Polyamine oxidase in rat hepatoma cells. Hepatoma tissue culture cells (HTC) were grown in spinner culture to a high density ($9 \cdot 10^5$ cells per ml), as previously described [12]. The culture was diluted to $1 \cdot 10^5$ cells per ml with Swim's 77 medium supplemented with horse serum. The cells were incubated for 3 days in the presence or absence of 5 mM D,L-α-difluoromethyl ornithine, an irreversible inhibitor of ornithine decarboxylase [7,13]. Then they were harvested by gentle centrifugation (200 × *g*) and resuspended in fresh medium ($1 \cdot 10^5$ cells per ml). Some of the cells were analyzed immediately (time zero),

the others were incubated again for varying times with or without difluoromethyl ornithine.

For the assay of polyamine oxidase $1 \cdot 10^6$ cells were combined, washed twice with phosphate-buffered saline and suspended in 400 µl of borate buffer, pH 9. Five cycles of freezing (liquid nitrogen) and thawing with intermittent homogenization in small all-glass homogenizers ensured reproducible cell homogenates. 100-µl aliquots were used for incubations.

Results

The enzymatic assay. A partially purified polyamine oxidase from rat liver [2] was used for exploratory experiments. This preparation was free of monoamine oxidase and diamine oxidase. It allowed us to establish that pargyline at 0.1 mM and aminoguanidine at 1 mM did not influence the degradation of N^1,N^{12}-diacetylspermine to N^1-acetylspermidine. Subsequently, the two inhibitors were added routinely to the assay medium in order to avoid degradation by these enzymes.

The K_m of N^1,N^{12}-diacetylspermine had been established previously to be 5 µM [2]. Using rat kidney homogenates, substrate concentrations between 0.1 mM and 2 mM showed saturation kinetics. Reaction rate was linear for at least 60 min with 0.56 mM substrate in respect to N^1-acetylspermidine formation, and the amount of reaction product formed was proportional to the amount of tissue, if it did not exceed 10 mg per incubation mixture (Fig. 2). Incubation times of 30 min with 10 mg tissue or less were, therefore, adequate for routine assays. The reproducibility of the method under these conditions was better than ±10% of the mean values, if the amount of the product formed was between 2—70 nmol per reaction mixture.

Polyamine oxidase in rat tissues. Polyamine oxidase activity was measured in a number of rat tissue homogenates. Table I summarized the results. All tissues showed relatively high enzyme activity. The highest activity was found in pan-

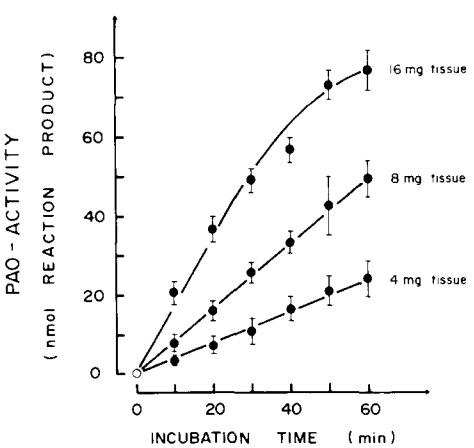

Fig. 2. N^1-acetylspermidine formation from N^1,N^{12}-diacetylspermine by rat kidney homogenates. The influence of incubation time and tissue amount on product formation. (Borate buffer pH 9.0; substrate conc. 0.56 mM; for futher details see Materials and Methods section). PAO, polyamine oxidase.

TABLE I

POLYAMINE OXIDASE ACTIVITY IN RAT TISSUES

Organ	nmol/g wet tissue per min	
Pancreas	381 ± 37	(3) *
Liver	202 ± 28	(14)
Spleen	152 ± 26	(7)
Kidney	149 ± 22	(6)
Small intestine	108 ± 15	(6)
Testes	98 ± 8	(5)
Thymus	93 ± 20	(3)
Prostate ***	69 ± 8	(3)
Brain	63 ± 21	(6)
Lung	43 ± 18	(4)
Heart	20 ± 3	(3)
Skeletal muscle	10 ± 3	(1)
Serum **	7 ± 0.3	(2)

* Number of animals in parentheses.

** nmol/ml serum per min.

*** Ventral prostate.

creas (381 nmol/g wet weight per min), and the lowest in skeletal muscle (10 nmol/g wet weight per min).

In order to allow the comparison of polyamine oxidase activity with the activity of polyamine synthesizing enzymes, we also carried out measurements in liver high-speed supernatant; 6 nmol N^1-acetylspermidine was formed per mg protein within 30 min. This value is about twice as high as spermidine synthase activity, measured in a similar preparation [14].

Effect of partial hepatectomy on polyamine oxidase activity. Polyamine oxidase was determined in the liver of partially (70%) hepatectomized rats at different times after hepatectomy. Sham-operated animals were used as controls for each time point. Fig. 3 summarizes the results. Each point represents the mean value of triplicate determinations from three animals. Although the values of the hepatectomized animals were always higher by about 20% than the corresponding controls, the difference was not statistically significant. The small fluctuations of polyamine oxidase activity were paralleled by analogous fluctuations of the RNA and protein values.

Polyamine oxidase activity in rat hepatoma cells during slow and rapid growth phases. The purpose of these experiments was to see whether polyamine oxidase activity is significantly influenced by cellular growth rates. Fig. 4 shows the results with cultured cells. Besides the comparison of the oxidase activity in slow-growing cells and cells in the logarithmic growth phase, a third aspect was studied, namely the activity of the oxidase in cells with decreased growth rates due to the depletion of cellular polyamines. The experimental conditions of retardation of cellular growth by difluoromethyl ornithine have been previously established [13]. From Fig. 4 it can be seen that the difference between polyamine oxidase activity in slow or rapidly-growing HTC cells is not impressive. A decrease of oxidase activity was observed during day one of incubation with a subsequent tendency to increase toward starting-point values. In contrast, difluoromethyl ornithine-treated cells did not show this increase.

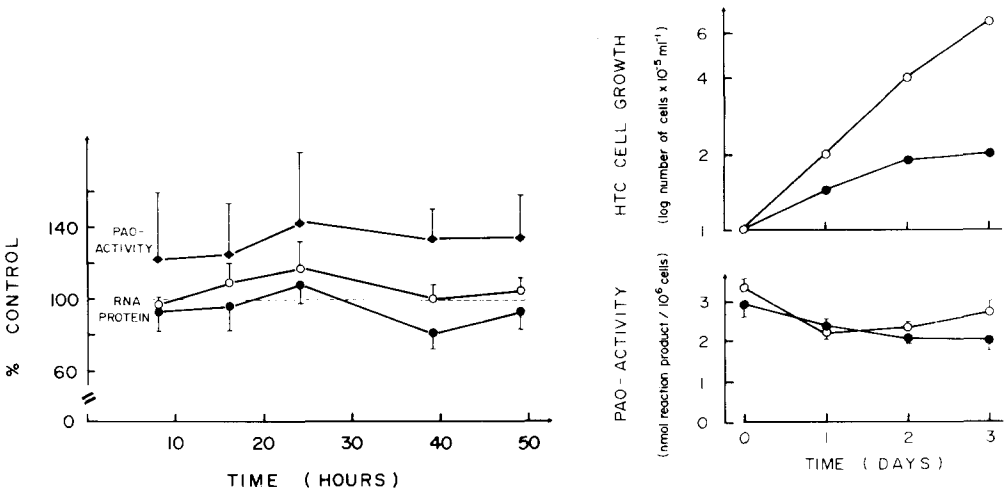

Fig. 3. Polyamine oxidase activity (PAO), and protein and RNA content in the liver of partially hepatectomized rats related to DNA content. Each point represents the mean ± S.D. of triplicate determinations of three animals. Three sham-operated rats were the controls at each time point. The average increase of polyamine oxidase above controls by about 20%, was not statistically significant.

Fig. 4. Growth rate and polyamine oxidase activity (PAO) in cultured rat hepatoma cells. HTC cells were first grown for 3 days in the presence or absence of D,L-difluoromethyl ornithine. (At this time they were approximately confluent). At time zero they were diluted with fresh medium with (dots) or without (circles) difluoromethyl ornithine and allowed to grow for a further 3 days. Polyamine oxidase was determined in duplicate in three batches. The vertical bars indicate S.D.

Polyamine oxidase activity in the skin of hairless mice (HRS/J strain). Attempts to isolate epidermis by the known heating procedure [16] resulted in partial or total inactivation of polyamine oxidase. It was therefore necessary to use whole skin preparations.

Skin homogenates prepared by extensive homogenization in all-glass homogenizers under vigorous cooling were less active than the 0.1 mm thick skin slices. The latter allowed us to measure polyamine oxidase activity with good reproducibility (S.D. ± 10% of the mean value).

Irradiation with a germicidal lamp (G8T5, General Electric, Cleveland, OH, U.S.A.) under standardized conditions induces polyamine turnover in mouse epidermis. A several-fold increase of putrescine, a doubling of spermidine and a decrease by about 50% of spermine levels have been observed at 24 h in the epidermis of hairless mice [16]. Under the same conditions, however, polyamine oxidase activity of skin was not significantly different from that in non-irradiated hairless mice (1.91 ± 0.17 nmol/mg skin per h), during the period of 48 h after exposure to ultraviolet light.

In order to obtain at least a crude estimate of the biological half-life of polyamine oxidase about 50 mg/cm^2 of an 1 : 10 (w/w) mixture of cycloheximide in an oil-in-water emulsion were applied to the dorsal skin of hairless mice every 24 h. Polyamine oxidase activity, protein and DNA were measured subsequently in the skin at different times. Animals treated with the oil-in-water emulsion for the same length of time were used as controls. In a pilot experiment it was established that a single treatment with cycloheximide inhibited (at 24 h) the incorporation of labelled leucine into proteins, and thymidine into

TABLE II

THE EFFECT OF A SINGLE TOPICAL DOSE OF CYCLOHEXIMIDE ON EPIDERMAL PROTEIN AND DNA METABOLISM AND ON POLYAMINE OXIDASE ACTIVITY AT 24 h

50 mg/cm^2 of a 1 + 10 (w/w) mixture of cycloheximide in an oil-in-water emulsion was applied to the dorsal skin of hairless mice. At 23 h 5 μCi of [^{3}H]leucine or 25 μCi of [^{3}H]thymidine were injected intraperitonally and the animals were sacrificed 60 min later. Control animals were treated with the oil-in-water emulsion. Radioactivity in proteins and DNA was determined by known procedures (16). Polyamine oxidase activity was measured from identically treated animals. Control skin was taken, however, from the contralateral side of the treated animals. (Mean values ± S.D., N = 5).

	Cycloheximide treated	Control
Protein (dpm/mg)	118 ± 31	2278 ± 174
DNA (dpm/nmol deoxyribose)	220 ± 390	6690 ± 1430
Polyamine oxidase (nmol/mg skin per h)	1.45 ± 0.34	2.17 ± 0.21

epidermal DNA by about 95%. Furthermore, only the cycloheximide-treated part of the skin showed a decrease of polyamine oxidase activity, not the contralateral, untreated skin. These results are summarized in Table II. The time-dependent effects of cycloheximide on polyamine oxidase activity in mouse skin are summarized in Table III. The treatment with cycloheximide is toxic to the animals, but since the local effects of the drug are more significant than the general toxicity elicited by diffusion of the drug into the circulation, the topical application allowed us to pursue polyamine oxidase activity for as long as 64 h. It appears from the values given in Table III that a time-dependent loss of polyamine oxidase activity can be observed in skin, after treatment with cycloheximide. From the polyamine oxidase activity values based on skin

TABLE III

EFFECT OF CYCLOHEXIMIDE ON POLYAMINE OXIDASE ACTIVITY AND PROTEIN AND DNA CONTENT OF THE DORSAL SKIN OF HAIRLESS MICE

50 mg/cm^2 of a 1 + 10 (w/w) mixture of cycloheximide in an oil-in-water emulsion was applied to the dorsal skin every 24 h. Polyamine oxidase activity, protein and DNA content of the treated skin was measured at various times. The values are the means of duplicate determinations of three animals ± S.D. Control animals (N = 9) were treated for varying times with the oil-in-water emulsion; the values of all control animals were combined in the 0 h value, since there was no difference between the three groups.

Polyamine oxidase activity			DNA	Protein
h	(nmol/mg skin per h)	(nmol/μmol deoxyribose per h)	(nmol deoxyribose /mg skin)	(μg/mg skin)
0	1.91 ± 0.17	883 ± 147	2.16 ± 0.15	136.2 ± 6.2
1	1.73 ± 0.13	800 ± 90	2.16 ± 0.07	135.0 ± 3.1
2	1.82 ± 0.25	—	—	—
6	1.80 ± 0.11	964 ± 201	1.87 ± 0.22	126.7 ± 9.9
18	1.31 ± 0.12	—	—	—
24	1.30 ± 0.14	697 ± 107	1.87 ± 0.07	114.7 ± 6.1
30	1.11 ± 0.12	—	—	—
48	1.05 ± 0.08	612 ± 107	1.72 ± 0.15	116.9 ± 4.0
64	0.74 ± 0.1	—	—	—

weight, a biological half-life of 40 h can be calculated. However, both DNA and protein content (based on fresh weight) of the skin decreased. The actual biological half-life of polyamine oxidase must therefore be considerably longer than it appears from the activity measurements based on skin weight. If enzyme activity is based on the DNA content of the skin, a biological half-life of polyamine oxidase exceeding 3 days can be estimated from the values given in Table III. A more precise determination is not feasible with the cycloheximide method, because of the toxicity of the drug and the long time periods needed for the significant decrease of polyamine oxidase.

Discussion

Previous attempts to measure polyamine oxidase activity relied on the formation of [^{14}C]spermidine from [^{14}C]spermine or of [^{14}C]putrescine from [^{14}C]spermidine. The reaction products were either isolated by electrophoresis [1] or [^{14}C]putrescine was transformed into Δ^1-pyrroline by diamine oxidase. Δ^1-Pyrroline was then determined [17] by the method of Kobayashi [27].

The method for measurement of polyamine oxidase activity presented here relies on the measurement of N^1-acetylspermidine formed by degradation from N^1,N^{12}-diacetylspermine. The further degradation of this compound to putrescine is avoided by high substrate concentrations and by a relatively short incubation time. Substantial putrescine formation can in fact be expected only in the case of high enzyme activities, as can be derived from our previous kinetic study [2].

The most conspicuous finding of this work is the high polyamine oxidase activity in most tissues. Although there is no direct correlation between the two enzyme activities, the polyamine oxidase pattern resembles, more or less, the organ distribution of spermidine synthase [14].

Polyamine oxidase is obviously not easily inducible, as is shown by the stability of the enzyme activity in regenerating liver, and in the ultraviolet-irradiated skin. Under both conditions it is known that ornithine decarboxylase and *S*-adenosylmethionine decarboxylase activities are increased and that polyamine biosynthesis is enhanced [16,18—20]. Partial hepactectomy, treatment with carbon tetrachloride, thioacetamide and growth hormone have already been tested by Hölttä [1] as growth stimuli with no significant changes of polyamine oxidase activity in rat liver. The obvious stability of enzyme activity is also demonstrated by the small changes observed in HTC cells at various growth rates. Quash et al. [17] reported similar findings for cultured normal, rat kidney cells. In virus-transformed kidney cells they found, however, a marked fluctuation of the enzyme with changing growth rates.

The small influence of difluoromethyl ornithine treatment on cellular polyamine oxidase activity is a further observation which fits into the general picture of polyamine oxidase as a stable enzyme. It seems not unlikely that depletion of difluoromethyl ornithine-treated cells of putrescine and spermidine, which occurs under the experimental conditions [13] could act as a signal for the adjustment of the polyamine degrading enzyme to a lower level. The small, but gradual decrease of the enzyme activity during incubation of the HTC cells with the difluoromethyl ornithine could be interpreted as a consequence of low

cellular polyamine levels, but the changes are too small to be considered as having physiological importance, unless the enzyme loss is restricted to a certain metabolic compartment.

These observations, together with the apparently long biological half-life of the enzyme, are in agreement with the notion that polyamine oxidase is not rate-limiting and thus not actively regulating cellular polyamine levels according to momentary needs. Its function seems to be rather the removal of excessive polyamines and presumably the maintenance of low cytoplasmic N^1-acetylspermidine levels. The latter compound is, however, a normal urinary, excretory compound [21–23]. The relative importance of degradation and excretion of N^1-acetylspermidine remains to be established.

A close analogy between polyamine oxidase and monoamine oxidase seems to exist not only with regard to FAD as cofactor, and the similar reaction mechanism, but also with regard to the passive role in the control of cellular amine levels. Since monoacetyl putrescine is a substrate of monoamine oxidase [24] it is even possible that the two enzymes fulfil similar functions in the metabolism of two closely related compounds N^1-acetylspermidine and N^1-acetylputrescine.

Acknowledgement

We would like to thank Miss M. Nüssli for technical assistance.

References

1 Hölttä, E. (1977) Bichemistry 16, 91–100
2 Bolkenius, F.N. and Seiler, N. (1980) Int. J. Biochem., submitted for publication
3 Seiler, N. (1980) in Polyamines in Biology and Medicine (D.R. Morris and L.H. Marton, eds.), Marcel Dekker Inc. New York
4 Tabor, H., Tabor, C.W. and DeMeis, L. (1971) Adv. Enzymol. 17B, 829–833
5 Seiler, N. and Knodgen, B. (1979) J. Chromatogr. 164, 155–168
6 Seiler, N. (1970) Meth. Biochem. Anal. 18, 259–337
7 Metacalf, B.W., Bey, P., Danzin, C., Jung, M., Casara, P. and Vevert, J.P. (1978) J. Am. Chem. Soc. 100, 2551–2553
8 Higgins, G.M. and Anderson, R.M. (1931) Arch. Pathol. 12, 186–202
9 Hartree, E.F. (1972) Anal. Biochem. 48, 422–427
10 Shibko, S., Koivistoinen, P., Tratnyek, C.A., Newhall, A.R. and Friedman, L. (1967) Anal. Biochem. 19, 514–528
11 Giles, K.W. and Myers, A. (1965) Nature 4979, 93
12 Hershko, A. and Tomkins, G.M. (1971) J. Biol. Chem. 246, 710–714
13 Mamont, P.S., Duchesne, M.C., Grove, J. and Bey, P. (1978) Biochem. Biophys. Res. Commun. 81, 58–66
14 Raina, A., Pajula, R.L. and Eloranta, T. (1976) FEBS Lett. 67, 252–255
15 Du Vivier, A. and Stoughton, R.B., Invest. Dermatol. (1975) 65, 235–237
16 Seiler, N. and Knödgen, B. (1979) Biochem. Med. 21, 168–181
17 Quash, G., Keoloungkhot, T., Gazzolo, L., Ripoll, H. and Saez, S. (1979) Biochem. J. 177, 275–282
18 Hölttä, E. and Jänne, J. (1972) FEBS Lett. 23, 117–121
19 Hölttä, E., Sinervirta, R. and Jänne, J. (1973) Biochem. Biophys. Res. Commun. 54, 350–357
20 Verma, A.K., Lowe, N.J. and Boutwell, R.K. (1979) Cancer Res. 39, 1035–1040
21 Nakajima, T., Zack, J.F. and Wolfgram, F. (1969) Biochim. Biophys. Acta 184, 651–652
22 Walle, T. (1973) in Polyamines in Normal and Neoplastic Growth (Russell, D.H., ed.), pp. 355–365, Raven Press, New York
23 Abdel-Monem, M.M. and Ohno, K. (1977) J. Pharm. Sci. 66, 1195–1197
24 Seiler, N. and Al-Therib, M.J. (1974) Biochem. J. 144, 29–35
25 Lowry, O.H., Rosebrough, W.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
26 Burton, K. (1956) Biochem. J. 62, 315–323
27 Kobayashi, Y. (1963) J. Lab. Clin. Med. 62, 699–702