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MODE OF ACTION OF 3-SUBSTITUTED PROPYLAMINE CYTOTOXICITY IN CULTURE CELLS

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Abstract—Cytotoxic effects on MDBK cells of various 3-substituted propylamines, including spermine and spermidine, were tested in culture in the presence of calf serum, and the possible mode of action was studied from the viewpoint of oxidative deamination leading to acrolein formation. The compounds were roughly classified in two groups with IC_{50} values of 0.1 and 0.4 mM under the conditions used. Phenyl derivatives of 3-substituted propylamines, 3-benzylaminopropylamine, and polyamines were included in the first group with IC_{50} values of 0.1 mM, and acrolein was liberated from these compounds after incubation with bovine plasma amine oxidase. Alkyl derivatives of 3-substituted propylamines (with IC_{50} values of 0.4 mM), on the other hand, were unable to release acrolein after the oxidative deamination, which was further confirmed by a lack of liberation of acrolein from the authentic 3-butoxypropanal. These observations indicated that both the acrolein originating from the 3-substituted propanals, and the propanal as such, were possibly involved in manifestation of the cytotoxicity of the 3-substituted propylamines. Thus, spermine and spermidine may exert their cytotoxic effects on the cells *in vitro* by a combination of two mechanisms involving acrolein and oxidized polyamines.

Our previous work with *S*-adenosyl-3-thiopropylamine (ATPA) suggested that the *in vitro* cytotoxic effect of ATPA might depend on acrolein formed from the corresponding aldehyde of ATPA via enzymic oxidative deamination [1]. A similar mechanism has been assumed by Alarcon [2, 3] for the cytotoxicity of the naturally occurring polyamines, spermidine and spermine. This mechanism, however, can not be simply accepted, since oxidized spermine, demonstrated by Tabor *et al.* [4] and chemically synthesized by Fukami *et al.* [5], interacts with DNA [6, 7] and seems to have its own mechanism of inhibiting cell proliferation [8, 9]. It was, therefore, of interest to investigate which one of these mechanisms involving the propanal or acrolein does actually function under physiological conditions.

Since acrolein is produced spontaneously from 3-substituted propanals [10–12], we first examined whether the culture cells were damaged by various 3-substituted propylamines and, then, considered a possible relationship between their cytotoxicity and the production of acrolein. Acrolein was measured by a modified method using cysteine diadducts [13, 14] formed under physiological conditions. The results may be useful in understanding the mechanism involved in the manifestation of the cytotoxicity of 3-substituted propylamines including polyamines.

MATERIALS AND METHODS

Materials

Some of the compounds tested in this study, such as 3-methoxypropylamine, 3-ethoxypropylamine, 3-methylaminopropylamine, *N*,*N*-bis(3-aminopropyl)amine (caldine), and semicarbazide were obtained from the Tokyo Kasei Kogyo Co., with purities of more than 98%; spermidine·3HCl and spermine·4HCl were from the Sigma Chemical Co. The other compounds, which were commercially unavailable, were synthesized in this laboratory and are summarized in Table 1. Synthetic methods used for some of them, designated as B or C in Table 1, and for two propanals are described below. 3-Bromopropionitrile (99%), 3-bromopropylamine·HBr (98%) and 3-phenoxypropylbromide (98%) were purchased from the Aldrich Chemical Co., Inc. Phenyltrimethylammonium hydroxide (20–25% in methanol), γ-bromobutyronitrile, DL-methionine, DL-ethionine, L-cysteine and acrolein were obtained from the Tokyo Kasei Kogyo Co. 4-Hydroxy-3-methoxyphenylacetic acid (HVA) and *o*-phthaldialdehyde were purchased from the Sigma Chemical Co. Peroxidase from horse radish roots (HRP) (105 guaiacol units/mg, Lot No. 7137) and amine oxidase from bovine plasma (28.2 units/g, Lot No. 7018) were purchased from Miles Laboratories (PTY) Ltd. All other chemicals and organic solvents were of the highest purity commercially available.

3-Phenoxypropylamine. Instead of reduction of 3-phenoxypropionitrile according to Soffer and Parrotta [15], the compound was prepared by the common method of hydrolysis of phthalimide derivatives [17]. A mixture of 25 mmoles of 3-phenoxypropylbromide and 27 mmoles of potassium phthalimide dissolved in 20 ml of dimethylformamide was heated up to 55°. After chloroform extraction, *N*-phthaloyl-3-phenoxypropylamine was obtained quantitatively and recrystallized from 95% ethanol. A mixture of 20 mmoles of the derivative dissolved in 36 ml of methanol and 3.86 ml of 80% hydrazine hydrate was refluxed for 1.5 hr. After evaporation of methanol, the residue was shaken with 20 ml of

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Table 1. Synthetic methods, melting point (or boiling point), and yields of 3-substituted propylamines*

Compounds	Synthetic methods†	Melting point or boiling point (°)	Yield‡ (%)
3-Propoxypropylamine	A [15]	37 (8 mm Hg)	20
3-Butoxypropylamine	A [15]	72 (12 mm Hg)	27
3-Phenoxypropylamine HCl	B	176	39
3-Methylthiopropylamine HCl	A [16]	143	18
3-Ethylthiopropylamine HCl	C	141	55
3-Propylthiopylamine HCl	C	143	8
3-Butylthiopropylamine HCl	C	136	20
3-Phenylthiopropylamine HCl	C	159	15
3-Ethylaminopropylamine 2HCl	B	222	30
3-Propylaminopropylamine 2HCl	B	282	50
3-Butylaminopropylamine 2HCl	B	296	61
3-Phenylaminopropylamine 2HCl	B	204	43
3-Benzylaminopropylamine 2HCl	B	281	72

* All compounds gave acceptable elemental analyses. N.M.R. and I.R. spectra were consistent with the proposed structure.

† (A) Known compound synthesized according to an established method (reference number in brackets). (B) Known compound synthesized by a modified method. (C) Newly synthesized compound.

‡ Yield of distilled or crystallized product.

conc ammonium hydroxide and 40 ml of chloroform for 1 hr. 3-Phenoxypropylamine, once extracted from the chloroform layer to 3 N acetic acid, was then re-extracted with ether from ammoniacal alkaline solution and was obtained as the HCl salt by introducing dry HCl gas to the dried ether solution. The reagent was finally recrystallized in absolute ethanol.

3-Alkyl-, phenyl-, and benzylaminopropylamine. Catalytic reduction of 3-substituted propionitrile [18, 19] and an application of Gabriel's method were reported for the synthesis of 3-alkyl- and benzylaminopropylamine, and 3-phenylaminopropylamine, respectively. We obtained all the compounds by mixing one equivalent of γ-bromopropylamine and 20 equivalents of each corresponding monoamine. The mixture was kept at room temperature for 3 days and extracted with ether. Ether and excess monoamines were removed by distillation *in vacuo*. The residues were applied on a Dowex-50 column and separated by stepwise elution of HCl. Title compounds were eluted with 3–4 N HCl. After evaporation, they were recrystallized from absolute ethanol as HCl salt.

3-Ethylthiopropylamine. The compound was prepared analogously to the method of Suyama and Kanao [16] for the synthesis of 3-methylthiopropylamine. Ethionine (0.05 mole) suspended in 50 ml of tetralin was heated at 215° for 30 min for decarboxylation. The solution was then distilled *in situ*. The distillate dissolved in 2 N HCl was evaporated to dryness. The compound, as the HCl salt, was recrystallized from acetone-ether.

3-Propyl-, butyl-, and phenylthiopropylamine. All the compounds were prepared by the method of Jamieson [20]. A mixture of 0.18 mole of each corresponding mercaptan and 0.24 mole of acrylonitrile dissolved in 25 ml of absolute ethanol was refluxed for 1 hr in the presence of 2.5 ml of 25% trimethylphenylammonium hydroxide. Boiling points for 3-propyl-, butyl-, and phenylthiopropionitrile were 121°/39 mm Hg, 97°/4 mm Hg, and 134°/4 mm Hg respectively. The nitriles were then reduced with $LiAlH_4$ and distilled *in vacuo*. Boiling points for 3-propyl-, butyl-, and phenylthiopropylamine were 72°/4 mm Hg, 74°/4 mm Hg, and 92°/1 mm Hg respectively. All the compounds, as HCl salts, were recrystallized from absolute ethanol-ether.

3-Butoxypropanal and 4-butoxybutanal. The compounds were prepared by the method of Suzuki and Okada [21] for the synthesis of *N*-nitrosamino aldehydes. A mixture of 7.9 mmoles of 3-butoxypropanol or 4-butoxybutanol and 13.5 mmoles of pyridinium chlorochromate dissolved in 26 ml of dichloromethane was well stirred at room temperature for 2.5 hr. After addition of 130 ml of dry ether, the reaction mixture was filtered through 35 g of Florisil. The filtrate evaporated *in vacuo* was dissolved in a small amount of hexane–ether–dichloromethane (4:3:2), applied on a Silica gel column (60 g), and eluted with the same solvent. Yields for 3-butoxypropanal and 4-butoxybutanal were 46 and 40%, respectively, and R_f values in thin-layer chromatography (Silica gel plate, benzene–ethylacetate = 20:1) were 0.41 and 0.42, detected as one spot by u.v. absorption and spraying with 2,4-dinitrophenylhydrazine. NMR data for 3-butoxypropanal in $CDCl_3$: $\delta = 0.90$ (3H, t, CH_3), 1.21–1.65 (4H, m, CH_2CH_2 in butyl), 2.62 (2H, t of w, CH_2CHO), 3.40 (2H, t, OCH_2 in butyl), 3.72 (2H, t, OCH_2 in propanal), and 9.73 (1H, t, CHO). NMR data for 4-butoxybutanal in $CDCl_3$: $\delta = 0.91$ (3H, t, CH_3), 1.2–1.65 (4H, m, CH_2CH_2 in butyl), 1.93 (2H, m, $CH_2CH_2CH_2$ in butanal), 2.52 (2H, t, CH_2CHO), 3.37 and 3.42 (4H, t each, CH_2OCH_2), and 9.75 (1H, t, CHO).

Cell culture

Mardin–Darby bovine kidney (MDBK) cells were grown in Eagle's minimum essential medium (Eagle's MEM) with 10% calf serum (Lot No. R265423, GIBCO, NY, U.S.A.) or fetal bovine serum (Lot No. 97951, M.A. Bioproducts, MD, U.S.A.). The cells were suspended at a concentration of 1×10^5 cells/ml in Eagle's MEM supplemented with 10% calf serum. The cell suspension in an aliquot of 5 ml was seeded in a disposable bottle (NUNCLON, 25 cm^2, Nunc, Denmark). After incubation at 37° for 3 days, 0.1 ml of each compound dissolved in phosphate-buffered saline (PBS) was added to the bottles through a millipore filter. After another incubation for 16 hr, cells were harvested by the usual 0.125% trypsin treatment and viable cells were estimated by the dye-exclusion test and counted microscopically.

Measurement of acrolein

Acrolein in incubation mixtures was measured by a high pressure liquid chromatography (HPLC) method, by a modification of amino acid analysis, after conversion to the cysteine diadduct, *S*-[1-(4-carboxy-2-thiazolidinyl)-2-ethyl]-cysteine [13]. Authentic acrolein (freshly distilled) or 3-substituted propylamine (0.3 μmole), dissolved in a final 3 ml of 0.1 M sodium potassium phosphate buffer (pH 7.8), was incubated at 37° for 1 hr in the presence of amine oxidase (200 μg). The incubation mixture, to which was added 20 μl of 0.12 M cysteine solution, was further incubated for 10 min for completion of cysteine diadduct formation. The reaction reached a plateau after incubation for 5 min. Conditions for HPLC* were as follows; column: TSK GEL IEX 215 (DVB 12.5%) from the Toyo Soda Co. Ltd.; column size: 5 mm i.d. × 10 cm stainless steel; column temperature: 60°; elution buffer: 0.35 N citrate buffer (pH 3.28) containing 0.01% *n*-caprylic acid, 0.5% 2-mercaptoethanol, 0.1% Brij-35 and 8% *n*-propanol; flow rate: 0.7 ml/min; and detection: fluorescent *o*-phthalaldehyde method. Retention time of the cysteine diadduct was 5.9 min, at which time no other compound was eluted. Detection limit was 50 pmoles. The cysteine diadduct that was eluted was measured from the peak height based on a calibration graph of standard.

Apparatus

NMR data were recorded on a Hitachi Perkin Elmer R-20A spectrometer (60 MHz) and fluorescence was measured with a Aminco SPF-125. High-pressure liquid chromatography equipped with an Aminco fluoro-microphotometer was constructed in our laboratory.

RESULTS

Effects of 3-substituted propylamines on MDBK cells

The cell lines, such as MDBK cells, C3H-2K cells [1] and SLE-A cells [1], responded similarly to the 3-substituted propylamines, the latter two cell lines being more susceptible to the cytotoxic effects of the compounds than were the MDBK cells. Since MDBK cells were more reproducible in their cultivation than the other cells, they were used in this experiment. Effects of 3-substituted propylamines on MDBK cells preincubated for 3 days were compared by counting viable cells after incubation for 16 hr in the presence of each propylamine. The compounds tested were roughly classified in two groups by their cytotoxic potency. As shown in Fig. 1, the first group, with IC_{50} values of around 0.1 mM under the experimental conditions, consisted of acrolein, 3-phenoxypropylamine, spermidine, and spermine, and the second group, with IC_{50} values around 0.4 mM consisted of the 3-alkyloxypropylamines. In addition to these compounds, 3-benzylaminopropylamine, 3-phenylaminopropylamine, 3-phenylthiopropylamine, and caldine belonged to the first group, and the 3-alkylthiopropylamines to the second group. A series of 3-alkylaminopropylamines was also tested for cytotoxicity, but their toxic effects were not observed up to 2 mM except for 3-butylaminopropylamine which had an IC_{50} of 0.7 mM.

When MDBK cells were grown in the medium supplemented with 10% fetal bovine serum in place of calf serum, the cytotoxic effects of 3-substituted propylamines were greatly reduced, and IC_{50} values of approximately 2.5 mM for 3-phenoxypropylamine and of more than 4 mM for 3-butoxypropylamine were observed. The toxic effect of 2.5 mM 3-phenoxypropylamine disappeared in the presence of 1.0 mM semicarbazide, an inhibitor of amine oxidase. 3-Butoxypropylamine did not show any toxic effect at 1.0 mM, but 3-butoxypropanal, an oxidized form of 3-butoxypropylamine, killed the cells completely at the same concentration. These results suggested that oxidative deamination of 3-substituted propylamines by amine oxidase in the serum was essential for the manifestation of cytotoxocity.

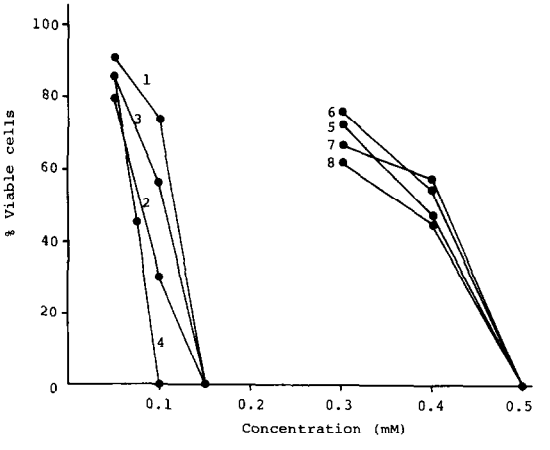

Fig. 1. Cytotoxicity of 3-substituted propylamines. Cytotoxicity was measured at the indicated concentrations of each compound under the conditions described in Materials and Methods. Percent viable cells represent ratios to control. Key: (1) acrolein, (2) 3-phenoxypropylamine, (3) spermidine, (4) spermine, (5) 3-methoxypropylamine, (6) 3-ethoxypropylamine, (7) 3-propoxypropylamine, and (8) 3-butoxypropylamine.

* Personal communication from Dr. H. Yoshida, Hiroshima University School of Medicine, Japan.

Oxidation of 3-substituted propylamines by amine oxidase

The eligibility of various propylamines as substrate for amine oxidase was next examined by determining the hydrogen peroxide (H_2O_2) liberated after incubation with purified amine oxidase from bovine plasma (Table 2). With both the 3-alkyloxypropylamine and 3-alkylthiopropylamine series, the liberation of H_2O_2 increased as the alkyl group became bulky, in that a relatively higher concentration of H_2O_2 was maintained with the compounds of the latter series compared with those compounds with the same alkyl group in the former series. The liberation of H_2O_2 from 3-butoxy- or 3-butylthiopropylamine was almost equivalent to that of the corresponding phenyl derivative. An appreciable amount of H_2O_2 was liberated from 3-benzylaminopropylamine; the concentration of H_2O_2 produced by oxidative deamination of 3-phenylaminopropylamine was roughly comparable with that from 3-phenoxy- or 3-phenylthiopropylamine. Spermidine and spermine were the most reactive substrates of amine oxidase. Compared with these compounds, the 3-alkylaminopropylamines were significantly poorer substrates of amine oxidase. It is possible to explain the weak cytotoxicity of 3-alkylaminopropylamines by this substrate specificity.

The relative concentrations of H_2O_2 liberated by oxidative deamination of these propylamines after incubation with 100-fold diluted calf serum were consistent with those obtained using purified amine oxidase. These results indicated that there was lack of full correlation between the IC_{50} values and the amounts of liberation of H_2O_2.

Formation of acrolein from 3-substituted propanal

The following studies were carried out to clarify whether acrolein was liberated from the resultant 3-substituted propanal and whether the propanal could also participate in the manifestation of cytotoxicity. Compounds used in this experiment were butyl and phenyl derivatives of 3-substituted propylamines, 3-benzylaminopropylamine, and polyamines.

Acrolein was measured by HPLC after reaction with cysteine. As an unknown substance in calf serum interfered with this method, experiments were performed using amine oxidase. The results are shown in Table 3. Acrolein could not be detected from incubation mixtures containing butyl derivatives of 3-substituted propylamines, while a significant concentration of acrolein was detected from the incubation mixtures containing 3-benzylaminopropylamine and phenyl derivatives of 3-substituted propylamines. Spermine and spermidine also released acrolein, and the concentrations were comparable to those estimated with 3-phenylaminopropylamine. All these acrolein-producing compounds were included in the group with IC_{50} values of 0.1 mM.

The inability to produce acrolein from 3-butoxypropylamine was further confirmed by using 3-butoxypropanal. The propanal did not produce any acrolein at 2 mM, while the cells were completely damaged at this concentration or even at 1 mM (Fig. 2). In addition, there was a remarkable difference between the observed cytotoxic effects of 3-butoxypropanal and of 4-butoxybutanal which had been chosen as a reference compound to consider the

Table 2. Oxidative deamination of 3-substituted propylamines by amine oxidase*

Compounds	H_2O_2	
	Liberated (μM)	Relative values (%)
3-Methoxypropylamine	4.17 ± 0.07	18
3-Ethoxypropylamine	6.13 ± 0.03	27
3-Propoxypropylamine	12.30 ± 0.07	54
3-Butoxypropylamine	14.67 ± 0.03	64
3-Phenoxypropylamine	15.57 ± 0.20	68
3-Methylthiopropylamine	4.73 ± 0.07	21
3-Ethylthiopropylamine	9.80 ± 0.07	43
3-Propylthiopropylamine	14.10 ± 0.03	61
3-Butylthiopropylamine	17.00 ± 0.10	74
3-Phenylthiopropylamine	17.60 ± 0.10	77
3-Methylaminopropylamine	0.20 ± 0.02	1
3-Ethylaminopropylamine	0.20 ± 0.02	1
3-Propylaminopropylamine	0.73 ± 0.02	3
3-Butylaminopropylamine	1.23 ± 0.10	5
3-Phenylaminopropylamine	13.87 ± 0.17	60
3-Benzylaminopropylamine	4.33 ± 0.13	19
Spermidine	19.50 ± 0.10	85
Spermine	23.00 ± 0.03	100

* Each amount of H_2O_2 liberated is the mean ± S.E. of four determinations. H_2O_2 was measured fluorometrically according to the method of Snyder and Hendley [22]. Incubation mixtures contained 40 μg of HRP, 250 μg of HVA, 0.3 μmoles of each compound (final concentration 0.1 mM), and 20 μg of amine oxidase in a final 3 ml of 0.1 M sodium potassium phosphate buffer (pH 7.8). Incubation was carried out at 37° for 1 hr. Fluorescence was measured at excitation (315 nm) and emission (425 nm).

Table 3. Measurement of acrolein from 3-substituted propylamines by cysteine diadduct-HPLC method*

Compounds	Cysteine diadduct	
	Formed (μM)	Relative values (%)
Acrolein	89 ± 2.3	100
3-Butoxypropylamine	ND†	0
3-Butylthiopropylamine	ND	0
3-Butylaminopropylamine	ND	0
3-Phenoxypropylamine	63 ± 2.6	70
3-Phenylthiopropylamine	41 ± 2.0	46
3-Phenylaminopropylamine	17 ± 4.8	19
3-Benzylaminopropylamine	10 ± 0.5	11
Spermidine	14 ± 0.5	16
Spermine	15 ± 0.5	17

* Acrolein liberated was determined. Each result is shown as the concentration of cysteine diadduct formed from 0.1 mM of each compound and is the mean ± S.E. of four determinations.
† Not detected.

cytotoxic effect of the aldehyde group. As seen from the figure, almost complete inhibition of cells was demonstrated with the propanal, whereas a viability of 85% was observed with the butanal at 1 mM.

DISCUSSION

MDBK cells in culture in the presence of calf serum were damaged by various 3-substituted propylamines, and these compounds could be roughly classified into two groups by their IC_{50} values. For the manifestation of cytotoxicity, an enzymatic oxidative deamination of these compounds was essential. This was demonstrated by several observations, such as an alleviation of cytotoxicity in fetal bovine serum with a marked effect of semicarbazide, a more potent cytotoxicity of 3-butoxypropanal than of 3-butoxypropylamine in fetal bovine serum, and a weak cytotoxicity of the 3-alkylaminopropylamines which were poor substrates for amine oxidase from bovine plasma. The results, however, showed lack of a full correlation between IC_{50} and H_2O_2 liberation taken as an index for oxidative deamination of each 3-substituted propylamine.

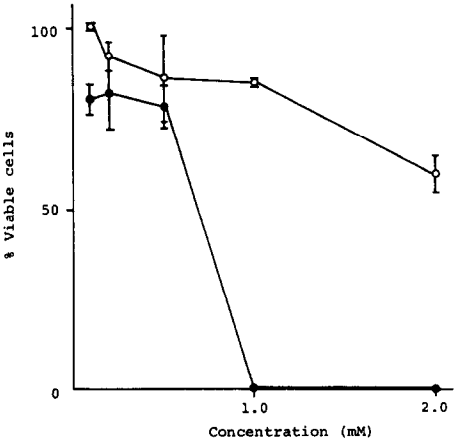

Fig. 2. Cytotoxicity of 3-butoxypropanal and 4-butoxybutanal. Conditions are described in the legend of Fig. 1. Key: (●—●) 3-butoxypropanal, and (○—○) 4-butoxybutanal.

Thus, the possibility of acrolein formation from the oxidized 3-substituted propylamines was examined as another mechanism involved. The cysteine diadduct-HPLC method was used for the detection and semiquantitation of acrolein under physiological conditions, which is simpler than the cold trap method [3]. By this method, it was possible to avoid artificial formation of acrolein during derivatization, since acrolein could be generated from various amino acids even at neutral pH and 100° in the presence of oxygen [23]. In fact, acrolein was liberated from the potent cytotoxic compounds with IC_{50} values of 0.1 mM, possibly in accordance with the eligibility for spontaneous β-elimination from their corresponding 3-substituted propanals, while not from the compounds with IC_{50} values of 0.4 mM. This clear correspondence between IC_{50} value and acrolein formation was interesting, and it strongly suggested that acrolein from 3-substituted propanal as well as the propanal moiety itself participated in the manifestations of cytotoxicity of the 3-substituted propylamines. The observed remarkable difference between the cytotoxic potencies of 3-butoxypropanal and 4-butoxybutanal also indicated that the propanal must have its own mode of action, different from that of the aldehyde group. Contrary to our expectations, 3-butoxypropanal was less cytotoxic than 3-butoxypropylamine in calf serum. The reason for this is unknown, since the additional possibility of the participation of H_2O_2 could be ignored at the concentrations used and on the basis of the recent report concerning a cytotoxic role of H_2O_2 liberated from spermine [24].

Although nothing is known about the actual interactions of acrolein or 3-substituted propanal with cells, it can be said that these two active forms may be involved in the manifestation of cytotoxicity of the 3-substituted propylamines, suggesting that spermine and spermidine may exert their cytotoxic effects on the cells through two mechanisms involving acrolein and the oxidized polyamines. The pres-

ent work was carried out focusing on the interactions of 3-substituted propylamines with amine oxidase in calf serum, but a similar mode of action would be expected to occur in the cells when these compounds are incorporated into cells and oxidatively deaminated by cellular amine oxidase. The demonstration of 3-substituted propanals as toxic compounds will be applied to other enzymatic reactions leading to the formation of 3-substituted propanals, such as dehydrogenation of 3-substituted propanol by alcohol dehydrogenase.

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