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EFFECT OF CATIONIC AMPHIPHILIC DRUGS ON THE HYDROLYSIS OF ACIDIC AND NEUTRAL PHOSPHOLIPIDS BY LIVER LYSOSOMAL PHOSPHOLIPASE A*

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Abstract—Rat liver lysosomal phospholipase A hydrolyzes both acidic and neutral phospholipids. Numerous cationic amphiphilic drugs including imipramine, propranolol, 4,4′-bis(diethylaminoethoxy)-α,β-diethyldiphenylethane and chloropromazine inhibit phospholipase A. Cationic amphiphilic drugs bind readily to acidic phospholipids but much less readily to neutral phospholipids. Formation of drug–lipid complexes is thought to be an important mechanism involved in the inhibition of lysosomal phospholipases. Therefore, we studied the effects of four cationic amphiphilic inhibitors on lysosomal phospholipase A using one acidic and two neutral phospholipid substrates. The concentration of the drugs required to produce 50% inhibition was much higher when phosphatidylinositol was used as substrate. The degradation of phosphatidylethanolamine and phosphatidylcholine was more readily inhibited by these agents than that of phosphatidylinositol. In drug-induced lipidosis, the predominance of acidic phospholipids may be due to redirection of phospholipid metabolism towards the formation of acidic phospholipids with a resultant increased delivery of these lipids to lysosomes. Based on our results, it does not appear to be due to decreased enzymatic hydrolysis of drug–acidic phospholipid complexes, at least when pure phospholipid substrates are used. Lysosomal storage of both acidic and neutral phospholipids appears to be caused by inhibition of lysosomal phospholipase action in view of the probable high intralysosomal levels of these agents.

With chronic use, cationic amphiphilic drugs induce phospholipidosis in humans and in experimental animals [1, 2]. The phospholipidosis is characterized by the occurrence of lamellated or crystalloid bodies consisting of cholesterol and polar lipids in cellular lysosomes of many tissues [3–6]. Acidic phospholipids, such as phosphatidylinositol, accumulate to a greater degree than neutral phosphoglycerides [2]. Lipid accumulation in tissues appears to be a consequence of drug-induced disturbances of phospholipid metabolism. Cationic amphiphilic drugs have been shown to concentrate in lysosomes [7] where they inhibit phospholipases A and C [8]. This sequence of events is proposed to be the major cause of the syndrome [8–10]. These agents also inhibit the cytoplasmic phosphatidate phosphohydrolase from rat liver [11, 12] and brain [13] and have been reported to redirect phospholipid synthesis toward the acidic phospholipids which are formed from phosphatidic acid and via the CDP-diacylglycerol pathway [14–16]. In addition, these agents inhibit pancreatic [17] and bee venom phospholipase A_2 [18]. A single unifying concept to explain the mechanism of drug inhibition of these phospholipases is difficult to formulate since these enzymes vary with respect to their pH optima and requirements for substrates and cofactors.

Cationic amphilic agents bind to polar lipids, forming drug–lipid complexes [19, 20]. It has been proposed that the binding of drugs to the phospholipids decreases their susceptibility to enzymatic hydrolysis by phospholipases [17–22]. Since the binding of cationic amphiphilic drugs to acidic lipids is greater than binding to neutral phospholipids, it has been suggested that the disproportionate increase in acidic lipids during chronic drug treatment may be due to greater resistance of drug–acidic lipid complexes to enzymatic degradation [21, 22].

To test this hypothesis, we studied drug-inhibition of lysomal phospholipase A which hydrolyzes both acidic and neutral phospholipids. Four drugs, imipramine, propranolol, 4,4′-bis(diethylaminoethoxy)α,β-diethyldiphenylethane (DH), and chlorpromazine, were selected for our studies, since they are agents which have been shown to alter tissue phospholipid metabolism both *in vitro* and in some cases *in vivo* [1, 2, 7, 8, 21–25]. The effects of these drugs on lysosomal phospholipase A were studied by measuring conversion of acidic and neutral phospholipids to their respective lyso derivatives. Contrary to prior suggestions, our results show that the degradation of neutral phospholipids is affected by cationic amphiphilic agents to a greater degree than that of acidic phospholipids.

MATERIALS AND METHODS

Preparation of delipidated lysosomal soluble protein: Male Sprague–Dawley rats were injected intraperitoneally with Triton WR-1339 (850 mg/kg body

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wt) in saline, and liver lysosomes were isolated by the method of Trouet [26]. Soluble lysosomal protein obtained by repeated freezing and thawing was delipidated with *n*-butyl alcohol as described earlier [27]. The protein concentration was determined by the method of Lowry *et al.* [28] using bovine serum albumin as standard. Aliquots of this soluble lysosomal fraction were stored at −60° until use.

Preparation of labeled lipid. [9,10-^{3}H]Dioleylphosphatidylcholine (DOPC) was synthesized chemically by the method of Warner and Benson [29] and purified by thin-layer chromatography. Phosphatidyl[^{3}H]inositol was prepared by incubating 10 μCi of [2-^{3}H]myoinositol for 48 hr with cultured MDCK cells. The cell monolayer was washed with phosphate-buffered saline, and the cells were harvested by scraping with a rubber policeman as described previously [30]. The lipids were extracted from the cells [31], and the labeled lipid was purified by two-dimensional thin-layer chromatography on silica gel H plates prepared with 1 mM magnesium acetate and developed with chloroform–methanol–conc. ammonia–water (60:30:0.5:3, by vol.) and chloroform–acetone–methanol–acetic acid–water (60:80:20:20:10, by vol.) as developing solvent systems [32]. ^{3}H-labeled phosphatidylinositol and phosphatidylcholine were used as tracers in the gel filtration experiments.

Preparation of substrates. Dispersions of the various phospholipids were prepared as follows. Chloroform solutions of lipids were taken to dryness under nitrogen. Residual lipids were dispersed in distilled water by vortexing. The lipid suspensions were sonicated three times for 5 sec with the microtip of a Heat Systems sonicator (model W-225R) to obtain a clear dispersion.

Separation of small unilamellar vesicle (SUV) and multilamellar vesicle (MLV) liposomes. Analytical procedure: to determine the relative proportions of SUV and MLV, phosphatidylcholine or phosphatidylinositol-containing liposomes, obtained by sonication under conditions identical to those described in Fig. 2, were applied to a Sepharose 4B-200 column (1 × 50 cm). The column was eluted with 50 mM sodium acetate buffer (pH 4.4), at a flow rate of 6 ml/hr. The lipid elution profile was determined by monitoring the radioactivity in the fractions.

For preparative isolation of substrate liposomes, SUV and MLV were separated as follows: the clear lipid dispersion prepared by sonication (30 μmoles phospholipid/ml containing 7 × 10^4 dpm/μmole) was applied to a 2.5 × 62 cm column of Sepharose 4B-200. The column was eluted with 5 mM Tris buffer (pH 7.4) containing 20 mM sodium chloride at a flow rate of 28 ml/hr. The elution profile of the respective lipids was monitored by determining the radioactivity in the respective fractions (phosphatidylcholine and phosphatydylinositol). The fractions containing relatively pure SUV or MLV were combined and concentrated using an Amicon ultrafiltration cell.

Assay procedures. The incubation medium contained 50 mM sodium acetate buffer (pH 4.4), 50 μg delipidated lysosomal soluble protein, lipid substrate, and drugs as noted in a final volume of 0.2 ml. The incubation was carried out at 37° for 20 min. The reaction was terminated by addition of 4 ml chloroform–methanol (2:1, v/v). Total lipids were extracted by the method of Folch *et al.* [31]. The individual phospholipids were separated by thin-layer chromatography on either HPTLC silica gel 60 precoated plates or silica gel H plates containing 1 mM magnesium acetate (0.25 mm thickness) using chloroform–methanol–water (65:35:5, by vol.) as a developing solvent. The areas corresponding to lysophospholipids were visualized by iodine vapors and scraped for phospholipid phosphorus determination by the method of Rouser *et al.* [33]. The recovery of the lipid from the plates was greater than 98%.

Materials. [9,10-^{3}H]Oleic acid (sp. act. 2.1 Ci/mmole) and myo[2-^{3}H]inositol (sp. act. 10–20 Ci/mmole) were obtained from the New England Nuclear Corp., Boston, MA. Phosphatidylcholine (egg), phosphatidylethanolamine (egg) and phosphatidylinositol (bovine brain) were purchased from Avanti Polar Lipids, Birmingham, AL. Imipramine, chlorpromazine, Tris buffer, bovine serum albumin, Sephadex G-25, and Sepharose 4B-200 were from the Sigma Chemical Co., St. Louis, MO. Propranolol–HCl was obtained from Ayerst Laboratories, Inc., New York, NY. 4,4′-Bis(diethylaminoethoxy)-α,β-diethyldiphenylethane was the gift of the Torii Pharmaceutical Co., Tokyo, and Dr. Y. Matsuzawa, Osaka, Japan. Triton WR-1339 was purchased from Supelco, Bellefonte, PA. Silica gel H and HPTLC silica gel 60 precoated plates were obtained from the EM Laboratories, Elmsford, N.Y. All other chemicals used were of analytical reagent grade. Chloroform and methanol were redistilled before use. Male rats of the Sprague–Dawley strain were purchased from the Charles River Breeding Laboratories, Wilmington, MA.

RESULTS

Phospholipase A catalyzes the removal of a fatty acid ester of glycerophospholipids, leading to formation of lysophospholipids. In rat liver lysosomes, phospholipase A_1 appears to be the major phospholipase while phospholipase A_2 and phospholipase C are less active [34–39]. However, in these studies, we measured the total phospholipase A activity of soluble lysosomal fraction by quantitative estimation of lysophospholipid formed (phospholipase $A_1 + A_2$).

Phospholipase A activity of soluble lysosomal fraction with various phospholipid substrates. Phospholipase A activity was measured by the release of lysophosphoglycerides at various concentrations of acidic and neutral phospholipids, as shown in Fig. 1. At low substrate concentrations, the soluble lysosomal protein fraction hydrolyzed the respective phospholipids to lyso derivatives at comparable rates. However, at substrate concentrations of 0.2 mM and above, phosphatidylinositol appeared to be the preferred substrate under these experimental conditions, the order of preference being phosphatidylinositol > phosphatidylcholine > phosphatidylethanolamine. Kinetic analysis of the data resulted in non-linear double-reciprocal plots; this may be due to the presence of an active lysophospholipase in the soluble lysosomal fraction which removes the

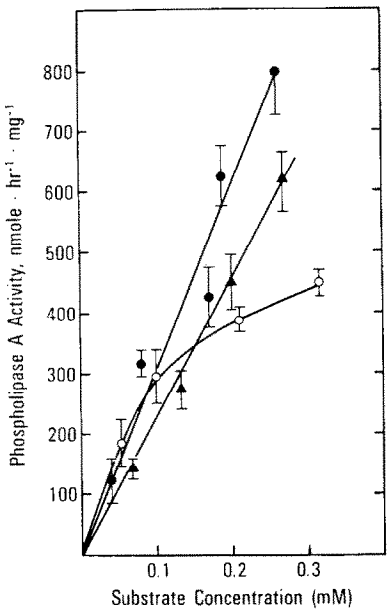

Fig. 1. Rat liver lysosomal phospholipase A activity using different substrates. The enzyme activity was determined by measuring the lysophospholipid formed. The incubation medium contained 50 mM sodium acetate, pH 4.4, 50 μg of delipidated soluble lysosomal protein and various concentrations of sonicated lipid substrate, in a final volume of 0.2 ml. The incubation, extraction and separation of lipids were described in Materials and Methods. The results are mean ± S.D. of three to six observations. Key: ○ phosphatidylethanolamine, (▲) phosphatidylcholine and (●) phosphatidylinositol.

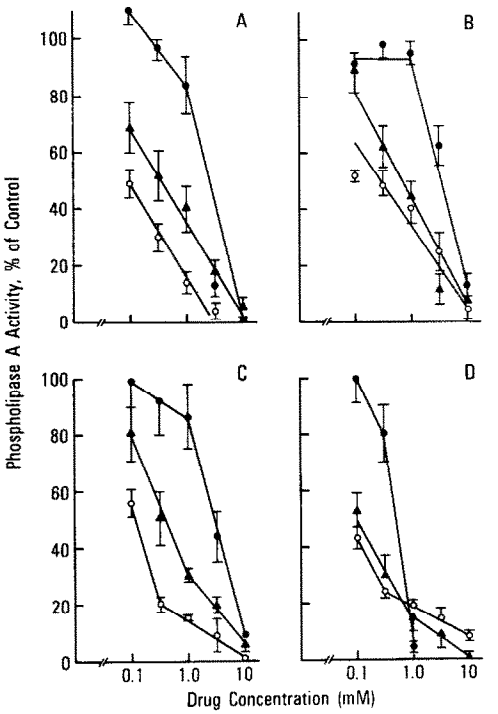

Fig. 2. Effect of cationic amphiphilic drugs on lysosomal phospholipase A using different substrates. The enzyme activity was determined at a substrate concentration of 0.175 mM. Incubation conditions were as described in the legend of Fig. 1. The results are expressed as percent of control. Control values (N = 9) were as follows: lysophosphatidylethanolamine, 325 ± 74 nmoles · mg^{-1} · hr^{-1}; lysophosphatidylcholine, 313 ± 65 nmoles · mg^{-1} · hr^{-1}; and lysophosphatidylinositol, 420 ± 50 nmoles · mg^{-1} · hr^{-1}. Key: (○) phosphatidylethanolamine, (▲) phosphatidylcholine, and (●) phosphatidylinositol. The drug concentrations shown on the ordinate are given in logarithmic scale. Panel A, imipramine; B, propranolol; C, 4,4′-bis(diethylaminoethoxy)α,β-diethyldiphenylethane; and D, chloropromazine.

lysophospholipid product of phospholipase A [39].

Inhibition of neutral and acidic phospholipid degradation by cationic amphiphilic drugs. The enzyme rates were measured at comparable concentrations of sonicated acidic and neutral phospholipids, in the presence or absence of four cationic amphiphilic drugs (Fig. 2). All inhibited the hydrolysis of neutral phospholipids more readily than the acidic phospholipid, phosphatidylinositol. The concentrations of the respective drugs required for 50% inhibition (IC_{50}) were calculated from the data in Fig. 2, and the results are shown in Table 1. The susceptibility of the soluble lysosomal phospholipase A to drug inhibition clearly depended on the substrate used; it was more pronounced with phosphatidylethanolamine, the order of susceptibility being phosphatidylethanolamine > phosphatidylcholine > phosphatidylinositol. The IC_{50} value of the respective drugs with phosphatidylinositol as substrate was four to eight times that required with phosphatidylcholine and six to twenty-five times that required with phosphatidylethanolamine respectively. The order of potency of drugs when phosphatidylcholine and phosphatidylinositol were used as substrates was chlorpromazine > imipramine > DH > propranolol. With phosphatidylethanolamine as substrate, the potencies were chloropromazine = imipramine = DH > propranolol.

Sepharose 4B-chromatography of sonicated phospholipid substrates. Since differences in the observed phospholipase A activity with or without in-

Table 1. Inhibition of lysosomal phospholipase A by cationic amphiphilic drugs using different substrates

Substrate	IC_{50}* (mM)			
	Imipramine	Propranolol	DH†	Chlorpromazine
Phosphatidylethanolamine	0.10	0.32	0.11	0.10
Phosphatidylcholine	0.36	0.71	0.41	0.10
Phosphatidylinositol	1.8	3.6	2.8	0.50

* Concentration of the drug required to produce 50% inhibition. The results are calculated from data shown in Fig. 2.
† 4,4′-Bis(diethylaminoethoxy)α,β-diethyldiphenylethane.

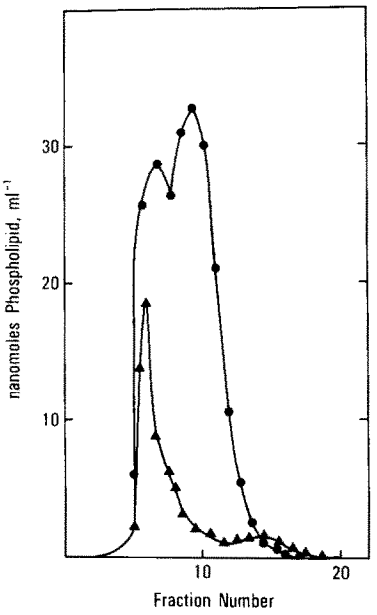

Fig. 3. Sephrose 4B chromatography of sonicated phospholipid substrates. Sonicates of phosphatidylcholine and phosphatidylinositol were prepared as described in the legend of Fig. 2 using the microtip of a Heat Systems sonicator (model W-225R) for short time intervals. The column was eluted according to the method described in the text (analytical procedure). Key: (▲) phosphatidylcholine, and (●) phosphatidylinositol.

hibitors might be a consequence of variations in the type of phospholipid vesicles present in the sonicated dispersions, we investigated this possibility by subjecting sonicates of phosphatidylcholine and phosphatidylinositol to gel filtration on columns of Sepharose 4B. The sonicates were prepared in a manner identical to that used for the inhibitor studies shown in Fig. 2. The liposomal elution profiles of the samples are given in Fig. 3. Sonication of phosphatidylcholine and phosphatidylinositol led to the formation of MLV which eluted at the void volume and SUV which eluted midway between the void and included column volume, as shown in Fig. 3. This type of sonication led to characteristic differences in vesicle species formed from phosphatidylcholine and phosphatidylinositol. Phosphatidylcholine sonicates contained 98% MLV and 2% SUV, while phosphatidylinositol sonicates consisted of 33% MLV and 67% SUV.

Activity of phospholipase A using SUV and MLV substrates. Since the observed differences in the rates of substrate hydrolysis by the enzyme may depend on the type of sonicated vesicle used, we examined the phospholipase A activity using equimolar concentrations of unfractionated sonicate, SUV or MLV substrates. The results are given in Table 2.

Crude soluble lysosomal phospholipase A hydrolyzed SUV of phosphatidylcholine or phosphatidylinositol at a rate 2.2 to 2.6-fold that of MLV. When sonicate of phosphatidylcholine, consisting primarily of MLV, was used as substrate (Fig. 3), the enzyme rate obtained was comparable to that obtained using purified MLV. However, using sonicates of phosphatidylinositol, consisting of 67% SUV and 33% MLV, the observed rate was intermediate to that obtained with purified populations of SUV or MLV substrates respectively.

Effect of imipramine on lysosomal phospholipase A activity with SUV and MLV substrates. To investigate the possiblity that the results in Fig. 2 could be explained by differing effects of cationic amphiphilic drugs on MLV versus SUV substrates, we examined the activity of lysosomal phospholipase A in the presence of increasing concentrations of imipramine, using purified SUV and unfractionated sonicates con-

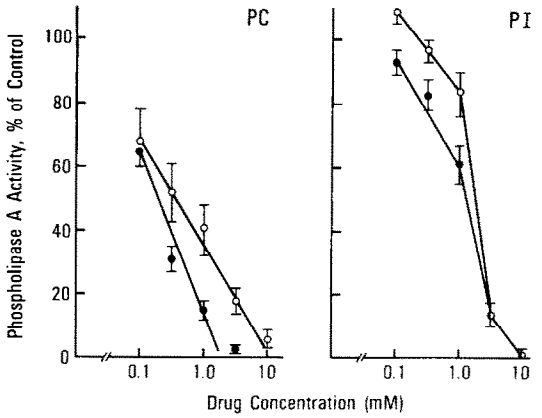

Fig. 4. Effect of imipramine on lysosomal phospholipase A using single and multilamellar vesicles of phosphatidylcholine and phosphatidylinositol. The enzyme activity was determined by using 0.175 mM substrate either as sonicates or as SUV. All other conditions were as described in the legend of Fig. 2. Key: (●) SUV substrate, and (○) sonicate containing SUV and MLV substrates. Panel A, phosphatidylcholine; and B, phosphatidylinositol.

Table 2. Phospholipase A activity using small unilamellar or multilamellar vesicles*

Substrate	Phospholipase A activity†	
	Phosphatidylcholine	Phosphatidylinositol
Sonicate (MLV + SUV)	394 ± 54	402 ± 18
MLV	413 ± 34	263 ± 17‡
SUV	925 ± 86‡	685 ± 55‡

* Phospholipase A activity was determined using 0.175 mM substrate either as unfractionated sonicates or purified MLV or SUV. The incubation conditions are described in the legend to Fig. 1. The results are mean ± S.D. of three to six observations. Abbreviations: MLV, multilamellar vesicles; and SUV, small unilamellar vesicles.

† Expressed as nmoles lysophospholipid · (mg protein)$^{-1}$ · hr^{-1}.

‡ Differs significantly from corresponding values for sonicate, $P < 0.01$.

sisting of phosphatidylcholine and phosphatidylinositol respectively (Fig. 4). The drug-induced inhibition of enzyme activity was essentially similar to that obtained previously with the sonicates of phosphatidylcholine and phosphatidylinositol. The IC_{50} values for imipramine with SUV substrates calculated from the data in Fig. 4 were lower, 0.2 and 1.25 mM, respectively, for both phosphatidylcholine and phosphatidylinositol than with the two unfractionated sonicates. However, the IC_{50} for imipramine when SUV phosphatidylinositol was used as substrate was still six times higher than that observed when SUV phosphatidylcholine was the substrate.

DISCUSSION

The substrate specificity of the soluble, delipidated lysosomal phospholipase A preparation appears to differ from that reported by others [37, 38] in that phosphatidylinositol and phosphatidylcholine were more actively hydrolyzed than phosphatidylethanolamine in our experiments (Fig. 1). However, the purified phospholipases A_1 from rat liver lysosomes also favor phosphatidylcholine and phosphatidylinositol over phosphatidylethanolamine [39]. The uninhibited rates for phospholipase A reported in this paper are lower than those reported by Kunze *et al.* [36] for the crude enzyme but agree well with our previous reports [9, 35]. This may be due to differences in the method for preparation of the crude enzyme fractions as noted by Kunze *et al.* [36], to differences in the nature of the sonicated substrate dispersion as shown in Fig. 3, and to the activity of acid lysophospholipase in the respective preparations.

The prevailing theory for cationic amphiphilic drug inhibition of phospholipase A activity hypothesizes that the complexes formed between the drug and the lipids render the phospholipid–drug complex less susceptible to hydrolysis [19–22]. Therefore, we expected to find that phosphatidylinositol hydrolysis would be inhibited to a greater degree since acidic phospholipids bind more cationic amphiphilic drug [20]. However, as the data in Fig. 2 and Table 1 indicate, 5- to 18-fold more drug was required to inhibit phosphatidylinositol catabolism as compared with phosphatidylcholine and phosphatidylethanolamine respectively.

Since the physical configuration of briefly sonicated dispersions of the phospholipids may vary considerably (Fig. 3), we isolated SUV of phosphatidylcholine and phosphatidylinositol and studied the effects of imipramine on phospholipase A hydrolysis of SUV and unfractionated sonicated substrates. The IC_{50} for imipramine with SUV substrates is lower than that for unfractionated sonicates, especially with phosphatidylcholine. However, the 6-fold increase in IC_{50} with phosphatidylinositol versus phosphatidylcholine substrates is still apparent. Thus, differences in the size and lamellar structure of the sonicated substrates cannot explain the higher IC_{50} with phosphatidylinositol.

Although it has been shown that acidic phospholipids form complexes with cationic amphiphilic drugs more readily than with neutral phospholipids, it does not appear that this can explain the predominance of acidic phospholipids in the stored material. There are probably at least two major intracellular effects of these agents. The predominance of acidic lipids is related to the increased synthesis of the acidic phospholipids which are derivatives of the CDP-diacylglycerol pathway [14–16]. This effect occurs at the endoplasmic reticulum and appears to be due to the inhibition of the conversion of phosphatidic acid to diacylglycerol which results in overproduction of CDP-diacylglycerol, the precursor of phosphatidylinositol and phosphatidylglycerol [40, 41]. The findings in this paper reemphasize the possible importance of this mechanism, which has been termed "redirection of phospholipid synthesis" [14–16]. A second effect occurs in lysosomes where the lysosomal storage of both neutral and acidic phospholipids is related to drug inhibition of phospholipid catabolism. The intralysosomal concentration of these agents may be over 50 mM in liver, and it seems clear that this level of DH would probably be sufficient to block all phospholipid breakdown [8]. The predominance of acidic phospholipids would, therefore, be related to increased synthesis and delivery of these acidic phospholipids to lysosomes. *In vivo* evidence for this sequence of events has been presented previously [42].

The molecular mechanisms involved in cationic amphiphilic drug inhibition are not well-defined at present and may be complex. Effects of these agents both on the enzyme and on the substrate are possible, and more detailed studies will be required to define the factors involved.

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