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The Relation between the Activity of a Lecithinase and the Electrophoretic Charge of the Substrate

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Fairbairn (1948) showed that the mycelium of the mould *Penicillium notatum* was a rich source of the enzyme phospholipase B which hydrolysed lysophosphoglycerides but not the parent diacylated compounds, e.g. lecithin, phosphatidylethanolamine. Dawson (1957) observed that preparations of this enzyme, although unable to hydrolyse pure lecithin, were able to attack the lecithin of the mixed phospholipid fractions isolated from liver. It was shown further that this property was due to the presence in the fractions of small quantities of monophosphoinositide and a polyglycerol phospholipid (Dawson, 1958a). When either of these substances was added to pure lecithin, the phospholipase B enzyme preparation rapidly hydrolysed the substrate with the formation of fatty acids and glycerylphosphorylcholine.

An examination of other lipid and non-lipid substances showed that only cardiolipin, a phospholipid structurally related to liver polyglycerol phospholipid, and the triglycerides tristearin and tripalmitin produced any appreciable activation of the enzymic attack upon lecithin. It was also observed that the addition of either monophosphoinositide or cardiolipin to lecithin caused a marked reduction in the extent to which the lecithin was extracted from its aqueous emulsion by organic solvents, e.g. ether and chloroform. This reduction in extractability of the lecithin ran

closely parallel to the degree of activation of the enzyme system and, moreover, it was found that both the extractability change and the activation could be reversed by the addition of Ca^{2+} ions. It was therefore suggested that the activation was connected with some alteration in the surface properties of the lecithin particles (Dawson, 1958b). It was thought that the most likely change would be found in the ionizable groups contributing to the electric double layer between lecithin particles and the bulk aqueous phase. It was decided therefore to examine the particles by microelectrophoresis to determine the sign and magnitude of their mobilities, a measure directly related to their ζ potentials.

Preliminary results showed that the naturally occurring activators (monophosphoinositide and cardiolipin), when added to pure lecithin, produced a net negative charge on the surface of the mixed lipid when emulsified in buffer at the pH optimum of the enzyme. A further study of this phenomenon showed that other anionic amphipathic molecules (substances having a hydrophobic portion and a polar group: *amphi*, dual; *pathic*, sympathy; Hartley, 1936) could be used as activators, but that always a minimum net charge was a prerequisite for the development of enzymic attack on the emulsified substrate irrespective of the anionic species tested. The inhibitory effect of various

multivalent-metal cations has been tested. The inhibitory effect of these multivalent-metal cations has been explained on the basis of counter-ion binding to the negative groups of the substrate essential for enzymic attack. A brief description of these findings has already been published (Bangham & Dawson, 1958).

EXPERIMENTAL

Preparation of emulsions. The lecithin was stored at -15° in chloroform solution. The activating amphipathic substances studied were usually dissolved in an appropriate organic solvent, e.g. chloroform, and mixed with the requisite amount of lecithin solution. The solvent was removed *in vacuo* and the lipid mixture emulsified by shaking at 37° with 0.02M-sodium formate-formic acid buffer, pH 3.3. In studying the activation produced by sodium dodecyl sulphate it was not found possible to obtain solutions in organic solvents of sufficient concentration; consequently this substance was dissolved in the formate buffer before emulsification of the lecithin. Sodium hexadecyl sulphate was also added to the aqueous phase.

Samples of the various emulsions were used to determine (a) the enzymic activity after the addition of a 20% volume of the enzyme preparation, (b) the electrophoretic mobility of the emulsion particles after the addition of either a 20% volume of water or of enzyme preparation. The addition of water ensured that the measurements were made at the same final ionic strength as those made in the presence of the dialysed enzyme.

Enzyme determinations. The preparation of the enzyme extract from *P. notatum* mycelium has been described by Dawson (1958a). No glycerol was used to stabilize the enzyme but it was stored frozen at -15° . The breakdown of lecithin by the enzyme preparation was followed by determining the release of water-soluble glycerylphosphorylcholine (Dawson, 1956).

Electrophoretic mobilities of the substrate particles. Mobilities were measured by direct observation of the particles in a horizontal electrophoresis cell mounted in a water bath at 37° . The apparatus used was of the type described by Bangham, Flemans, Heard & Seaman (1958).

The velocities of the emulsion particles in the d.c. electric field are expressed in terms of $\mu/\text{sec.}/\text{v}/\text{cm.}$ Electrophoretic mobilities can be converted into ζ potentials by using the Helmholtz-Smoluchowski equation, $\zeta = 4\eta\pi\bar{V}/\epsilon$, where η and ϵ are the viscosity and the dielectric constants respectively of the suspending medium and $\bar{V}$ is the electrophoretic mobility in $\mu/\text{sec.}/\text{v}/\text{cm.}$ To avoid excessive polarization of the electrodes and undesirable thermal effects in the electrophoresis cell, the current passed was limited to 0.5 ma. A potential of 90 v across a 14 cm. tube with a bore of 2 mm. was suitable. With this apparatus, measurements could be made within 45 sec. after the addition of the enzyme to the substrate emulsion and on as little as 1 ml. of material.

Electrophoresis of the enzyme. Initial experiments showed that the lysolecithinase activity of the enzyme preparation was lost after application to acid-washed filter paper before conventional paper electrophoresis. This inactivation was largely overcome by impregnating the filter paper with

lecithin. Impregnation was achieved by dipping the filter paper in a solution containing 3.5 mg. of lecithin/ml. of chloroform and drying. Before electrophoresis the treated paper was wetted with the appropriate buffer by spraying. Parallel strips were run with inositol markers to correct for endosmosis, the location of the inositol being determined by spraying with the silver nitrate-alkali reagents of Trevelyan, Procter & Harrison (1950). The position of the lysolecithinase was determined by cutting strips 1 cm. wide and incubating them individually with lysolecithin solution and estimating the release of glycerylphosphorylcholine.

The electrophoresis of the enzyme preparation on starch gel was performed according to Smithies (1955), with either a 2 mM-sodium formate-formic acid buffer, pH 3.3, or a 2 mM-sodium acetate-acetic acid buffer, pH 4.4. Identification of the fractions was made by staining part of the gel with naphthalene black, the proteins being eluted from strips (2.5 mm. wide) of the remainder by the Smithies technique. Yields of activity recovered were low but were sufficient for the present purposes.

Materials. Ovolecithin was isolated from mixed egg phospholipids by chromatography on alumina and silicic acid columns, and monophosphoinositide from guinea-pig liver phospholipids (Dawson, 1958a). Cardiolipin was given by Dr M. G. Macfarlane. A sample of sodium hexadecyl sulphate and the palmitoyl- and stearoyl-anisidine sulphates were gifts from Professor N. K. Adam, F.R.S.; sodium taurocholate was likewise a gift from Professor Haslewood; dicetylphosphoric acid was obtained from Albright and Wilson (Mfg) Ltd.; all the reagents were of A.R. grade and the water was from a Pyrex still.

The formate buffer (pH 3.3) was prepared by mixing 63 ml. of 0.02M-formic acid and 37 ml. of 0.02M-sodium formate solution.

RESULTS

Effect of monophosphoinositide and cardiolipin on electrophoretic mobility of lecithin emulsions. Initial observations of the mobilities of pure lecithin emulsified in 0.145M-sodium chloride at various pH

Fig. 1. pH-mobility curves for lecithin (O) and lecithin plus monophosphoinositide (●) emulsions. The lipids were emulsified in 0.145M-NaCl, the pH was adjusted by addition of 0.145M-HCl and 0.145M-NaOH and mobilities were measured at 25° . The phospholipid mixture contained 8.2 moles of monophosphoinositide/100 moles of lecithin.

values between 1.0 and 13.0 showed that the isoelectric span ranged from pH 3.0 to 13.0. This result was to be expected since the lecithin molecule contains both strong acid and basic groups. On the other hand, when the lecithin was mixed with monophosphoinositide (100 moles:8.2 moles respectively), the particles showed a plateau of a net negative mobility between pH 3.5 and 12.0 (Fig. 1). This result indicates that the unesterified ionizable group of the monophosphoinositide appear on the surface of the lecithin micelle. It seemed possible that there was a connexion between the presence of such a surplus of negative groups and the ability of the enzyme to attack the lecithin. In support of this it was found that cardiolipin, another activator of the enzyme system, likewise produced a net negative charge on the emulsion surface. It was therefore expected that any negatively charged amphipathic molecule might confer on a lecithin micelle a net negative charge and make it susceptible to enzyme attack.

Activation of the enzyme system with synthetic amphipathic molecules. So far the following compounds have been investigated and found both to activate the enzyme system and to produce particles having a net negative mobility at pH 3.3: dicetylphosphoric acid, sodium dodecyl sulphate, sodium taurocholate, sodium 1-stearoyl-4-anisidine-2-sulphonate, sodium 1-palmitoyl-4-phenetidine-2-sulphonate, sodium 1-palmitoyl-4-anisidine-2-sulphonate and sodium hexadecyl sulphate.

Fig. 2 shows the effect of adding increasing amounts of dicetylphosphoric acid to lecithin on both the electrophoretic mobility and the extent to which the lecithin is hydrolysed. As was previously shown for cardiolipin and monophosphoinositide (Dawson, 1958*b*), the enzyme activity does not commence until a certain critical concentration of the activator has been reached. Lecithin breakdown then rapidly reaches a maximum and declines as further dicetylphosphoric acid is added. The electrophoretic mobility of pure lecithin particles in formate buffer at pH 3.3 is net positive. This contrasts with the apparent lack of electrophoretic charge of the particles in 0.145*M*-NaCl (Fig. 1),

where the positive charge has been neutralized by much higher concentration of counter Cl^- ions. On adding activator to lecithin particles, the charge rapidly changes its sign and the particles then show a steadily increasing negative charge, ultimately reaching one identical with that for pure dicetylphosphoric acid. In the absence of enzyme there is no apparent point of inflexion in the curve at the

Fig. 2. Electrophoretic mobilities and the degree of enzymic hydrolysis on the addition of dicetylphosphoric acid to lecithin. The mixed lipid containing 4.2 μ moles of lecithin was emulsified in 4.5 ml. of 0.02*M*-sodium formate-formic acid buffer, pH 3.3. A sample (1.5 ml.) was incubated with 0.3 ml. of enzyme for 30 min. at 37°. Electrophoretic mobilities were measured on the remainder of the emulsion.

Table 1. *Minimum concentration of various amphipathic substances necessary to activate enzymic hydrolysis of lecithin, and the electrophoretic mobilities of such mixtures*

Activator	Anion species	Critical activation mobility (μ /sec./v/cm.)		Critical activation molarity (molar % of lecithin)
		With enzyme	Without enzyme	
Dicetylphosphoric acid	PO_4^-	-0.90	-1.6	8.5
Cardiolipin	PO_4^-	-1.00	-1.9	5.5
Monophosphoinositide	PO_4^-	-1.2	-1.4	6.0
Sodium 1-palmitoyl-4-anisidine-2-sulphonate	SO_3^-	-1.05	-1.6	36.0
Sodium dodecyl sulphate	SO_4^-	-1.10	-1.6	0.0011 % in bulk aqueous phase
Sodium hexadecyl sulphate	SO_4^-	-1.10	-1.7	0.00065 % in bulk aqueous phase

concentration of activator at which hydrolysis begins. The presence of enzyme reduces the negative charge of the particles, especially above those concentrations of activator which support enzymic hydrolysis. It is not known at present whether this reduction is due to the interaction of enzyme with substrate or the adsorption of non-specific protein contaminating the enzyme preparation.

Table 1 shows that in a 0.02M-formate buffer system, activation of the enzyme commenced at an approximately constant electrophoretic mobility (critical activation mobility) irrespective of the type of anion group attached to the amphipathic molecule mixed with the lecithin. The reduction of the charge on the particles in the presence of the enzyme results in the critical activation mobilities being less in the presence of the enzyme than in its absence. With occasional enzyme preparations this difference is very marked, a result which may be due to excess of contamination with protein which is positively charged at pH 3.3. If the preparation

is purified by ammonium sulphate fractionation this discrepancy disappears.

When sodium dodecyl sulphate was used as activator, it was observed that above the concentration producing maximum hydrolysis, the activity rapidly disappeared. The same loss of activity was also apparent if lysolecithin was used as substrate; this suggests that the sodium dodecyl sulphate is causing inhibition by denaturation and explains why previous attempts to obtain activation with a high concentration of sodium dodecyl sulphate (0.04%) in the aqueous phase were unsuccessful (Dawson, 1958b).

Physicochemical mechanisms which alter the activated lecithin surface and inhibit the enzyme. The results reported in Table 1 suggest that the enzymic hydrolysis of lecithin was dependent on a critical negative charge at the plane of shear of the particle, and it was expected therefore that any physicochemical mechanism that could reduce the magnitude of this charge would inhibit the enzyme reaction. Two such approaches have been made: the first consisted in adding a counter cation to the bulk aqueous phase, and the second in matching the negatively charged activator molecule by the

Fig. 3. Effect of the addition of UO_2^{2+} ions on the electrophoretic mobility and enzymic hydrolysis of lecithin plus dicetylphosphoric acid particles. Lecithin ($8.4 \mu\text{moles}$) plus dicetylphosphoric acid ($2.4 \mu\text{moles}$) was emulsified in 8.5 ml. of 0.02M-sodium formate-formic acid buffer, pH 3.3, containing increasing amounts of $\text{UO}_2(\text{NO}_3)_2$. Samples (1.5 ml.) were incubated with 0.3 ml. of enzyme at 37° for 20 min. Electrophoretic mobilities were measured on the remainder of the emulsion.

Fig. 4. Effect of the addition of Th^{4+} ions on the electrophoretic mobility and enzymic hydrolysis of lecithin plus dicetylphosphoric acid particles. Experimental conditions were the same as described for Fig. 3 except that $\text{Th}(\text{NO}_3)_4$ was added to the system.

addition of a positive amphipathic molecule. Figs. 3–5 illustrate the effect of varying the concentration of a counter ion, e.g. UO_2^{2+} , Th^{4+} or Ca^{2+} , on both the electrophoretic mobility and the enzymic hydrolysis of a lecithin emulsion activated with dicetylphosphoric acid.

Each type of counter ion completely inhibited the hydrolysis at a concentration just sufficient to reduce the net negative mobility to zero, although the actual concentration at which this occurred varied widely with each cation (UO_2^{2+} , 0.19 mN; Th^{4+} , 2.2 mN; Ca^{2+} , 7.0 mN). With both UO_2^{2+} and Th^{4+} ions it was consistently observed that low concentrations caused a slight stimulation of the hydrolysis. This stimulated activity changed to complete inhibition over a very narrow concentration range, e.g. for Th^{4+} , 1.5–2.2 mN.

Fig. 6 shows the effect on both the enzymic activity and the electrophoretic mobility of adding increasing concentrations of cetyltrimethylammonium bromide to a lecithin–dicetylphosphoric acid mixture. Here inhibition of hydrolysis was complete at approximately the critical activation mobility.

Electrophoresis of Penicillium notatum phospholipase B. When the enzyme preparation was subjected to electrophoresis at pH 3.3 on paper impregnated with lecithin, that portion of the

proteins which hydrolysed lysolecithin remained at the point of application. As endosmosis on this treated paper was negligible, this result indicates that the enzyme is isoelectric at the pH for optimum hydrolysis of lecithin, i.e. pH 3.3.

After electrophoresis of the crude enzyme preparation on starch gel at pH 4.4, staining revealed the presence of at least 14 protein bands. On elution of the bands the activity towards a lysolecithin substrate was found to lie in a minor negatively charged protein band. The activity towards a lecithin plus dicetylphosphoric acid substrate resided in the identical band, which is good evidence for believing that phospholipase B is responsible for both lecithinase and lysolecithinase activity. Electrophoresis on starch gel at pH 3.3 confirmed that the phospholipase B activity was isoelectric at this pH.

Fig. 5. Effect of the addition of Ca^{2+} ions on the electrophoretic mobility and enzymic hydrolysis of lecithin plus dicetylphosphoric acid particles. Experimental conditions were the same as described for Fig. 3 except that CaCl_2 was added to the system.

Fig. 6. Electrophoretic mobility and inhibition of enzymic hydrolysis upon the addition of cetyltrimethylammonium bromide to lecithin plus dicetylphosphoric acid particles. A mixture of lecithin (8.4 μ moles) plus dicetylphosphoric acid (2.4 μ moles) and varying amounts of cetyltrimethylammonium bromide was emulsified in 0.02M-sodium formate–formic acid buffer, pH 3.3. Enzyme activity and electrophoretic mobility were determined as described for Fig. 3.

DISCUSSION

The present enzyme preparation, which possesses powerful phospholipase B activity, is clearly not pure. The end result of the activated lecithin breakdown is the removal of both fatty acids which is equivalent to saying that the system shows both phospholipase A and B activity. As emphasized before (Dawson, 1958*b*), no concrete assumption can be made that phospholipase B is responsible for the hydrolysis of both acyl fatty acid bonds in lecithin, although this is suggested by certain evidence such as the identical pH optima and behaviour of inhibitors when the enzyme preparation is attacking both substrates. The observation that after starch-gel electrophoresis (with its high resolving power of protein mixtures) the activities towards lysolecithin and lecithin substrates lie in the same protein band provides more certain evidence that the B enzyme is responsible for attacking both substrates.

The present results clearly show that a minimum net negative electrophoretic charge is necessary before a lecithin particle is attacked by the phospholipase B preparation. It must be emphasized that the electrophoretic charge (ζ potential) does not represent the actual charge on the surface of the particle or micelle, as each will move in the electrical field with an associated cloud of counter ions which are in equilibrium with those present in the bulk aqueous phase. The actual mobility will therefore depend on the charge existing at the plane of shear between the particles and the solution, i.e. the algebraic sum of the surface charge and those counter ions within the plane of shear. It is also apparent from our results that the negative charge required for activation can be produced by addition to the lecithin surface of a number of differing anionic groups, e.g. phosphate, sulphate, sulphonate. On the other hand, little activation was observed on the addition of amphipathic molecules with carboxylic acid groups, e.g. fatty acids (Dawson, 1958*b*). This exception can be explained by the fact that at the low pH (3.3) at which the enzyme functions, most of the carboxyl groups would be un-ionized. It also should be noted in this connexion that the apparent pK of a fatty acid at an interface is higher than when it is in free solution (Peters & Wakelin, 1938). Stearic acid particles have an observed pK 4.8 (Bangham, Pethica & Seaman, 1958), which reduces to 4.2 when correction is made for its orientation at an interface (Verwey & Overbeek, 1948).

The question arises whether the necessity for a minimum net negative charge on the lecithin substrate is merely due to a more efficient emulsification of the lecithin, or to some specific action of the charge in orientating the active group of the enzyme

or the surface of the substrate or both. Evidence is presented in a later paper which favours such a theory of orientation, and this conception will be used to discuss the present results.

In the experiments in which the enzymic activity was inhibited by the addition of counter metal ions (Ca^{2+} , UO_2^{2+} , Th^{4+}) it is apparent that inhibition is complete only at zero mobility of the particles. This contrasts with the minimum mobility necessary for the hydrolysis to begin in systems not containing inhibitor or when inhibition is brought about by the addition of positive groups to the micelle surface. One explanation of this difference is that cations added to the bulk aqueous phase may bind to anionic sites on the enzyme as well as to the anionic sites on the mixed lecithin-dicetylphosphoric acid surface. This could well be maintaining a potential differential between the enzyme becoming more positive, and the lecithin surface becoming less negative, so that loss of orientation and enzyme activity would then occur only when the particles lose all effective negative charges. This conception might also explain why low concentrations of Th^{4+} and UO_2^{2+} ions actually stimulate the system. Thus it is possible that with low concentrations of these ions, preferential binding occurs on the anion sites on the enzyme molecule, which being isoelectric at pH 3.3 would gain a net positive charge. At first sight, this is contradictory to the evidence that UO_2^{2+} ion binds phosphate groups more effectively than carboxyl groups (Kruyt, 1949), but it must be remembered that although at this pH most of the carboxyl groups are un-ionized, the total number of ionized carboxyl groups available from the soluble enzyme would be considerably greater than the number of phosphate groups in the surface of the lecithin particle.

When cetyltrimethylammonium bromide is added to lecithin-dicetylphosphoric acid mixtures, complete inhibition of enzymic attack on an emulsion of the mixed lipids occurs when the particles still have a net negative mobility, which is very close to the critical activation mobility required for lecithin-activator systems. This could be due to the fact that, in this instance, the inhibitor cation is present only in the particle surface and is not available in the bulk phase to bind with the enzyme.

There are a limited number of examples reported in the literature where surface-active agents have been used to increase the rate of an enzymic reaction. For example, it has been known for some years that the rate of hydrolysis of triglycerides by pancreatic lipase can be stimulated by the addition of bile salts. Recently, Wills (1955) has shown that cationic and to a limited extent anionic and non-ionic detergents increase the velocity of pancreatic

lipase action. Similarly, Kates (1957) has shown that anionic detergents stimulate the rate of hydrolysis of lecithin by the phospholipase D (C in American terminology) of chloroplasts. The present system, however, differs markedly from these previous instances in that the presence of the activator is an absolute prerequisite for the commencement of enzymic action.

It is likely that there may be other examples of phospholipases being activated by naturally occurring amphipathic substances. Epstein & Shapiro (1957) have recently shown that the lecithinase of intestinal mucosa can be activated by the addition of fatty acids to the substrate and inhibited by bivalent cations. It seems possible that as this enzyme is working at near neutral pH, the carboxyl groups would impart a negative charge on the lecithin particles and that this might play a part in the activation phenomena.

One significant fact emerging from the present study of metal-ion inhibition is the delicate poise achieved between the hyperactivation produced by low concentrations of cation and the very slightly higher concentrations which produce complete inhibition. It is not difficult to speculate how such a sensitive 'trigger' mechanism might be employed for the control of enzyme systems in the living cells, e.g. those of excitable tissues. Moreover, the principle shown by the present studies where the surface charge of the substrate controls the enzymic attack might equally well apply to the converse situation, that is, an enzyme on a surface and a soluble substrate in the bulk phase.

SUMMARY

1. Aqueous emulsions of pure lecithin and lecithin containing various lipids (monophosphoinositide, cardiolipin), known to activate the hydrolysis of lecithin by a phospholipase B preparation, have been examined electrophoretically.

2. Pure lecithin particles were electrophoretically net positive at the optimum pH of the enzyme, whereas the activating lipids conferred on them a net negative surface.

3. Other anionic amphipathic molecules (dicytylphosphoric acid, sodium dodecyl sulphate, sodium hexadecyl sulphate, sodium 1-palmitoyl-4-anisidine-2-sulphonate and sodium taurocholate) added to the lecithin were found to confer a net negative charge on its surface and also to activate the enzyme system.

4. In a buffer of constant ionic strength, the enzymic activity commenced at a constant ζ potential, independent of the species of anionic amphipathic molecule used to activate the system.

5. Inhibition of the enzyme system was produced by the addition to the bulk aqueous phase of various multivalent counter cations (Ca^{2+} , UO_2^{2+} , Th^{4+}) and was complete when the observed ζ potential became zero.

6. Addition of a cationic amphipathic molecule (cetyltrimethylammonium bromide) to the activated lecithin emulsion produced complete inhibition of the enzyme when the mobility of the emulsion particles fell below the critical activation value.

7. Electrophoresis of *Penicillium notatum* phospholipase B on paper and starch gel showed it was isoelectric at its pH optimum (3.3). After starch-gel electrophoresis, lysolecithinase and lecithinase activity resided in the same protein band, which provides additional evidence that these enzymes are identical.

8. It is concluded that a prerequisite for the attack of *P. notatum* phospholipase B on lecithin is that the substrate particles carry a minimum net negative ζ potential.

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