

Some Properties of Purified Phospholipase D and especially the Effect of Amphipathic Substances

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1. The soluble phospholipase D of cabbage was purified by heat treatment, acetone precipitation and electrophoresis on a density gradient of aqueous glycerol. 2. The purified enzyme slowly attacked a lecithin suspension whereas ultrasonically treated lecithin was hydrolysed more rapidly. 3. Diethyl ether stimulated the hydrolysis of both the lecithin suspension and ultrasonically treated lecithin. 4. Ca^{2+} was essential for the hydrolysis (optimum about 0.04 M); it could not be replaced by Mg^{2+} or cationic amphipathic substances. 5. The reaction had a sharp pH optimum at pH 5.4, irrespective of the physical form of the lecithin substrate or the activator used. 6. Anionic amphipathic substances such as dodecyl sulphate, phosphatidic acid, triphosphoinositide and monocetyl phosphoric acid, were potent activators of the reaction: other acidic lipids were relatively inactive. 7. Cationic amphipathic substances inhibited the hydrolysis; however, they also reversed the inhibition caused by using an excess of anionic amphipathic substance as activator. 8. The activation produced by amphipathic substances could not be correlated with their effect on the ζ -potential or size of the substrate particles. 9. The addition of activating anionic amphipaths to lecithin induces the latter to adsorb enzyme from solution. In the absence of Ca^{2+} the enzyme is denatured on the highly negatively charged surface, but in the presence of Ca^{2+} (or Mg^{2+}) it is protected from denaturation. It is suggested that this adsorption is an essential prerequisite for ready enzymic hydrolysis. 10. The hydrolysis of lecithin by the enzyme was strongly inhibited by protamine sulphate (0.1 mg./ml.) and by choline and ethanolamine. 11. Ultrasonically treated phosphatidylethanolamine, or mixed particles of phosphatidylethanolamine plus dodecyl sulphate, were slowly attacked by the enzyme provided that Ca^{2+} was present.

Phospholipase D (EC 3.1.4.4) occurs widely in the tissues of higher plants and especially in those of the genus *Brassica* (Davidson & Long, 1958; Einset & Clark, 1958). It appears to exist in the plant cells in an insoluble form associated with the plastids (Kates, 1954) but a soluble form can readily be demonstrated in cabbage (Davidson & Long, 1958), carrot (Einset & Clark, 1958) and cottonseed (Tookey & Balls, 1956).

It has been reported that the activities of the insoluble plastid enzyme and the soluble enzyme of the carrot and cabbage are stimulated by the addition of various organic solvents such as linear aliphatic ethers (especially diethyl ether), ketones and esters (Kates, 1957; Davidson & Long, 1958; Einset & Clark, 1958), although the phospholipase D of cottonseed is not activated by diethyl ether (Tookey & Balls, 1956). As well as showing this solvent activation, the plastid enzyme from spinach

is stimulated to a smaller extent by certain anionic detergents (Kates, 1957), and the soluble enzyme from cabbage by the acidic phospholipid, phosphatidylinositol (Weiss, Spiegel & Titus, 1959). In addition, the plastid enzyme can be inhibited by cationic detergents (Kates, 1957). These observations on the stimulatory and inhibitory effects of solvents and surface-active lipids suggest that the rate of enzymic hydrolysis is largely dependent on the physical chemistry of the lipid substrate, as has been observed with many other lipolytic enzymes (Bangham & Dawson, 1959; Dawson, 1964). In the present investigation a study has been made of the effect of various amphipathic substances on the enzymic activity of phospholipase D and on the physical chemistry of its substrate. In addition, the opportunity has been taken to investigate other properties of the purified enzyme.

METHODS

Purification of enzyme. The enzyme was prepared from the inner, yellowish-white, leaves of Savoy cabbages purchased locally. The initial purification, employing heat treatment and acetone precipitation, was carried out as described by Davidson & Long (1958). Attempts to purify the enzyme further by calcium-gel adsorption, as described by these authors, led to large losses of activity. Consequently, after acetone precipitation, the enzyme was fractionated in a density-gradient electrophoresis apparatus (LKB Produktor, Stockholm, Sweden) (Svensson, 1960) by using similar techniques to that described by Dawson (1963a) for the isolation of pure phospholipase A from cobra venom. The density gradient was prepared by using 0.01 M-sodium acetate-acetic acid buffer, pH 5.0, in water and the same buffer in glycerol-water (9/11, v/v) as the light and heavy components respectively. The acetone-precipitated enzyme was dissolved in water (0.1 ml./g. of initial leaves). Glycerol was added so that the density of the solution was just less than that of the heavy component and acetate buffer (pH 5.0) so that the final concentration was 0.01 M. This enzyme solution (25 mg. of protein) was layered on to the heavy buffer contained in the electrophoresis column and the gradient was then formed, starting with a 'light-heavy' buffer containing glycerol-water (2/3, v/v). Electrophoresis was continued for 15-17 hr. (900 v, 10 mA) at the temperature of running tap water (10-12°) with the anode at the top (light) end of the column. The column was separated into fractions by moving a glass funnel, connected to the exterior by glass tubing sliding in a polythene plug, through the heavy buffer to the bottom of the gradient. Twelve 5 ml. samples were withdrawn, followed by 12

7.5 ml. samples and then 12 10 ml. samples. The E_{280} values of the fractions were measured and their phospholipase D activities were assayed with lecithin as substrate. In later runs only those fractions that corresponded to the approximate position of the phospholipase D peak, as deduced from the E_{280} measurements, were examined for enzymic activity.

The phospholipase D peak was well separated from the bulk of the proteins, which were negatively charged at pH 5 (Fig. 1). In no run was there found an E_{280} peak that coincided with the peak of enzymic activity. The fractions constituting the enzyme peak were combined, giving a water-clear solution (approx. 30 ml.) containing very little protein but about 80-90% of the enzymic activity applied to the column. Crystalline serum albumin (10 mg./ml.) was added. The solution was stored at -15°: a small loss of enzymic activity occurred over several months.

Assay of enzymic activity. The phospholipase D activity was usually estimated by measuring the base liberated in the reaction, although in some cases the lipid products (phosphatidic acid, phosphatidylglycerol) were determined. A suitable portion (150 μ g. of P) of a chloroform solution of egg lecithin or egg phosphatidylethanolamine (both prepared as described by Dawson, 1963a) was evaporated to dryness in a tube *in vacuo*. Where appropriate the amphipathic substance in organic solvent solution was mixed with the phospholipid beforehand, although certain of these were added as aqueous 'solutions'. The dried lipid was then shaken with 0.3 ml. of 0.05 M-sodium acetate-acetic acid buffer, pH 5.4 or 5.8, 0.2 ml. of 0.225 M-CaCl₂ and water was added to 1.125 ml. producing a turbid suspension of lecithin particles. These particles vary in size between about 1 and 5 μ and are subsequently referred to as 'large particles' to distinguish them from the much smaller particles (< 0.3 μ) present in ultrasonically treated lecithin preparations. A 0.075 ml. portion of the enzyme solution (unless otherwise stated) was then added and the mixture incubated at 27° for an appropriate time (usually 15-30 min.).

When ultrasonically treated lecithin or phosphatidylethanolamine was used as substrate, the phospholipid (31 μ moles), suspended in 2 ml. of water, was treated with ultrasonics under N₂ in an ice bath for 10 min. with an MSE ultrasonic disintegrator (60 w). A portion of the ultrasonically treated solution (0.3 ml., 150 μ g. of P) was mixed with acetate buffer, CaCl₂ and enzyme and incubated as described above for the large lecithin particles. The 'water-soluble' amphipathic substances (sodium dodecyl sulphate, Koch-Light Ltd.; cetyltrimethylammonium bromide, Hopkin and Williams Ltd.) were added to the incubation mixture in aqueous solution. With dodecyl sulphate it was essential to introduce the CaCl₂ before the enzyme, or else extensive denaturation of the latter occurred.

After incubation, the method of stopping the reaction varied both with the substrate employed and the hydrolysis product being measured. When measuring the liberation of choline from lecithin, 0.2 ml. of 5% (w/v) serum albumin followed by 0.5 ml. of 20% (v/v) HClO₄ were added to the mixture cooled in ice. After centrifuging, the supernatant was filtered and 1 ml. was used for choline assay. If ether had been added, the incubation mixture was shaken for a few minutes with 0.8 ml. of CHCl₃, centrifuged, and the upper aqueous layer filtered and warmed at 50° to remove dissolved CHCl₃. Such an extraction effectively removes

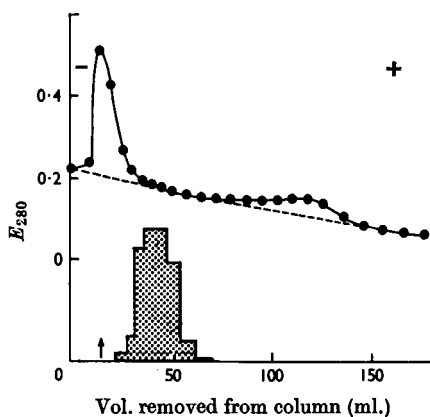


Fig. 1. Density-gradient electrophoresis of partially purified phospholipase D. The point of insertion of the enzyme is indicated by the arrow. After electrophoresis, serial samples were removed from the column, beginning at the bottom (cathode) end, as described in the Methods section. Their E_{280} values were recorded (●). The interrupted line shows the approximate E_{280} of the gradient without enzyme. The histogram represents the ability of the samples to liberate choline from lecithin, expressed in arbitrary units.

any dissolved ether, traces of which can interfere with the subsequent choline estimation.

When the release of ethanolamine from phosphatidyl-ethanolamine was being measured, 0.3 ml. of cold 20% (w/v) trichloroacetic acid was added and, after cooling in ice, the mixture was spun. The supernatant was washed four times with 2 vol. of ether at each extraction to remove the trichloroacetic acid, 1 ml. of the resultant aqueous phase being used for ethanolamine determination.

Sometimes added substances (e.g. taurocholate) interfered with the determination of the base and then the lipid hydrolysis products (phosphatidic acid, phosphatidyl-glycerol) were assayed. The incubation medium was shaken vigorously with 5 vol. of CHCl_3 -methanol (2:1, v/v) and, after centrifuging, the lower layer was washed with an equal volume of a mixture containing methanol-0.05% CaCl_2 - CHCl_3 (45:47:3, by vol.). The chloroform-rich layer was taken and the phospholipids were assayed by the method of Dawson, Hemington & Davenport (1962).

Determinations. Choline was determined by the method of Appleton, La Du, Levy, Steele & Brodie (1953) with the precautions described by Dawson (1956). A linear response was obtained with choline chloride standards (27-186 μg .). Ethanolamine was estimated by its ninhydrin reaction based on the procedure of Yemm & Cocking (1955). A 1 ml. portion of the aqueous phase was mixed with 0.5 ml. of 0.2M-trisodium citrate-citric acid buffer, pH 5.0, and then with 1.2 ml. of the KCN-methylCellosolve-ninhydrin reagent described by these authors. The solution was heated at 100° for 15 min., cooled and made up to 5 ml. with 60% (v/v) ethanol and the colour read at 570 μm . With ethanolamine standards (10-50 μg .) a linear curve passing through the origin was obtained.

Electrophoretic mobilities. The mobilities of substrate particles were measured at 27° in the microelectrophoresis apparatus of Bangham, Flemans, Heard & Seaman (1958) as described by Dawson (1963a). To see the lipid particles clearly, these had to be diluted fourfold compared with the concentration used in the enzyme assay. However, the ionic environment was kept constant by diluting with incubation medium.

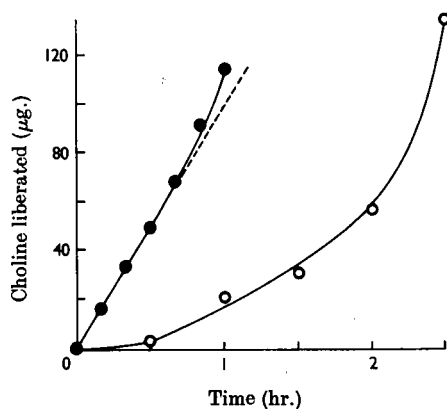


Fig. 2. Hydrolysis of lecithin by phospholipase D. ○, Large lecithin particles, 0.2 ml. of enzyme solution; ●, ultrasonically treated lecithin, 0.08 ml. of enzyme solution.

Preparation of phosphatidic acid. Egg lecithin (4.8 mg of P) was suspended in 18 ml. of 0.017M-sodium acetate-acetic acid buffer, pH 5.4, and 4 ml. of 0.225M- CaCl_2 , 2 ml. of acetone-precipitated phospholipase D preparation and 4 ml. of diethyl ether were added. After being shaken well, the mixture was incubated for 2 hr. at 30°. It was then extracted four times with equal volumes of ether and the combined ethereal extracts were evaporated to dryness. The residue was dissolved in CHCl_3 -methanol (19:1, v/v) and introduced on to a silicic acid column (2 cm. diam. \times 10 cm. long) washed with the same solvent. The column was eluted with the same solvent and to the first 50 ml. emerging was added 20 ml. of methanol and 15 ml. of 0.1N-HCl. After shaking, the lower chloroform-rich phase was collected, neutralized to pH 8 with ammonia and evaporated to dryness. The resulting ammonium phosphatide was taken up in a small volume of CHCl_3 . The yield was 80% and alkaline degradation (Dawson *et al.* 1962) indicated that phosphatidic acid was the only phospholipid present.

RESULTS

Adsorption of the purified enzyme on glass surfaces. The specific activity of the purified phospholipase D could not be measured because all the methods of protein estimation available were far too insensitive. Nevertheless, it must have been extremely high and this caused great difficulties in the determination of its activity. Thus on pipetting the purified enzyme a number of times with separate clean pipettes appreciable losses of activity occurred, the extent of the loss depending on the number of pipettings. This effect can almost certainly be related to irreversible adsorption of the enzyme on glass surfaces. Very inconsistent results were in fact obtained until a certain proportion of another protein (serum albumin) was added to the preparation to block the effect, presumably by direct competition for the charged surface. Thus the enzyme preparations used to obtain most of the present results had a small amount of added foreign protein introduced but, as far as could be ascertained, were substantially free from native proteins such as other enzymes or lipoproteins. No substantial difference was noted between the properties of the electrophoretically purified enzyme with added serum albumin and the preparation at the acetone-precipitation stage.

Hydrolysis of lecithin by phospholipase D. When large lecithin particles were incubated in the presence of calcium chloride and a high concentration of phospholipase D at pH 5.8, a slow release of choline was observed, the rate of which increased with time (Fig. 2). If the lecithin preparation was treated with ultrasonics to give a water-clear colloid (Saunders, Perrin & Gammack, 1962), the initial rate of the reaction was very much faster although it again increased as the hydrolysis proceeded (Fig. 2). This increase in the rate of

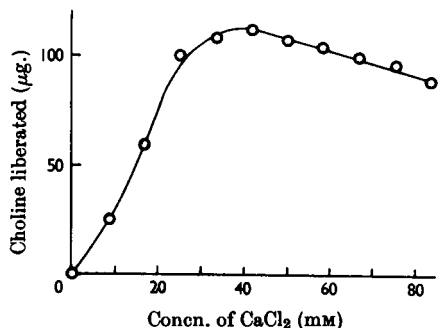


Fig. 3. Effect of Ca^{2+} on the hydrolysis of ultrasonically treated lecithin by phospholipase D at pH 5.8.

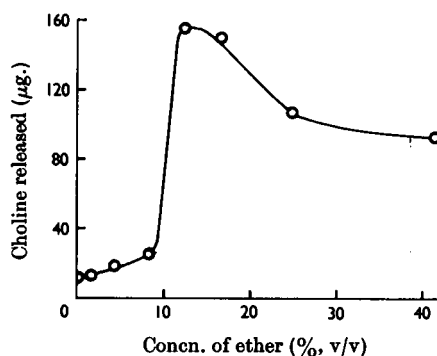


Fig. 4. Effect of ether on the hydrolysis of large lecithin particles by phospholipase D at pH 5.4. Ether concentration represents the volume of ether added to 1.2 ml. of the incubation medium in a stoppered tube of 12 ml. total volume.

hydrolysis is probably due to the reactions being stimulated by the acidic phospholipids produced in the reaction (see the Discussion section).

Ca^{2+} was obligatory for the enzyme reaction. It could not be replaced by Mg^{2+} or low concentrations of cationic amphipathic substances, both of which can substitute for the Ca^{2+} requirement of phospholipase C (Bangham & Dawson, 1962). In fact, the addition of Mg^{2+} to an incubation medium containing Ca^{2+} produced an inhibition of the hydrolysis. Ba^{2+} and Sr^{2+} , over a range of concentrations, produced no activation of the enzyme (cf. Davidson & Long, 1958).

With ultrasonically treated lecithin as substrate the optimum Ca^{2+} concentration was 0.039 M between pH 5.4 and 5.8 (Fig. 3): at higher Ca^{2+} concentrations the rate of reaction became slower. The optimum was not affected by doubling the substrate concentration (cf. Davidson & Long, 1958); however, decreasing the pH of the buffer to 5.2 slightly increased the concentration required to produce optimum activity. At about the optimum concentration of Ca^{2+} (0.0375 M) the rate of reaction was linear with time; above the optimum (0.104 M) it was also linear, whereas at a low Ca^{2+} concentration (0.021 M) the rate of reaction tended to decrease somewhat with time. No lag phase was observed with any Ca^{2+} concentration.

Ether activated the hydrolysis of large lecithin particles by the enzyme at all pH values at which the enzyme was active. However, little activation occurred until the aqueous incubation medium was saturated with the solvent (about 9% by volume) and at just above this concentration the activity began to decrease again (Fig. 4). Ether (16%, v/v) also increased the hydrolysis rate of ultrasonically treated lecithin particles by the enzyme; the increase was, however, only twofold so that the absolute rate was of the same order as that of large lecithin particles activated with the same concentration of ether.

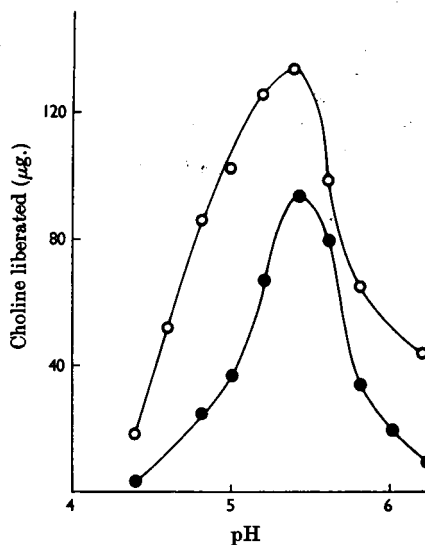


Fig. 5. Effect of pH on the hydrolysis of lecithin by phospholipase D. Activity was measured in sodium acetate-acetic acid buffers (0.04 M), except for pH 6 and above when NaOH - $\beta\beta'$ -dimethylglutaric acid buffers (0.04 M) were used. O, Large lecithin particles (5 μmoles); 16% (v/v) ether added, 0.054 ml. of acetone-precipitated enzyme. ●, Large lecithin particles (5 μmoles); 2.5 μmoles of sodium dodecyl sulphate; 0.02 ml. of acetone-precipitated enzyme.

The optimum pH of the enzyme was at 5.4 when it was attacking either an ultrasonically treated lecithin substrate or large lecithin particles in a system activated by ether or dodecyl sulphate (Fig. 5).

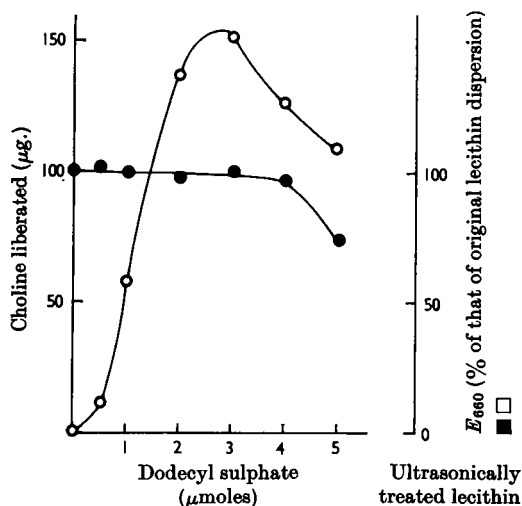


Fig. 6. Effect of sodium dodecyl sulphate on the extinction of a lecithin suspension and its hydrolysis by phospholipase D. The incubation medium contained $5\mu\text{moles}$ of egg-lecithin and CaCl_2 (0.0375M) was present. ○, Choline liberated; ●, E_{660} of the lipid suspension before incubation as a percentage of that of the original lecithin dispersion. For comparison, the amounts of choline liberated when an ultrasonically treated lecithin preparation was hydrolysed by phospholipase D under the same conditions are given to the right of the Figure (□). The E_{660} of this system before its incubation is also given as a percentage of that of the medium containing the lecithin suspension without ultrasonic treatment (■).

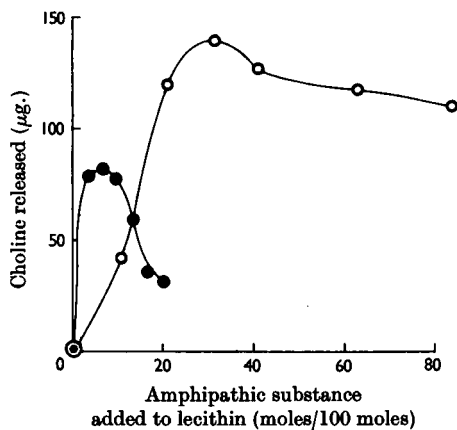


Fig. 7. Effect of adding two anionic amphipathic substances on the hydrolysis of lecithin by phospholipase D: ○, phosphatidic acid; ●, monocetylphosphoric acid.

Effect of anionic amphipathic substances. Certain anionic amphipathic substances produced a large stimulation of the hydrolysis of lecithin by phospholipase D, often considerably greater than that

produced by ether. Fig. 6 shows the effect at a very low enzyme concentration of adding sodium dodecyl sulphate to the incubation medium. The maximum amount of hydrolysis occurred when the dodecyl sulphate and lecithin substrate were about in the molar proportions 1:2; further addition of the detergent caused a fall in activity. The light-transmission of the lecithin suspension did not change appreciably on adding these amounts of dodecyl sulphate (Fig. 6). Presumably the dispersive effects that this detergent can have on lecithin particles (Dawson, 1963b) are largely nullified by the Ca^{2+} present in the incubation medium. Both at the concentration of dodecyl sulphate required to produce optimum activity and below this (lecithin/dodecyl sulphate molar ratio 5:1) the reaction was linear with time up to 20% substrate hydrolysis. Again Fig. 6 shows that the marked dispersion produced by ultrasonic treatment of the lecithin only stimulates the hydrolysis to a limited extent compared with the effect of the dodecyl sulphate. Further, the hydrolysis of ultrasonically treated lecithin itself was greatly accelerated by the addition of dodecyl sulphate, the rate being equivalent to that obtained with large lecithin particles and the same added molar percentage of dodecyl sulphate.

Fig. 7 shows the stimulatory effect of two other anionic amphipaths, phosphatidic acid and monocetylphosphoric acid, on the enzymic activity. It again indicates that the addition of too much activator causes a fall off in the rate of hydrolysis and emphasizes that the substrate/activator molar ratio required to produce optimum activity varies with each anionic amphipathic substance. As with dodecyl sulphate, turbidity measurements gave no evidence that these substances had produced an increased dispersion of the substrate in the presence of the Ca^{2+} required for enzymic activity.

A number of other anionic amphipaths were examined for their ability to activate phospholipase D. The relative activities of some of these at various enzyme concentrations are given in Table I, the choline release being that with the optimum amount of activator present. Phosphatidylserine had an activity similar to that of dicetylphosphoric acid. Cardiolipin, saponin, sodium deoxycholate, sodium taurocholate, phosphatidylethanolamine, and phosphatidylinositol did not stimulate the reaction.

Diphenyl phosphate, described by Davidson & Long (1958) as an activator of phospholipase D, did not stimulate the breakdown of large lecithin particles but large amounts (0.03M) did increase the breakdown of ultrasonically treated lecithin three- to five-fold. It is weakly surface-active and at these high concentrations it did produce a negative ζ -potential on lecithin particles. Deoxy-

Table 1. *Lecithin hydrolysis by phospholipase D with various anionic amphipathic activators*

Purified enzyme present (ml.)	Choline released ($\mu\text{g.}$) in 15 min.					
	No addition	+ Dicetyl-phosphoric acid	+ Triphospho-inositide	+ Monocetyl-phosphoric acid	+ Phosphatidic acid	+ Sodium dodecyl sulphate
0.033	0.5	5.6	28.3	49.2	91.5	132
0.066	0.5	7.1	61.4	76.4	163.0	160
0.133	2.7	47.3	115.7	157.5		

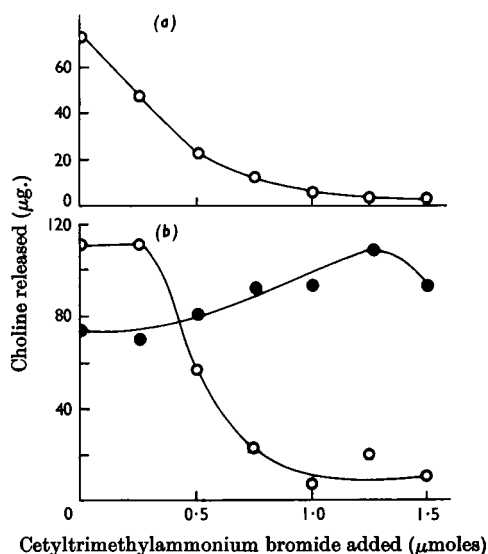


Fig. 8. Effect of cetyltrimethylammonium bromide on the hydrolysis of lecithin by phospholipase D. (a) Ultrasonically treated lecithin substrate ($5\mu\text{moles}$); (b) \circ , large lecithin particles ($5\mu\text{moles}$) with dodecyl sulphate ($2\mu\text{moles}$) added; \bullet , large lecithin particles ($5\mu\text{moles}$) with dodecyl sulphate ($5\mu\text{moles}$) added.

cholate, although without effect with large lecithin particles as substrate, did stimulate the hydrolysis of ultrasonically treated lecithin by about 30% at low concentration (lecithin/deoxycholate molar ratio 10:1); higher concentrations (molar ratio 5:2), however, completely inhibited the hydrolysis.

Effect of cationic amphipathic substances. Cationic amphipathic substances were, in general, potent inhibitors of phospholipase D action. Cetyltrimethylammonium bromide added to the standard assay system with ultrasonically treated lecithin as substrate caused inhibition of the hydrolysis that became complete with $1.4\mu\text{moles}$ (Fig. 8a). With large lecithin particles as substrate, together with sufficient dodecyl sulphate to produce approximately optimum breakdown, cetyltrimethylammonium bromide again inhibited (Fig. 8b).

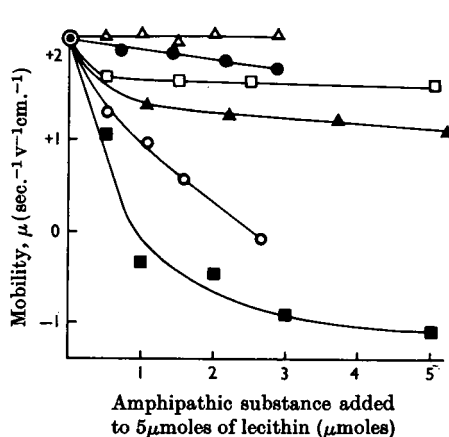


Fig. 9. Effect of amphipathic substances on the electrophoretic mobility of large lecithin particles in the presence of Ca^{2+} : Δ , monocetylphosphoric acid; \bullet , triphosphoinositide; \square , dicetylphosphoric acid; \blacktriangle , phosphatidic acid; \circ , phosphatidylinositol; \blacksquare , sodium dodecyl sulphate. The buffer was 0.0125M -sodium acetate-acetic acid, pH 5.6.

However, with greater amounts of dodecyl sulphate present ($5\mu\text{moles}$), such that the system was inhibited by the excess of anionic detergent (see Fig. 6), the addition of cetyltrimethylammonium bromide reversed the inhibition (Fig. 8b).

Stearylamine ($1\mu\text{mole}$) and palmitoylcholine ($1.5\mu\text{moles}$) also produced nearly complete inhibition of the enzymic hydrolysis of large lecithin particles ($5\mu\text{moles}$) in the presence of dodecyl sulphate ($2.5\mu\text{moles}$).

Effect of amphipathic activators and inhibitors on the electrophoretic mobility of lecithin particles. Since it has been shown that the ζ -potential of phospholipid particles is often a factor that determines the rate at which they are attacked by phospholipases (Bangham & Dawson, 1959; Dawson, 1964), measurements were made of the change of electrophoretic mobility of lecithin on adding anionic amphipathic substances in the same ionic environment as the enzymic incubation. Fig. 9 shows that the effect of the anionic amphipaths was very

Table 2. *Effect of anionic amphipathic substances on the electrophoretic mobility of lecithin particles (5 μ moles) in the presence and absence of Mg^{2+} and Ca^{2+}*

Anionic amphipathic substance added	Electrophoretic mobility, μ (sec. ⁻¹ v ⁻¹ cm. ⁻¹)		
	No bivalent ion	+0.037 M- $CaCl_2$	+0.037 M- $MgCl_2$
None	- 0.56	+1.98	+1.56
Sodium dodecyl sulphate (2.5 μ moles)	-13.1	-0.53	-0.55
Phosphatidic acid (1.5 μ moles)	-11.6	+1.22	+0.74
Monocetylphosphoric acid (1 μ mole)	- 8.1	+2.12	+1.43
Triphosphoinositide (0.57 μ mole)	- 7.52	+1.69	+1.43
Dicetylphosphoric acid (2 μ moles)	- 8.48	+1.58	+1.08
Phosphatidylinositol (1 μ mole)	-10.0	+1.35	-

variable. Lecithin particles on their own had a positive ζ -potential due to counter-ion-binding or salt formation or both with the Ca^{2+} present. The amphipaths usually produced a decrease in the positive ζ -potential but this decrease could in no way be correlated with their efficiency in activating the enzyme (Table 1). Thus, of the good activators, monocetylphosphoric acid and triphosphoinositide produced little change in the mobility of the lecithin substrate particles whereas phosphatidic acid gave some decrease and dodecyl sulphate a big decrease with charge reversal. On the other hand, dicetylphosphoric acid and phosphatidylinositol caused a decrease in the mobility well within this range but activated the enzyme very little or not at all.

Table 2 shows the electrophoretic mobility of lecithin particles with certain activating or non-activating amphipathic substances, the former tested at or near their optimum activating concentration. In the absence of bivalent metal ions, the amphipathic substances produce very negative ζ -potentials on the lecithin particles. Of the good activators, the efficiency of activation of the enzyme (Table 1) roughly correlates with this ζ -potential. However, phosphatidylinositol and dicetylphosphoric acid produce a negative ζ -potential at least as great as the analogous triphosphoinositide and monocetylphosphoric acid, yet they are poor activators of the enzyme. In addition, Table 2 compares the effect of Ca^{2+} on the mobility of the substrate particles with that of Mg^{2+} . The Mg^{2+} is almost as efficient at masking the high negative ζ -potential produced by the activators as Ca^{2+} and consequently the effect of these ions on the electrokinetic potential of the substrate particles cannot explain the specific necessity for Ca^{2+} in the enzymic reaction.

In the presence of Ca^{2+} , concentrations of cetyltrimethylammonium bromide that cause substantial inhibition of the enzyme barely altered the electrophoretic mobility of mixed lecithin-dodecyl sulphate particles (Fig. 10). Without Ca^{2+} a small

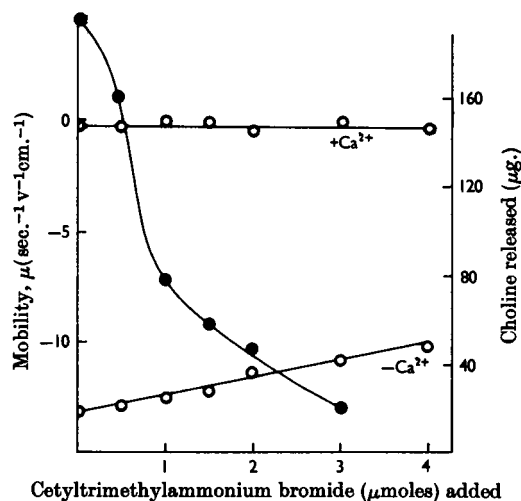


Fig. 10. Effect of cetyltrimethylammonium bromide on (○) the electrophoretic mobility of mixed lecithin (5 μ moles) and dodecyl sulphate (2.5 μ moles) particles and (●) their susceptibility to hydrolysis by phospholipase D.

reduction in the negative ζ -potential occurred (Fig. 10).

Adsorption of phospholipase D on lecithin substrate. When phospholipase D was mixed with lecithin and dodecyl sulphate in the absence of Ca^{2+} , little hydrolysis occurred when Ca^{2+} was subsequently added and the mixture incubated (Table 3). This presumably indicates that the protein had been denatured, because of its adsorption on the surface of the highly negatively charged substrate. Ca^{2+} protected the enzyme against denaturation when added to the lecithin-dodecyl sulphate mixture before the enzyme or simultaneously with it. This protective effect of Ca^{2+} was not specific and Mg^{2+} fulfilled the same function.

A loss of activity was also observed when lecithin in admixture with other activators (e.g. phosphatidic acid, triphosphoinositide, monocetyl-

phosphoric acid) was mixed with the enzyme 1 min. before adding Ca^{2+} . However, this deactivation was not nearly so substantial as observed with the lecithin-dodecyl sulphate mixture, presumably because of the less negatively charged surfaces produced by these phosphorus-containing amphipaths (Table 2).

Experiments were made to test whether enzyme was adsorbed on to the lecithin substrate in the presence of Ca^{2+} . The basic procedure was to mix the substrate at 0° with enzyme in the presence of calcium chloride and acetate buffer and then to spin down the lecithin by high-speed centrifugation and assay the sediment and the supernatant for enzyme activity. In a number of experiments it was found that, although little enzyme appeared in the sediment when lecithin alone was used, a considerable amount did so if the lecithin was mixed

with activating amounts of dodecyl sulphate (Table 4), phosphatidic acid or triphosphoinositide. Little activity appeared to have left the supernatant, but the small amount of enzyme adsorbed on to the lecithin-dodecyl sulphate was very effective in hydrolysing the lecithin when the sediment was incubated with calcium chloride and acetate buffer in the absence of additional enzyme (Table 4). The enzyme adsorbed was roughly proportional to the amount of enzyme added (Fig. 11): it was slowly lost from the sedimented material when this was repeatedly washed with the incubation media and recentrifuged (48% of the activity was lost after three washes). Experiments were made to test whether the enzyme was adsorbed on to the substrate in the presence of Mg^{2+} rather than Ca^{2+} . On recovering such particles by centrifuging and incubating them (after washing with Ca^{2+} -acetate buffer) in the presence of Ca^{2+} , hydrolysis of the lecithin occurred but this was usually slightly less than in equivalent experiments in which the enzyme had been adsorbed in the presence of Ca^{2+} .

Inhibitors of phospholipase D. As expected from its requirement for Ca^{2+} , the reaction was very sensitive to the presence of EDTA; 8.3 mM-EDTA completely inhibited the standard assay system with ultrasonically treated lecithin as substrate and 37.5 mM-calcium chloride. The bases, ethanolamine and choline, inhibited the hydrolysis of lecithin by phospholipase D at pH 5.4 and 5.8 both when ultrasonically treated lecithin and lecithin-dodecyl sulphate were the substrates (Fig. 12). Experiments were made with [Me^{14}C]choline to see whether the inhibition due to choline was due to the reversibility of the enzymic reaction and the mass-action effect of choline. After incubation of the enzyme with labelled choline and phosphatidic acid or with labelled choline and lecithin

Table 3. Denaturation of phospholipase D by mixtures of lecithin and dodecyl sulphate

Incubation tubes containing lecithin (5 μmoles) and sodium dodecyl sulphate (2.5 μmoles) were set up. Ca^{2+} was added at various times before or after the enzyme with the tube at room temperature. Immediately Ca^{2+} had been added, the tube was incubated at 27° to determine the enzymic activity.

Time elapsing between addition of enzyme and addition of Ca^{2+} (min.)	Choline released ($\mu\text{g.}$)
- 1 (Ca^{2+} before enzyme)	120.6
+ 1	9.5
+ 2	14.0
+ 4	9.2
+ 6	4.2
+ 8	3.5
+ 10	5.2

Table 4. Adsorption of enzyme on lecithin in the presence of Ca^{2+}

Lecithin (20 μmoles) in the presence and absence of 10 μmoles of dodecyl sulphate was shaken at 0° for 2-3 min. with 4.8 ml. of an incubation medium composed of 37.5 mM- CaCl_2 in 12.5 mM-sodium acetate-acetic acid buffer, pH 5.8, and 0.4 ml. of the purified enzyme solution. The mixtures were centrifuged (100 000 $g_{av.}$) at 0° for 30 min.: with lecithin alone, 87-88% of the lecithin was recovered as a loosely packed sediment; with lecithin and dodecyl sulphate, 93-94% of the lecithin was recovered as a tightly packed sediment. The precipitates and supernatants were assayed for enzymic activity as described in the Methods section, dodecyl sulphate, lecithin and CaCl_2 being added where appropriate. N.M., Not measured.

Lipid mixture	Enzyme activity ($\mu\text{g.}$ of choline released)			
	Lecithin		Lecithin + dodecyl sulphate	
	Supernatant	Sediment	Supernatant	Sediment
Expt. no. 1	104	9.5	96	290
2	108	1	102	92
3	N.M.	2	N.M.	111

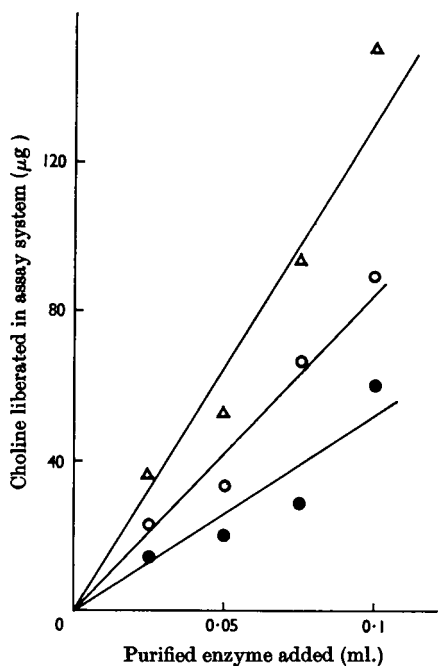


Fig. 11. Effect of enzyme concentration on the adsorption of enzyme on lecithin-dodecyl sulphate in the presence of Ca^{2+} . Lecithin ($5\ \mu\text{moles}$) and dodecyl sulphate ($2.5\ \mu\text{moles}$) were suspended in CaCl_2 -acetate buffer at 0° and treated with various amounts of enzyme as described in the legend to Table 5. After centrifuging, enzyme was assayed in the sediment and in the supernatant: \circ , enzyme in sediment of lecithin-dodecyl sulphate; \bullet , enzyme in supernatant; Δ , total activity recovered.

+phosphatidic acid, the lecithin reisolated from the reaction mixture showed a minimum of radioactivity. The amount of choline incorporated was insufficient to account for its inhibitory action on the enzymic reaction. The inhibition due to ethanolamine could be partially reversed by increasing the concentration of Ca^{2+} in the incubation medium.

The hydrolysis of lecithin suspension in the presence of dodecyl sulphate by purified phospholipase D was very sensitive to the presence of protamine sulphate: $0.8\ \text{mg./ml.}$ in the standard assay system produced complete inhibition, and $0.1\ \text{mg./ml.}$ inhibited the reaction by about 90%. At the lower concentration the electrophoretic mobility of the substrate particles was scarcely affected, but both in the presence and absence of Ca^{2+} the higher concentration of protamine caused the ζ -potential to become more positive. The hydrolysis of ultrasonically treated lecithin was also strongly inhibited by the addition of protamine sulphate.

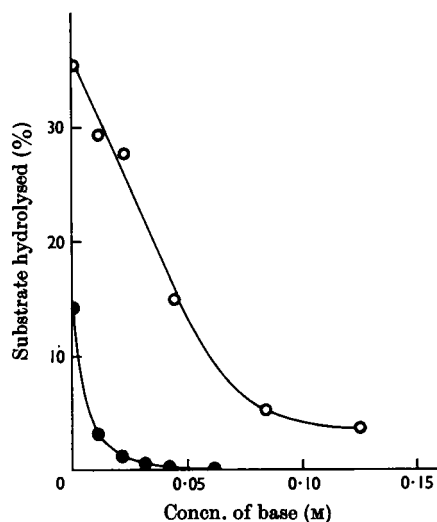


Fig. 12. Inhibition of phospholipase D by choline and ethanolamine. The substrate was lecithin ($5\ \mu\text{moles}$) + dodecyl sulphate ($2.5\ \mu\text{moles}$). Expt. 1 (\circ): choline ($0.029\ \text{ml.}$ of enzyme; 20 min. incubation time; release of lipid products determined). Expt. 2 (\bullet): ethanolamine ($0.022\ \text{ml.}$ of enzyme; 15 min. incubation time; release of choline determined).

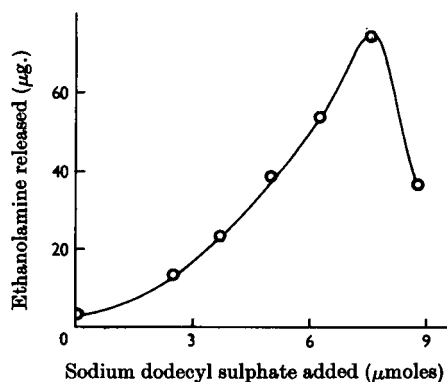


Fig. 13. Effect of sodium dodecyl sulphate on the hydrolysis of phosphatidylethanolamine by purified phospholipase D. Phosphatidylethanolamine, $5\ \mu\text{moles}$. Incubation time 30 min., enzyme $0.13\ \text{ml.}$; otherwise experimental details were as for standard assay.

Hydrolysis of phosphatidylethanolamine by phospholipase D. Under the standard assay conditions used for determining the hydrolysis of a lecithin substrate, ethanolamine was liberated from phosphatidylethanolamine particles by the purified enzyme at a rate that was much slower than the equivalent lecithin breakdown. Ca^{2+} was again

essential for the hydrolysis and it was stimulated by the addition of dodecyl sulphate (Fig. 13). However, the maximum activity occurred with considerably higher concentrations of the detergent than was found necessary for the equivalent hydrolysis of lecithin. This might be due to the prevention of the coalescence of the substrate particles with the detergent micelles due to the negative charge present on the phosphatidyl-ethanolamine even at low pH values (Dawson, 1963a). Ultrasonically treated phosphatidyl-ethanolamine was also slowly attacked by the enzyme, but a lower Ca^{2+} concentration was required for optimum activity than with ultrasonically treated lecithin. This is probably due to the action of this bivalent ion in causing precipitation of the ultrasonically treated phosphatidyl-ethanolamine substrate. Ultrasonically treated lecithin was not affected in this way.

DISCUSSION

The present investigation has confirmed the large stimulatory effect of diethyl ether on the hydrolysis of lecithin by phospholipase D as described by Davidson & Long (1958) for the soluble cabbage enzyme and by Kates (1954) for the insoluble plastid enzyme. Maximum stimulation was observed at about the point when sufficient ether had been added to form a two-phase system and is thus very similar to the stimulation of purified venom phospholipase A by ether (Dawson, 1963a).

The stimulation of hydrolysis produced by certain anionic amphiphathic substances, and in particular dodecyl sulphate, was much greater than that produced by ether. This is in contrast with the results of Kates (1957) with the insoluble plastid enzyme where dodecyl sulphate was inferior to diethyl ether as an activator. The most potent anionic amphiphathic activators found in the series tested were dodecyl sulphate and three compounds containing one or more phosphomonoester groups, namely monocetylphosphoric acid, phosphatidic acid and triphosphoinositide. Other substances, including naturally occurring acidic phospholipids with only phosphodiester groups, were much less effective. In contrast, cationic amphiphathic substances were potent inhibitors of the enzymic reaction, both when the enzyme was attacking ultrasonically treated lecithin and in a system activated by dodecyl sulphate.

No evidence could be obtained from the turbidity of the suspensions that the anionic amphiphaths were producing an activation through an increased dispersion of the substrate. The large concentration of Ca^{2+} that was necessary for maximum enzymic activity effectively prevented the activators from dispersing the substrate by a detergent-like action.

In fact, although dispersing the lecithin substrate by ultrasonics increased the rate of enzyme attack this was still very much less than that found in the presence of the effective anionic amphiphathic activators.

The activation produced by anionic amphiphathic substances and the inhibition produced by cationic amphiphaths suggested a parallelism with the phospholipase B enzyme of *Penicillium notatum*, where the rate of deacylation is controlled by the ζ -potential of the lecithin (Bangham & Dawson, 1959). However, in the present case the activation appears to bear no relationship to the ζ -potential of the substrate particles. Although in the absence of Ca^{2+} the activators produced a large negative ζ -potential, in the incubation medium this effect was minimized or even nullified altogether by the Ca^{2+} present. Thus the addition of monocetylphosphoric acid to the system does not change the ζ -potential of the lecithin particles detectably, yet the hydrolysis was stimulated. Similarly, cetyltrimethylammonium bromide produced inhibition of this activated system well before any change in the ζ -potential could be observed. Moreover, monophosphoinositide and dietylphosphoric acid, although relatively ineffective as activators, produced as big a change in the ζ -potential as some of the effective activators, both in the absence and presence of Ca^{2+} .

The addition of an activator did promote the adsorption of the enzyme on the surface of a lecithin substrate. In the absence of Ca^{2+} this led to denaturation of the enzyme, presumably due to an unfolding of its protein chains on the highly negatively charged surface. With Ca^{2+} present, adsorption still occurred but the decrease of the ζ -potential protected the enzyme from denaturation. This adsorption of the enzyme is probably an essential prerequisite for the formation of an enzyme-substrate complex and may explain the mechanism of the activation by anionic amphiphaths. Thus, although limited adsorption probably occurs with pure lecithin, it would be greater on ultrasonically treated lecithin, because of the larger surface area, and very much greater when the highly negatively charged sulphate or phosphomonoester groups of the activators were introduced on to the surface to attract electrostatically the positive amino groups of the enzyme. The system can be compared with the model experiments of Frazer, Kaplan & Schulman (1955), who examined the adsorption of catalase on paraffin oil/water interfaces stabilized by the addition of a C_{21} anthracenesulphonate. With low concentrations of enzyme the catalase was completely adsorbed and denatured on the highly negatively charged surface. With higher concentrations, although complete adsorption still occurred, the layer of unfolded protein produced initially seemed to

protect the additional enzyme from complete unfolding. Thus a certain proportion of the active enzyme could be released by changing the pH or by adding dodecyltrimethylammonium bromide. In the present system the phospholipase D was protected from denaturation by the Ca^{2+} present, which presumably minimized the interaction between the positive groups on the protein and the negative groups on the substrate and prevented a complete unfolding of the enzyme molecule.

Matalon & Schulman (1949) have shown that the relative signs and magnitudes of the electrical charges are important factors for promoting the adsorption of blood proteins on to unimolecular lipid films. In the present experiments the enzyme is only adsorbed on to lecithin whose surface potential has been made very negative by the introduction of strongly anionic amphipathic substances. Owing to the masking effect of the high concentration of Ca^{2+} present, the ζ -potential of such surfaces is slightly positive, as is that of a pure lecithin surface in the presence of calcium. This suggests therefore that the enzyme must have a marked affinity for the negative charges on the substrate's surface so it can compete with the large number of Ca^{2+} ions present. Presumably the dramatic inhibition produced by protamine is due to this basic protein, which would be very positively charged at the pH used in the enzyme assay, successfully competing with the enzyme for the negative sites on the substrate's surface.

There is nothing in the observations to explain the specific requirement for Ca^{2+} in the hydrolysis. If the only role of Ca^{2+} was in the initial attraction between substrate and enzyme, one might expect that Mg^{2+} or cationic amphipathic substances would substitute for it as they do in the phospholipase C hydrolysis (Bangham & Dawson, 1962). An equivalent concentration of magnesium chloride has almost the same masking effect on the negative ζ -potential of the activated substrate as Ca^{2+} . Consequently, it is not surprising that Mg^{2+} is as effective at preventing denaturation of the enzyme on the negatively charged substrate. Moreover, reversible adsorption of the enzyme occurs almost as well in the presence of Mg^{2+} as with Ca^{2+} . Presumably this latter ion must play an essential subsequent role, either in the formation of the enzyme-substrate complex or its hydrolysis.

The enzyme is unique in that one of the products of hydrolysis activates the reaction whereas the other can inhibit. The main lipid product was

phosphatidic acid, although a small percentage of phosphatidylglycerol was formed by a transferase reaction with the glycerol present in the enzyme preparation (R. M. C. Dawson & N. Hemington, unpublished work). Phosphatidic acid is, of course, an activator of the reaction and this is probably why the hydrolysis rate increased with time. The inhibition of the enzyme produced by the bases, choline and ethanolamine, would not be of importance in the present enzyme assays since fairly high concentrations of base are needed before the inhibition becomes substantial. The mechanism of the inhibition is not understood, although the fact that it can be partially reversed by increasing the Ca^{2+} concentration could indicate that the base is competing with this bivalent ion for negative charges at the site of the enzymic reaction.

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