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EFFECTS OF INHIBITORS OF SPERMIDINE SYNTHASE AND SPERMINE SYNTHASE ON POLYAMINE SYNTHESIS IN RAT TISSUES

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Abstract—Several inhibitors of aminopropyltransferases, developed recently in this laboratory, were tested for their specificity by measuring their effects on six enzyme activities related to polyamine biosynthesis and interconversion. Two of them, *trans*-4-methylcyclohexylamine (4MCHA) and *N*-(3-aminopropyl)cyclohexylamine (APCHA), selectively and potently inhibited the activities of spermidine synthase and spermine synthase, respectively. They were subjected to *in vivo* studies using rats. Oral administration of 4MCHA or APCHA dissolved in drinking water (0.02 and 0.1%) available *ad lib.* for a period of 10 days or 4 months caused a specific and marked decrease in spermidine or spermine in tissues (such as a 95% decrease) with a compensatory increase of spermine or spermidine, respectively, but without any observable change in the growth of the treated rats. Also, with extreme reduction of spermidine or spermine, when their sum was approximately constant, the activity of *S*-adenosylmethionine decarboxylase in these tissues was enhanced significantly with no change in the activity of ornithine decarboxylase. These results suggested a separate role for spermidine or spermine in the *in vivo* enhancement of *S*-adenosylmethionine decarboxylase activity.

During the last two decades, specific inhibitors of polyamine biosynthetic enzymes have been used to deplete cellular polyamine and to clarify the relationship between the polyamine level and cell growth or differentiation processes [1–3]. Studies using such inhibitors have shown that the polyamines spermidine and spermine and their precursor putrescine are essential to the processes. Spermidine and spermine are synthesized by enzymatic aminopropylation of putrescine and spermidine, respectively, using decarboxylated *S*-adenosylmethionine. The two polyamine precursors, putrescine and decarboxylated *S*-adenosylmethionine, are formed by the decarboxylation of ornithine and *S*-adenosylmethionine with ornithine decarboxylase and *S*-adenosylmethionine decarboxylase, respectively. Most inhibitors of polyamine biosynthesis developed thus far have been directed toward the two decarboxylases, because both decarboxylation steps are thought to be rate-limiting ones in polyamine biosynthesis [3, 4]. However, as those inhibitors could not deplete spermidine or spermine selectively in cells or tissues over a long period, it is still unclear whether or not each polyamine has its own specific function *in vivo*. To find the answer, compounds which interfere directly and selectively with one of the two aminopropylation steps, i.e. the reactions of spermidine synthase and spermine synthase, should be useful for learning more about the physiological function of each polyamine.

Most of the known inhibitors of aminopropyltransferases are competitive ones directed at decarboxylated *S*-adenosylmethionine binding sites of the enzymes. Recent studies [5, 6] suggest that the potency of such inhibitors could be weakened with a significant increase of decarboxylated *S*-adenosylmethionine, which is usually observed in biological systems when polyamines are depleted. Hence, the inhibitors directed at the binding site of putrescine or spermidine were expected to be of practical use *in vivo*.

Recently, we found that several alkylamines and *N*-(3-aminopropyl)alkylamines were potent competitive inhibitors *in vitro* to the putrescine binding site of spermidine synthase from pig liver and to the spermidine binding site of spermine synthase from rat brain, respectively [7, 8]. The inhibitory effects of all those compounds to purified spermidine synthase or spermine synthase were not influenced by an increase in the decarboxylated *S*-adenosylmethionine concentration in the assay mixtures. In the present paper, we show that among them, *trans*-4-methylcyclohexylamine (4MCHA)†

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† Abbreviations: 4MCHA, *trans*-4-methylcyclohexylamine; APCHA, *N*-(3-aminopropyl)cyclohexylamine; AdoDato, *S*-adenosyl-1,8-diamino-3-thiooctane; AdoDatad, *S*-adenosyl-1,12-diamino-3-thio-9-azadodecane; ODC, ornithine decarboxylase; AdoMetDC, *S*-adenosylmethionine decarboxylase; SpdSyn, spermidine synthase; SpmSyn, spermine synthase; SAT, spermidine/spermine N^1-acetyltransferase; and PAO, polyamine oxidase.

and *N*-(3-aminopropyl)cyclohexylamine (APCHA) were the most potent *in vivo*, leading to specific inhibition of spermidine or spermine synthesis, respectively, and that they could be used to study the regulation of *S*-adenosylmethionine decarboxylase in rat tissues.

MATERIALS AND METHODS

Materials. *S*-Adenosyl-L-[*methyl*-^{3}H]methionine (81 Ci/mmol), *S*-adenosyl-L-[*carboxyl*-^{14}C]methionine (52 mCi/mmol), and DL-[1-^{14}C]ornithine (55 mCi/mmol) were purchased from NEN (Boston, MA). Hydrochloride forms of 4MCHA [8], *exo*-2-aminonorbornane [7], *N*-(3-aminopropyl)-*n*-butylamine [7], *N*-(3-aminopropyl)-*exo*-2-aminonorbornane [7], APCHA [7] and *N*-(3-aminopropyl)cadaverine [9] were synthesized or prepared as described previously. The hydrochloride forms of spermidine and spermine were obtained from Sigma and purified by ion-exchange chromatography with Dowex 50WX4 and by recrystallization. The purity of both preparations was over 99.5% when determined by the HPLC described below.

Analysis of polyamines and inhibitors. Tissues were homogenized with 0.1 N hydrochloric acid solution containing *N*-(3-aminopropyl)cadaverine as an internal standard and deproteinized with trichloric acid (final concentration 10%). The supernatant of the mixture after centrifugation was directly injected into the high performance liquid chromatograph (HPLC) for analysis. The HPLC conditions (a modification of Ref. 10) were as follows: column, cation exchange resin, JEOL LC-R-2, 4 mm × 8 cm; elution buffer, a mixture of 10% (v/v) of methanol and 90% (v/v) of 0.28 M sodium citrate buffer, pH 5.5, containing 2.0 M sodium chloride, 0.8 mL/min; column temperature, 80°; post-column reagent solution, 6 mM *o*-phthaldialdehyde in 0.4 M potassium borate buffer, pH 10.4, containing 0.2% mercaptoethanol, 0.1 mM EDTA and 0.1% Brij 35, 0.4 mL/min at 80°; fluorescence detection, excitation 365 nm, emission 450 nm. Retention times of polyamines and inhibitors were as follows: N^1-acetylspermidine, 5.7 min; putrescine, 6.7 min; spermidine, 13.7 min; spermine, 29.4 min; *N*-(3-aminopropyl)cadaverine, 22.6 min; 4MCHA, 38.0 min; and APCHA, 42.6 min.

In vitro *assay of polyamine biosynthetic enzymes.* Preparation and assay of crude ornithine decarboxylase and *S*-adenosylmethionine decarboxylase were carried out according to the method of Kameji *et al.* [11] and Pegg and Williams-Ashman [12], respectively. Spermidine synthase and spermine synthase were purified from rat liver [13] and brain [14], respectively, as previously described. The assay was carried out according to a method previously described [7]. The labeled substrate, decarboxylated *S*-adenosylmethionine, was enzymatically prepared from *S*-adenosyl-L-[*methyl*-^{3}H]methionine [15]. Crude extract of polyamine oxidase was prepared from rat liver by the method of Holtta [16]. The assay was carried out by measuring the produced spermidine by HPLC as described above. Crude enzyme preparation of spermidine/spermine N^1-acetyltransferase was prepared by the method of Matsui and Pegg [17] with some modifications. The assay was carried out by measuring the N^1-acetylspermidine produced from the spermidine substrate by HPLC as described above.

Administration of inhibitors to rats. Male Sprague–Dawley rats were used. They were housed under a constant 12 hr light–12 hr dark lighting schedule and received standard rodent chow (CE-2, Clea Japan Inc.). In the single administration experiment, inhibitors (200 μmol/mL) dissolved in 0.9% saline were injected intraperitoneally to rats fasted for 24 hr. In the oral administration experiment, inhibitors were given as a solution in drinking water. The pH of the solution was adjusted with 5 N NaOH to 6.0.

Measurement of ornithine decarboxylase or S-adenosylmethionine decarboxylase activity in tissues. Tissue extracts for ornithine decarboxylase [11] or *S*-adenosylmethionine decarboxylase [12] were prepared as previously described, and the activities were measured as described in the section on *in vitro* assay of polyamine biosynthetic enzymes. Protein was determined by the method of Bradford [18] using bovine serum albumin as a standard. One unit of activity was defined as the release of 1 nmol $^{14}CO_2$/min.

RESULTS

Effects of inhibitors of aminopropyltransferases on enzyme activities involved in polyamine biosynthesis or interconversion. Two inhibitors of spermidine synthase, 4MCHA and *exo*-2-aminonorbornane, and three for spermine synthase, APCHA, *N*-(3-aminopropyl)-*exo*-2-aminonorbornane and *N*-(3-aminopropyl)-*n*-butylamine, were chosen because of their low IC_{50} values to the respective enzyme [7]. As it was likely that these inhibitors, being structurally similar to putrescine or spermidine, might act as substrate, product or activator with other enzymes involved in polyamine biosynthesis or interconversion, their specificities were first examined by comparing their *in vitro* effects on the activities of six polyamine biosynthetic enzymes, ornithine decarboxylase, *S*-adenosylmethionine decarboxylase, spermidine synthase, spermine synthase, spermidine/spermine-N^1-acetyltransferase, and polyamine oxidase, in rat tissues. As shown in Figs. 1 and 2, each inhibitor showed specific inhibition of its target enzyme, spermidine synthase or spermine synthase. 4MCHA or *exo*-2-aminonorbornane at 100 μM inhibited spermidine synthase activity over 98% in the presence of 1 mM putrescine substrate, but did not affect the other enzyme activities with the exception of *S*-adenosylmethionine decarboxylase, slightly decreasing its activity with increase of the inhibitor concentration under the assay conditions (Fig. 1). Similarly, 10 μM APCHA, *N*-(3-aminopropyl)-*exo*-2-aminonorbornane or *N*-(3-aminopropyl)-*n*-butylamine inhibited spermine synthase activity over 98% in the presence of 1 mM spermidine substrate, but did not affect the other enzyme activities (Fig. 2). These results obtained using such a crude enzyme source as the supernatant of rat liver

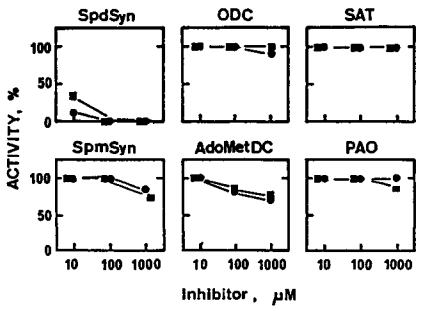

Fig. 1. Inhibition of enzymes involved in the biosynthesis and interconversion of polyamines with inhibitors of spermidine synthase. Assays using purified or crude enzyme preparation were performed as described in Materials and Methods. Results are expressed as a percentage of the activity in the absence of inhibitor. Each point represents the average of duplicate assays for 4MCHA (●) and *exo*-2-aminonorbornane (■). Activities of spermidine synthase (SpdSyn), spermine synthase (SpmSyn), ornithine decarboxylase (ODC), *S*-adenosylmethionine decarboxylase (AdoMetDC), spermidine/spermine N^1-acetyltransferase (SAT) or polyamine oxidase (PAO) in the absence of the inhibitors in the assay tube were 10, 10, 30, 25, 40 or 240 pmol product/min, respectively.

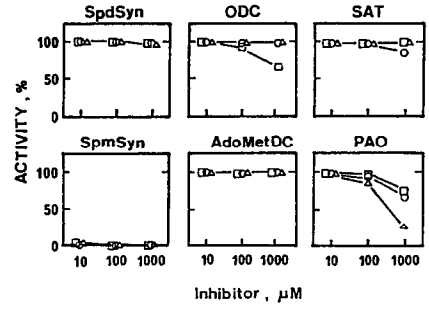

Fig. 2. Inhibition of enzymes involved in the biosynthesis and interconversion of polyamines with inhibitors of spermine synthase. Results are expressed as a percentage of the activity in the absence of inhibitor. Each point represents the average of duplicate assays for *N*-(3-aminopropyl)-*n*-butylamine (△), *N*-(3-aminopropyl)-*exo*-2-aminonorbornane (□), and APCHA (○). Assay conditions and abbreviations are the same as given in the legend of Fig. 1.

homogenate suggested that the inhibitors could be used in *in vivo* experiments.

Effects of a single intraperitoneal administration of inhibitors on polyamine contents in rat tissues. To examine the *in vivo* effects of the inhibitors, polyamines in rat tissues were measured 24 hr after a single intraperitoneal administration of 400 μmol/kg body weight of the inhibitors. Of the two inhibitors for spermidine synthase, 4MCHA seemed to be effective for decreasing spermidine content in prostate (Table 1). Of the three inhibitors of spermine synthase, APCHA seemed to be the most effective for decreasing spermine content in prostate (Table 1). However, the compounds were not accumulated significantly in the tissues examined and they did not lead to significant changes in the polyamine pools (data not shown). These results suggested that continuous administration of 4MCHA or APCHA for a prolonged period of time would be necessary to decrease the content of spermidine or spermine which has a long half-life in the tissues [19].

Effect of oral administration of 4MCHA or APCHA on polyamine content in rat tissues. Oral administration was adopted for a continuous supply of each inhibitor to the rats. Experiments were carried out by treating rats for 10 days with *ad lib.* access to drinking water containing 0.02% or 0.1% of either 4MCHA or APCHA. No significant difference in body weights was noted between the control and the treated rats with the exception that the rats given a 0.1% solution of 4MCHA lost 25% of their body weight while control rats gained 45% (data not shown). As shown in Figs 3 and 4, this oral treatment had clearer effects on polyamine content in the tissues than the single intraperitoneal administration. A significant dose-dependent decrease of spermidine content was observed with 4MCHA treatment in accordance with a compensatory increase of spermine in liver and kidney and of spermine and putrescine in the prostate (Fig. 3). Upon treatment with a 0.1% solution of 4MCHA, the spermidine content decreased to 28% in the prostate, 21% in the liver and 33% in the kidney, in comparison with the control. In the brain, the polyamine content seemed to be decreased slightly. In these tissues, no marked change in the total polyamine (putrescine, spermidine and spermine) content was observed during the treatment. As shown in Fig. 4, with the treatment of APCHA, spermine content decreased dose-dependently in the three tissues but not in the brain, showing a compensatory increase of spermidine. Upon treatment with a 0.1% solution of APCHA, the spermine content decreased to 5% in the prostate, 48% in the liver and 55% in the kidney as compared with control levels. There was no change in the polyamine content in the brain. Again, the total polyamine content did not seem to fluctuate strikingly with the treatment.

The finding that the treatment of rats with drinking water containing 4MCHA or APCHA resulted in a significant decrease of spermidine or spermine content, respectively, in the three tissues led us to examine the effects of the selective decrease of spermidine or spermine on the activities of ornithine decarboxylase and *S*-adenosylmethionine decarboxylase in prostate and liver. As shown in Table 2, the activity of *S*-adenosylmethionine decarboxylase increased significantly when either spermidine or spermine content in the prostate decreased markedly, while the activity of ornithine decarboxylase changed only slightly. Similar induction of *S*-adenosylmethionine decarboxylase activity was also observed in the liver as shown in Table 3. When the treatment period with 0.1% APCHA was

Table 1. Effects of inhibitors on polyamine content in rat prostate

Treatment*	Polyamine content† (μmol/g prostate)		
	Putrescine (%)	Spermidine (%)	Spermine (%)
Control (saline)	0.45 (100)‡	8.38 (100)	3.38 (100)
4MCHA	0.59 (131)	6.09 (73)	3.38 (100)
exo-2-Aminonorbornane	0.62 (138)	9.28 (111)	3.34 (99)
N-(3-Aminopropyl)-*n*-butylamine	0.32 (71)	10.6 (126)	2.36 (70)
N-(3-Aminopropyl)-*exo*-2-aminonorbornane	0.19 (42)	9.52 (114)	1.89 (56)
APCHA	0.23 (51)	9.84 (117)	1.83 (54)

* Rats (180–200 g) were injected intraperitoneally with inhibitors (400 μmol/kg) or saline 24 hr before being killed.

† Values are means for groups of three rats. The standard deviation for each group was within 15% of the mean value.

‡ Numbers in parentheses represent percent of control.

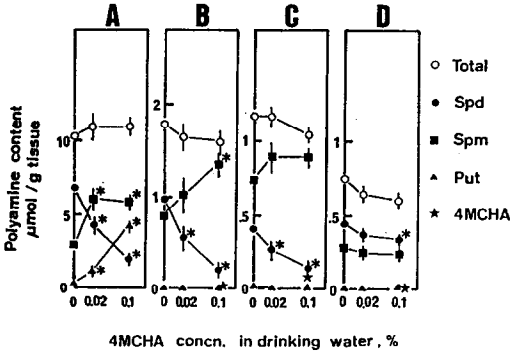

Fig. 3. Effect of treatment with drinking water containing 4MCHA on polyamine content in rat tissues. 4MCHA was administered to rats (200–220 g when the treatment was started) as a 0.02% or 0.1% solution (pH 6.0) in drinking water. Total exposure time to the inhibitor was 10 days. Each point represents the mean of three rats and the vertical bar indicates ± SD. Asterisks denote a significant difference compared with the mean control values, $P < 0.05$. Results are shown in A for prostate, B for liver, C for kidney and D for brain. Key: (▲) putrescine, (●) spermidine, (■) spermine, (★) 4MCHA, and (○) total polyamine.

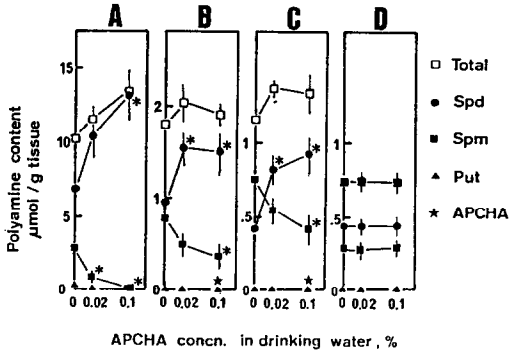

Fig. 4. Effect of treatment with drinking water containing APCHA on polyamine content in rat tissues. APCHA was administered to rats (200–220 g when the treatment was started) as a 0.02% or 0.1% solution (pH 6.0) in drinking water. Total exposure time to the inhibitor was 10 days. Each point represents the mean of three rats and the vertical bar indicates ± SD. Asterisks denote a significant difference compared with the mean control values, $P < 0.05$. Results are shown in A for prostate, B for liver, C for kidney and D for brain. Key: (▲) putrescine, (●) spermidine, (■) spermine, (★) APCHA, and (□) total polyamine.

extended to 4 months, the enzyme activity increased significantly with a profound decrease of spermine content.

DISCUSSION

Previous studies in this laboratory led to the discovery of five compounds inhibiting spermidine synthase activity with IC_{50} values of less than 10 μM and four compounds inhibiting spermine synthase activity with IC_{50} values of less than 1 μM under the established conditions [7]. These compounds were shown to selectively inhibit either of the two purified enzymes, but their selectivity was not examined using enzymes other than aminopropyltransferase. Hence, the present study began with estimating the inhibitory effects of the five compounds exhibiting low IC_{50} values on the enzymes related to polyamine biosynthesis and interconversion, since these enzymes functionally have a putrescine or polyamine binding site to which these compounds may bind. The results in Figs. 1 and 2 suggested that they were sufficiently selective, and almost complete inhibition of spermidine synthase or spermine synthase could be achieved with the respective compounds at one-tenth to one-hundredth of the concentration of each polyamine substrate in the tissues.

Of these compounds, 4MCHA or APCHA, with the lowest IC_{50} value and with demonstrated effects on the polyamine content in rat prostate (Table 1),

Table 2. Effects of treatment with 4MCHA or APCHA on activities of ornithine decarboxylase and *S*-adenosylmethionine decarboxylase in rat prostate

Treatment*	Polyamine content† (μmol/g tissue) Spermidine	Spermine	Enzyme activity‡ (mU/mg protein) ODC	AdoMetDC
Control	6.89 (100)§	2.96 (100)	6.5 ± 1.2 (100)	85 ± 12 (100)
0.02% 4MCHA	4.31‖ (63)	6.04‖ (204)	ND¶	87 ± 14 (102)
0.10% 4MCHA	1.95‖ (28)	5.38‖ (181)	6.9 ± 0.8 (106)	190 ± 51‖ (223)
0.02% APCHA	10.3 (149)	0.92‖ (31)	ND	129 ± 59 (151)
0.10% APCHA	13.2‖ (191)	0.15‖ (5)	8.6 ± 2.3 (132)	232 ± 48‖ (272)

* Inhibitors were administered to rats (200–220 g when the treatment was started) as a 0.02% or 0.1% solution (pH 6.0) in drinking water. Total exposure time to the inhibitors was 10 days.
† Values are means for groups of three rats.
‡ Values are means ± SD for groups of three rats.
§ Numbers in parentheses represent percent of control.
‖ Significantly different from control, $P < 0.05$.
¶ Not determined.

Table 3. Effect of treatment with 4MCHA or APCHA on activities of *S*-adenosylmethionine decarboxylase in rat liver

Treatment*	Period (days)	Polyamine content† (μmol/g tissue) Spermidine	Spermine	AdoMetDC activity‡ (mU/mg protein)
Control	10	0.90 (100)§	0.71 (100)	1.33 ± 0.14 (100)
0.10% 4MCHA	10	0.28‖ (31)	1.22‖ (171)	1.79 ± 0.66 (134)
0.10% APCHA	10	1.17 (130)	0.50‖ (70)	1.35 ± 0.09 (101)
Control	120	0.61 (100)	0.89 (100)	1.05 ± 0.09 (100)
0.10% APCHA	120	1.29‖ (211)	0.19‖ (21)	3.60 ± 0.29‖ (342)

* Inhibitors were administered to rats (200–220 g for 10-day treatment or 400–450 g for 4-month treatment, when the treatment was started) as a 0.1% solution (pH 6.0) in drinking water.
† Values are means for groups of three rats.
‡ Values are means ± SD for groups of three rats.
§ Numbers in parentheses represent percent of control.
‖ Significantly different from control, $P < 0.05$.

was used for *in vivo* experiments as the inhibitor of spermidine synthase or spermine synthase, respectively. The effect of 4MCHA observed in the present experiment on spermidine content in rat tissues (Fig. 3) was much greater than that of cyclohexylamine, a known competitive inhibitor with putrescine, administered to hepatectomized rats [20]. Marked depletion of spermine with APCHA (Fig. 4) was achieved for the first time in the mammalian tissues. As it has been reported that polyamines in mammalian tissues including tumors can be supplied from food in addition to biosynthesis [21], the polyamine depletion with 4MCHA or APCHA might be more profound if rats were fed polyamine-deficient chow.

Inhibition of aminopropyltransferases has been well studied in cell culture systems using several inhibitors including *S*-adenosyl-1,8-diamino-3-thio-octane (AdoDato) [22] and *S*-adenosyl-1,12-diamino-3-thio-9-azadodecane (AdoDatad) [6], multi-substrate analogs of spermidine synthase and spermine synthase, respectively, and *n*-butyl-1,3-diaminopropane [designated as *N*-(3-aminopropyl)-*n*-butylamine, in the present paper], a reported spermine synthase inhibitor [7, 23]. These studies revealed that the potent inhibition of spermidine biosynthesis caused a compensatory increase of spermine, and that of spermine biosynthesis a compensatory increase of spermidine, keeping the total polyamine content approximately constant in various cultured cells. The compensation of spermine or spermidine could be explained by the accumulation of decarboxylated *S*-adenosylmethionine accompanied by the inhibition of polyamine biosynthesis. The results of 4MCHA or APCHA in the present *in vivo* studies agreed with these observations. A marked increase of putrescine observed in the prostate with 4MCHA treatment (Fig. 3A) might reflect the fact that the prostate displays much higher activity of ornithine decarboxylase than the other tissues of normal rats [24].

In contrast with the treatment of 4MCHA, a decrease of putrescine in the prostate was observed with the APCHA treatment (Fig. 4A). Pegg *et al.*

[6] also observed a similar decrease of putrescine in cultured cells treated with AdoDatad and discussed that it might be mediated via a decrease of ornithine decarboxylase activity in response to the elevation of spermidine since ornithine decarboxylase was strongly repressed by spermidine. However, this was not true in the present case because the treatment of APCHA did not change the activity of ornithine decarboxylase in the prostate, as shown in Table 2. The decrease of putrescine could be similarly explained by the acceleration of aminopropyltransfer reaction to putrescine due to accumulation of decarboxylated *S*-adenosylmethionine as described above.

Pegg *et al.* [3, 6] have suggested that the accumulation of decarboxylated *S*-adenosylmethionine in cells depleted of polyamine with inhibitors for ornithine decarboxylase or aminopropyltransferases is caused by both failure of the aminopropyltransfer reaction and induction of *S*-adenosylmethionine decarboxylase which is known to be negatively regulated with polyamines. It would, therefore, be interesting to know whether or not the treatment with 4MCHA or APCHA changes the activity of *S*-adenosylmethionine decarboxylase in the tissues and how the enzyme is regulated with spermidine or spermine. The results in Tables 2 and 3 showed that significant depletion of either spermidine or spermine, with the total polyamine content kept approximately constant, markedly increased the activity, suggesting that spermidine and spermine do not operate on the same regulation point to repress the enzyme, since the total polyamine content was similar in all the treatments including the control. On the whole, the present *in vivo* results agree with those of recent studies [5, 6, 22, 25], showing that the treatment with aminopropyltransferase inhibitors including AdoDato and AdoDatad led to an increase in *S*-adenosylmethionine decarboxylase but not in ornithine decarboxylase in cultured cells.

The relatively low toxicities of 4MCHA and APCHA are worth reporting. The LD_{50} values of these compounds in rats are more than 0.25 g/kg of weight for 4MCHA and 0.5 g/kg of weight for APCHA. Reduction of the body weight gain of rats was not significant during the 10-day treatment with drinking water containing the inhibitors (within 5% as compared with control rats) except for rats treated with 0.1% 4MCHA solution. Their weight loss may be due to the 70% reduction of the water intake containing 0.1% 4MCHA during the treatment. APCHA could be used to treat the rats for 4 months (Table 3), keeping the tissue spermine content very low without affecting body weight gain or tissue weight gain in the prostate and liver during the treatment (data not shown). These observations suggest that a very small fraction of cellular spermine might be necessary for supporting tissue growth, or spermidine might be substituted for spermine for the growth as has been discussed for cell growth of a number of cell lines [5, 6, 23, 26].

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