Biochem. J. (1977) **166**, 89–94
Printed in Great Britain

Polyamines and their Biosynthetic Enzymes in Ehrlich Ascites-Carcinoma Cells

MODIFICATION OF TUMOUR POLYAMINE PATTERN BY DIAMINES

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(*Received* 10 *January* 1977)

1. Ehrlich ascites-carcinoma cells contained relatively high concentrations of spermidine and spermine, but the putrescine content of the washed cells was less than 10% of that of higher polyamines. 2. Ascites-tumour cells likewise exhibited high activities of L-ornithine decarboxylase (EC 4.1.1.17), *S*-adenosyl-L-methionine decarboxylase (EC 4.1.1.50), spermidine synthase (EC 2.5.1.16) and spermine synthase. 3. During the first days after the inoculation, the polyamine pattern of the ascites cells was characterized by a high molar ratio of spermidine to spermine, which markedly decreased on aging of the cells. 4. Various diamines injected into mice bearing ascites cells rapidly and powerfully decreased ornithine decarboxylase activity in the carcinoma cells, apparently through a mechanism that was not a direct inhibition of the enzyme *in vitro*. Cadaverine (1,5-diaminopentane) and 1,6-diaminohexane were the most potent inhibitors of ornithine decarboxylase among the amines tested. 5. Chronic treatment of the mice with diamines resulted in a virtually complete disappearance of ornithine decarboxylase activity, and after 24h a significant decline in spermidine accumulation. 6. Cadaverine appeared to be an especially suitable compound for use as an inhibitor of the synthesis of higher polyamines, at least in Ehrlich ascites cells, since this diamine also acted as a competitive inhibitor for putrescine in the spermidine synthase reaction without being incorporated into the higher polyamines.

The significance of the enhanced biosynthesis and accumulation of natural polyamines (putrescine, spermidine and spermine) under conditions of rapid growth remains intriguingly elusive. A large body of evidence indicates that these compounds exert a great number of effects on diverse systems *in vitro* (Tabor & Tabor, 1972) that might or might not be relevant to their functions *in vivo*. The physiological roles of polyamines can only be determined through a selective decrease of these compounds in living cells, whereafter the metabolic consequences resulting from the polyamine deficiency may be properly investigated.

A number of more or less specific inhibitors, mainly congeners of L-ornithine or inhibitors of *S*-adenosylmethionine decarboxylase, have been developed. These include 1,1′-[(methylethanediyldidine)-dinitrilo]diguanidine [methylglyoxal bis-(guanylhydrazone)] (Williams-Ashman & Schenone, 1972), α-hydrazino-ornithine (Harik *et al.*, 1974), α-hydrazino-δ-aminovaleric acid (5-amino-2-hydrazinopentanoic acid) (Inoue *et al.*, 1975), unsaturated derivatives of L-ornithine and putrescine (Relyea & Rando, 1975) and α-methylornithine (Mamont *et al.*, 1976). All these compounds are inhibitors of ornithine decarboxylase or *S*-adenosylmethionine decarboxylase acting directly on the enzymes also *in vitro*.

An alternative approach towards specific inhibition of polyamine synthesis is based on the fact that mammalian ornithine decarboxylase appears to be regulated through an indirect repression-type mechanism, not only by natural polyamines (putrescine and spermidine) (Kay & Lindsay, 1973; Clark, 1974; Jänne & Hölttä, 1974), but also by other amines not normally found in animal tissues, such as 1,3-diaminopropane (Pösö & Jänne, 1976*a*) and cadaverine (1,5-diaminopentane) (Pösö, 1977).

These inhibitors, both directly and indirectly acting, have been used to prevent the enhanced accumulation of polyamines during lymphocyte activation (Fillingame *et al.*, 1975), in mouse parotid gland after isoproterenol administration (Inoue *et al.*, 1975) and during rat liver regeneration (Pösö & Jänne, 1976*b*). In every instance the inhibition of polyamine biosynthesis appeared to result in, or at least to be accompanied by, a remarkable anti-proliferative effect, mainly shown as decreased DNA synthesis.

In the present work we have exploited the possibilities to modify the pattern of natural polyamines (putrescine, spermidine, spermine) in Ehrlich ascites cells by using the repression-type inhibition of ornithine decarboxylase exerted by various amines. It is shown that ornithine decarboxylase activity of the tumour cells, just like that of rat liver, can be

decreased *in vivo* by a variety of diamines. The inhibition of ornithine decarboxylase by cadaverine, which also acted as an inhibitor of spermidine synthesis, led to a marked decrease in the concentration of tumour spermidine.

Experimental

Materials

Ehrlich ascites-carcinoma cells were maintained in the peritoneal cavity of female albino mice. The mice were inoculated with 0.25ml of the ascites fluid diluted with 0.9% NaCl (1:1, v/v). Under these conditions the animals died after 8–10 days. The cells were harvested at day 6 after the inoculation if not otherwise indicated.

L-[1-^{14}C]Ornithine (sp. radioactivity 59mCi/mmol) was purchased from The Radiochemical Centre (Amersham, Bucks., U.K.). *S*-Adenosyl-L-[1-^{14}C]-methionine was prepared enzymically from L-[^{14}C]-methionine (The Radiochemical Centre) essentially as described by Pegg & Williams-Ashman (1969).

[1,4-^{14}C]Putrescine (sp. radioactivity 17.5mCi/mmol), [1,4-^{14}C]spermidine (sp. radioactivity 12.4mCi/mmol) and [1,5-^{14}C]cadaverine (sp. radioactivity 3.27mCi/mmol) were purchased from the New England Nuclear Corp. (Dreieichenhain, West Germany).

Decarboxylated adenosylmethionine (*S*-adenosylmethylhomocysteamine) was prepared enzymically with *S*-adenosylmethionine decarboxylase partially purified from cells of *Escherichia coli* by the method described in detail by Pösö *et al.* (1976).

Putrescine, cadaverine, spermidine and spermine (as their hydrochloride salts) were purchased from Calbiochem (San Diego, CA, U.S.A.). 1,3-Diaminopropane was obtained from Fluka A.G. (Buchs SG, Switzerland) and 1,6-diaminohexane (as the hydrochloride salt) from BDH Chemicals Ltd. (Poole, Dorset, U.K.). The amines were neutralized with NaOH (or HCl for diaminopropane) and injected intraperitoneally in small volumes (0.2–0.25ml).

Preparation of the cell extracts

The tumour cells were harvested and rapidly washed twice at 0°C with 25mM-Tris/HCl buffer (pH7.4) containing 0.1mM-EDTA and 1mM-dithiothreitol. The cells were disintegrated by ultrasonication with a Branson Sonifier (model B-30; Branson Ultrasonic Corp., Danbury, CT, U.S.A.) at half-maximal power (6×5s), in an ice/ethanol bath for cooling. After samples for polyamine and protein determinations were taken, the sonicated preparations were centrifuged at 100000$g_{max.}$ for 30min in a Spinco ultracentrifuge. The resultant cytosol fractions were used as the source of enzymes.

Spermidine synthase and spermine synthase were partially purified from the cytosol fractions of ascites cells by $(NH_4)_2SO_4$ fractionation. The proteins precipitated between 0.3 and 0.6 saturation of $(NH_4)_2SO_4$ were dialysed overnight against the above buffer and used as the source of the enzymes in inhibition studies.

Analytical methods

The activities of ornithine decarboxylase (EC 4.1.1.17) (Jänne & Williams-Ashman, 1971*a*), *S*-adenosylmethionine decarboxylase (EC 4.1.1.50) (Jänne & Williams-Ashman, 1971*b*), spermidine synthase (EC 2.5.1.16) (Jänne *et al.*, 1971) and spermine synthase (Hannonen *et al.*, 1972) were assayed by methods used as a routine in our laboratory.

Polyamines were measured after alkaline butanol extraction (Raina, 1963) and paper electrophoresis by staining the fractions with ninhydrin (Raina & Cohen, 1966). Putrescine, 1,3-diaminopropane and cadaverine were separated from each other by using sulphosalicylic acid buffer (pH3.2) in the electrophoresis (Raina, 1963; Pösö & Jänne, 1976*b*). The extraction recoveries for putrescine, spermidine and spermine were 86, 92 and 95%, appropriate corrections being applied. Corresponding recoveries for diaminopropane and cadaverine were 67 and 89%.

Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard.

For statistical analyses, Student's *t* test (two-tailed) was used.

Results

Polyamine concentrations in Ehrlich ascites cells during tumour growth

The concentrations of putrescine, spermidine and spermine were measured in extracts of ascites-carcinoma cells at different times after the inoculation. As shown in Table 1, only minor changes were found in the concentration of cellular putrescine during tumour growth, whereas both spermidine and spermine concentrations sharply increased between days 5 and 6 after the inoculation (Table 1). The concentrations of spermidine and spermine were surprisingly constant from experiment to experiment, but that of putrescine varied greatly. The molar ratio of spermidine to spermine appeared to be remarkably high during the early days of tumour growth, decreasing as the cells aged (Table 1).

Biosynthetic enzymes of polyamines in Ehrlich ascites cells during tumour growth

Ornithine decarboxylase and *S*-adenosylmethionine decarboxylase exhibited highest activities at

Table 1. *Polyamine concentrations in Ehrlich ascites cells at different times after inoculation*
The concentrations of polyamines are expressed as nmol (±S.D. or ranges) per mg of protein. The molar ratios have been computed by using individual amine concentrations. The number of mice in each group is given in parentheses. The significance of the differences (compared with the 5-day group) was: $^{**}P<0.01$; $^{***}P<0.001$.

Time after inoculation (days)	Putrescine	Spermidine	Spermine	Molar ratio of spermidine/spermine
4 (2)	2.50 (2.1–2.9)	18.15 (15.6–20.7)	8.80 (8.1–9.5)	2.04 (1.92–2.17)
5 (6)	2.28±0.65	12.68±1.13	6.99±1.00	1.82±0.14
6 (4)	2.91±0.88	21.47±5.78**	15.01±4.23**	1.42±0.11**
7 (3)	2.83±0.50	18.76±2.52***	11.79±2.28**	1.60±0.11

Table 2. *Activities of polyamine-biosynthetic enzymes in Ehrlich ascites cells at different times after inoculation*
The enzyme activities are expressed as nmol (±S.D. or ranges) of product formed/30min per mg of protein. The number of mice in each group is given in parentheses. The significance of the differences (compared with the 5-day group) was: $^{*}P<0.05$; $^{***}P<0.001$.

Time after inoculation (days)	Ornithine decarboxylase	*S*-Adenosylmethionine decarboxylase	Spermidine synthase	Spermine synthase
4 (2)	0.38 (0.34–0.43)	0.28 (0.26–0.31)	3.79 (3.07–4.51)	0.58 (0.52–0.65)
5 (6)	0.53±0.25	0.31±0.10	3.11±0.37	0.47±0.10
6 (4)	0.44±0.17	0.28±0.06	4.84±1.30*	0.75±0.09***
7 (5)	0.31±0.15	0.22±0.05	4.76±0.74***	0.66±0.09*

Table 3. *Effect of various diamines on ornithine decarboxylase and adenosylmethionine decarboxylase activities in vivo*
Neutralized amines (150μmol/100g body wt.) were injected intraperitoneally into mice, inoculated with cells 6 days earlier, 1h and 2h before death. The enzyme activities are expressed as nmol of CO_2 (±S.D.)/30min per mg of protein. There were three or four animals in each group. The significance of the differences (compared with the NaCl-injected group) was: $^{*}P<0.05$; $^{**}P<0.01$.

Treatment	Ornithine decarboxylase	*S*-Adenosylmethionine decarboxylase
0.9% NaCl	0.23±0.08 (100%)	0.55±0.06 (100%)
1,3-Diaminopropane	0.11±0.02* (48%)	0.44±0.08 (80%)
Putrescine (1,4-diaminobutane)	0.10±0.01* (43%)	0.70±0.20 (127%)
Cadaverine (1,5-diaminopentane)	0.07±0.02** (30%)	0.62±0.06 (113%)
1,6-Diaminohexane	0.09±0.05* (39%)	0.81±0.14* (147%)

day 5 after inoculation; these activities thereafter gradually decreased (Table 2), although these changes were not statistically significant. Just like the concentrations of the higher polyamines (Table 1), the activities of spermidine synthase and spermine synthase showed marked and significant increases between days 5 and 6 after the inoculation (Table 2).

As in many other mammalian tissues (Raina *et al.*, 1976) the activity of spermidine synthase in Ehrlich ascites cells appeared to be more than 10 times the activities of the two decarboxylases and of spermine synthase (Table 2). It is thus likely that both ornithine decarboxylase and *S*-adenosylmethionine decarboxylase function as the rate-controlling enzymes in the synthesis of higher polyamines in these cells.

Inhibition of ornithine decarboxylase by various diamines

Two subsequent injections (1 and 2h before death of the mouse) of various diamines (150μmol/100g body wt.) resulted in a marked inhibition of ornithine decarboxylase activity in Ehrlich ascites cells (Table 3). The longer diamines (1,5-diaminopentane and 1,6-diaminohexane) appeared to be more potent inhibitors of the enzyme than the shorter ones (1,3-diaminopropane and 1,4-diaminobutane). In striking contrast with ornithine decarboxylase, the activity of *S*-adenosylmethionine decarboxylase only showed minor changes in response to the amine injections (Table 3). Diaminopropane slightly inhibited and diaminohexane significantly stimulated tumour *S*-adenosylmethionine decarboxylase activity (Table 3).

It should be emphasized that the inhibition of ornithine decarboxylase activity by the diamines most likely involved an inhibition of the accumulation of the enzyme protein, since none of the compounds tested exerted any inhibition on ornithine

Table 4. *Effect of putrescine and diaminopropane on the activities of ornithine decarboxylase and S-adenosylmethionine decarboxylase and on the concentrations of polyamines in Ehrlich ascites cells during chronic treatment*

The mice received 150 μmol of the amines/100 g body wt. (or 0.9% NaCl) every 3 h as intraperitoneal injections for 12 h starting on day 6 after inoculation of cells. The enzyme activities are expressed as nmol of CO_2 (±S.D.)/30 min per mg of protein and the concentrations of polyamines as nmol (±S.D.)/mg of protein. There were three or four animals in each group. The significance of the differences (compared with the NaCl-injected group) was: $*P<0.05$; $**P<0.01$; $***P<0.001$.

Treatment	Ornithine decarboxylase	*S*-Adenosylmethionine decarboxylase	Putrescine	Spermidine	Spermine
NaCl	0.34±0.09	0.70±0.11	0.75±0.15	12.7±2.0	8.9±0.6
Putrescine	0.01±0.02***	0.56±0.10	4.97±1.6**	12.8±1.1	7.1±0.5**
Diaminopropane	0.00†***	0.45±0.12*	0.57±0.08	10.9±0.4	7.8±0.6

† Not detectable.

Table 5. *Effect of putrescine and cadaverine on the activities of ornithine decarboxylase and S-adenosylmethionine decarboxylase and on the concentrations of polyamines in Ehrlich ascites cells during chronic treatment*

The mice received 150 μmol of the amines/100 g (or 0.9% NaCl) every 3 h as intraperitoneal injections for 24 h starting on day 6 after inoculation of cells. The enzyme activities are expressed as nmol of CO_2 (±S.D.)/30 min per mg of protein and the concentrations of polyamines as nmol (±S.D.)/mg of protein. There were four to six animals in each group. The significance of the differences (compared with the NaCl-injected group) was: $**P<0.01$; $***P<0.001$.

Treatment	Ornithine decarboxylase	*S*-Adenosylmethionine decarboxylase	Putrescine	Spermidine	Spermine
NaCl	0.25±0.10	0.26±0.10	0.62±0.14	10.3±2.2	6.0±0.5
Putrescine	0.01±0.01***	0.18±0.04	1.72±0.44**	10.3±0.9	4.9±0.4**
Cadaverine	0.02±0.01***	0.22±0.06	0.00†***	6.0±1.5**	7.9±0.7**

† Not detectable.

decarboxylase activity *in vitro* under the assay conditions used (results not shown).

Effect of chronic administration of diamines on the polyamine pattern of Ehrlich ascites cells

Putrescine and diaminopropane injected at every 3 h for 12 h completely abolished ornithine decarboxylase activity of the tumour cells, as shown in Table 4. Putrescine treatment did not change the concentration of spermidine, but significantly decreased the concentration of cellular spermine (Table 4). Injections of diaminopropane slightly decreased both spermidine and spermine concentrations, but these changes were not statistically significant.

With cadaverine instead of diaminopropane and by also using somewhat longer periods of treatment (24 h), it was possible to inhibit ornithine decarboxylase completely, 'remove' putrescine and markedly decrease the concentration of tumour spermidine (Table 5). As also shown in Table 5, similar treatment with an equimolar dose of putrescine increased tumour putrescine, did not change the concentration of spermidine, but significantly decreased the concentration of spermine. Continuous administration of cadaverine, however, markedly increased the concentration of spermine (Table 5). The fact that both putrescine and cadaverine were actively taken up by the tumour cells and that these closely related diamines exerted strikingly different effects on the accumulation of higher polyamines (spermidine and spermine) make it unlikely that the changes in polyamines observed were attributable to unspecific actions, such as inflammatory reactions or changes in the number of viable cells.

Effect of diamines on spermidine synthase and spermine synthase activities in ascites cells

The different effect of putrescine and cadaverine on the tumour concentrations of higher polyamines (spermidine and spermine), regardless of their similar action on ornithine decarboxylase, raised the possibility that some of the amines might exert a direct influence on spermidine and spermine synthases.

As illustrated in Fig. 1, cadaverine acted as a competitive inhibitor for putrescine in the synthesis of spermidine catalysed by partially purified

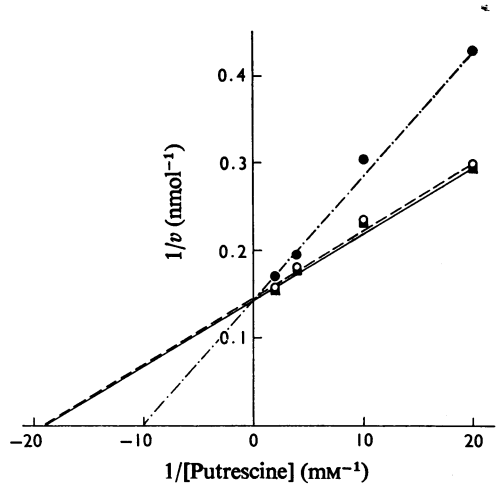

Fig. 1. *Effect of cadaverine (1,5-diaminopentane) and 1,6-diaminohexane on spermidine synthase reaction*
Spermidine synthase activity was measured at various concentrations of putrescine in the absence (■) or in the presence of 0.5mM-cadaverine (●) or 0.5mM-diaminohexane (○). Partially purified spermidine synthase (0.66mg of protein) was used as the source of the enzyme.

spermidine synthase from ascites cells. A K_i of about 0.6mM was obtained for cadaverine as competitive inhibitor for putrescine. Interestingly, the longer-chain analogue of cadaverine, namely 1,6-diaminohexane, had no effect on spermidine synthase whatsoever (Fig. 1). 1,2-Diaminoethane and 1,3-diaminopropane also appeared to have very little influence on the synthesis of spermidine from putrescine and decarboxylated adenosylmethionine (results not shown).

Cadaverine likewise slightly inhibited the synthesis of spermine from spermidine by partially purified spermine synthase from ascites cells; however, putrescine was apparently a much more potent inhibitor of this reaction (Table 6).

The decrease in spermidine concentration as a result of a chronic treatment with cadaverine (Table 5) might thus be based both on an inhibition of ornithine decarboxylase activity and on a direct decrease in spermidine synthesis from putrescine, especially under conditions where the concentration of putrescine approaches zero. The increase in the concentration of spermine during cadaverine treatment, in turn, might just reflect the release of the inhibition by putrescine in the synthesis of spermine, cadaverine being less inhibitory than putrescine in the latter reaction (Table 6).

Also, in no case was any radioactivity incorporated from [^{14}C]cadaverine into spermidine or spermine molecules (results not shown).

Table 6. *Effect of cadaverine and putrescine on spermine synthase activity in vitro.*
Spermine synthase activity was assayed by using a partially purified enzyme preparation as described in the Experimental section with 0.35mM-[^{14}C]-spermidine as the substrate.

Addition	Concn. (mM)	Spermine synthase activity (nmol/mg of protein)
None	—	0.39 (100%)
Cadaverine	0.5	0.25 (65%)
Putrescine	0.5	0.08 (19%)

Discussion

The fact that mammalian ornithine decarboxylase can be repressed by a variety of unphysiological amines, such as diaminopropane (Pösö & Jänne, 1976*a*), cadaverine (Pösö, 1977) and diaminohexane (Table 3), which cannot serve as substrates in the synthesis of higher polyamines, offers certain possibilities for studying whether a given polyamine is specifically required during periods of rapid growth. The inhibition of ornithine decarboxylase activity and spermidine accumulation by diaminopropane in regenerating rat liver (Pösö & Jänne, 1976*b*) and the concomitant decrease in hepatic DNA synthesis might indicate that an increased accumulation of the natural polyamines (putrescine and spermidine) is specifically needed for the enhancement in DNA synthesis elicited by partial hepatectomy. If this is correct, the increased accumulation of putrescine and spermidine could not be replaced by high concentrations of exogenous 1,3-diaminopropane.

Similarly, Mamont *et al.* (1976) found that the inhibition of DNA synthesis by α-methylornithine in hepatoma cells could be reversed by addition of putrescine, spermidine or spermine, but not of cadaverine or 1,3-diaminopropane. The inhibition of DNA synthesis by α-hydrazino-δ-aminovaleric acid (Inoue *et al.*, 1975) in mouse parotid glands could likewise be prevented by putrescine, but not by spermidine or 1,7-diaminoheptane.

The fact that Ehrlich ascites-carcinoma cells contain relatively high concentrations of spermidine and spermine (Siimes & Jänne, 1967; Andersson & Heby, 1972; Noguchi *et al.*, 1976) as well as high activities of their biosynthetic enzymes makes them suitable for studies devoted to the elucidation of the specific functions of these compounds. As shown here, ornithine decarboxylase in Ehrlich ascites cells, just like in regenerating rat liver, can be repressed by a variety of diamines. However, unlike in rat liver (Pösö, 1977), longer diamines (cadaverine and diaminohexane) appeared to be more potent inhibitors of the enzyme than shorter ones (Table 3).

This most likely reflects the penetration ability of the compound into the ascites cell.

The rate of degradation of spermidine and spermine in ascites cells appears to be rather slow. As shown in the present results, the concentration of spermidine decreased less than 50% in 24h after a complete inhibition of ornithine decarboxylase activity. Thus any metabolic changes associated with inhibition of polyamine synthesis are expected to occur much later than in circumstances, such as rat liver regeneration, where an abrupt stimulation of the polyamine synthesis can be prevented at the very beginning. In any case, it appears to us that the use of repression-type inhibition of ornithine decarboxylase offers several advantages in comparison with conventional direct enzyme inhibitors. This approach could be called an 'amine-replacement treatment', where the natural polyamines (putrescine and spermidine) will be replaced by unphysiological amines probably unable to take over the specific metabolic functions of their physiological counterparts.

The skilful technical assistance of Mrs. Riitta Sinervirta and Miss Merja Kärkkäinen is gratefully acknowledged. We also thank Dr. P. Hannonen and Dr. G. Scalabrino for their help at various stages of the present study. H. P. is a recipient of a fellowship from the Natural Sciences Research Council of the Academy of Finland, and S. K. G. is a recipient of a research fellowship from the Sigrid Jusélius Foundation (Helsinki, Finland).

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