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The Physicochemical Requirements for the Action of *Penicillium notatum* Phospholipase B on Unimolecular Films of Lecithin

BY A. D. BANGHAM AND R. M. C. DAWSON
Agricultural Research Council, Institute of Animal Physiology, Babraham, Cambridge

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Previous work has shown that emulsions of lecithin are attacked by the phospholipase B of *Penicillium notatum* only when they are activated by certain other lipids such as monophosphoinositide or cardiolipin (Dawson, 1958*a*). It was found that such activators do not operate as typical coenzymes but rather as substances which produce distinct physical changes on the surface of the lecithin particles (Dawson, 1958*b*). A study of the system by electrophoresis has indicated that the phospholipase activity can be correlated with the presence on the lecithin particles of a net negative surface (Bangham & Dawson, 1959*a*). The enzyme activity occurred only when the net negative potential of the lipid particles had reached a certain critical value. This could be obtained not only by the introduction into the lecithin of naturally occurring lipids such as monophosphoinositide but also by the use of synthetic anionic amphipathic molecules (substances having a hydrophobic portion and a polar group), e.g. dicetylphosphoric acid, hexadecyl sulphate.

The enzyme system has also been studied by using surface films of [^{32}P]lecithin floating on a buffer and observing the loss of radioactive counts coming from the surface when enzyme was introduced into the buffer (Dawson & Bangham, 1959). Here again, enzyme activity occurred only when the surface film of lecithin contained a certain minimum proportion of anionic amphipathic molecules. However, in these experiments the lecithin was added in much greater quantities than was necessary to form a unimolecular film, and consequently the excess existed in the form of 'rafts' or liquid crystals presumably separated by a monolayer at its collapse pressure (Adam, 1941).

In the present experiments the enzyme system has been studied by using unimolecular films of [^{32}P]lecithin of high specific activity spread on a Langmuir-type trough with arrangements for continuously recording not only the loss of surface counts (enzyme activity) but also the pressure of the film and the surface potential. With these techniques it has been shown that at low-surface pressures the enzyme can hydrolyse a pure lecithin surface which possesses no apparent surface charge. On increasing the pressure this activity first increases and then at a pressure of about 30 dynes/cm. is sharply abolished. With high-pressure films it can be obtained only if negative groupings are introduced into the surface, e.g. by the addition of dicetylphosphoric acid.

EXPERIMENTAL

Preparation of [^{32}P]*lecithin.* The biosynthetic preparation of [^{32}P]lecithin with *Saccharomyces cerevisiae* previously described (Dawson & Bangham, 1959) has been modified to simplify the procedure for the purification of the extracted yeast lecithin. The yeast was grown in 2 l. of the same nutrient medium with 5 mc of $^{32}PO_4^{3-}$ added. The yeast was harvested at the point when no inorganic P, as measured by the Fiske & Subbarow (1925) method, was left in the medium; Fig. 1 shows that this gives a maximum yield of yeast phospholipid. The extraction of the lipids from the yeast was as described in the previous method (Dawson & Bangham, 1959). The dehydrated lipids were thoroughly extracted with 100 ml. of diethyl ether, and after insoluble matter had been removed by filtration the filtrate was evaporated to dryness under reduced pressure

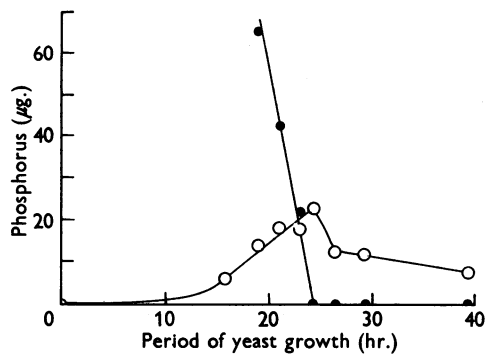

Fig. 1. Accumulation of phospholipid in bakers' yeast during growth in a medium containing limited inorganic phosphate. The phospholipid was extracted from the yeast cells with $CHCl_3$–methanol and purified by the procedure of Folch, Lees & Sloane-Stanley (1957). ○, Yeast phospholipid P in 40 ml. of medium; ●, inorganic P in 2 ml. of medium.

and the lipid residue extracted with 8 ml. of $CHCl_3$–methanol (1:1, v/v). The extract, together with any insoluble matter, was transferred to an activated alumina column (15 cm. long × 0·8 cm. diam.; Hopkins and Williams Ltd.). Solvent ($CHCl_3$–methanol; 1:1, v/v) was passed down the column; the lecithin emerged almost immediately and was detected by monitoring the effluent with a Geiger–Müller counter. Kephalins and insoluble matter remained on the column. The lecithin solution was evaporated to dryness *in vacuo* and chromatographed on a silicic acid column (10 cm. long × 0·8 cm. diam.; Mallinckrodt Ltd.) with $CHCl_3$–methanol (68:32, v/v). Fractions of 5 ml. were collected with an automatic fraction collector; the neutral lipids appeared almost immediately, and the lecithin, now free from lysolecithin, emerged in (approximately) tubes 18–33 with some tailing afterwards. Its position was easily located by monitoring the ^{32}P in the fractions. The resulting [^{32}P]lecithin was stored at −15° in $CHCl_3$. Analysis by a new hydrolytic method (Dawson, 1960) showed that the only phospholipid present was lecithin; choline plasmalogen could not be detected.

For use, a sample of the solution of [^{32}P]lecithin in $CHCl_3$ was evaporated to dryness *in vacuo* and redissolved in the appropriate volume of light petroleum (b.p. 60–80°) to give a solution containing about 1·2 μmoles/ml. Dicetylphosphoric acid (Albright and Wilson Ltd.) was dissolved in the minimum amount of $CHCl_3$ and diluted with light petroleum before mixing it with the lecithin solution.

Preparation of enzyme. The enzyme was extracted from the *P. notatum* and purified with ammonium sulphate according to a previously described method (Dawson, 1958*a*). The final dialysis was against distilled water at 4° for 3 days. The purified enzyme was more effective in attacking negatively charged monolayers of lecithin than the original extract; evidence was obtained during the investigation that this may be due to the inhibitory effect of positively charged proteins in the original extract.

Apparatus for continuously recording the enzymic hydrolysis, surface pressure and potential of a unimolecular film of lecithin. The basis of the apparatus (Fig. 2) was a trough milled from a block of polytetrafluoroethylene (Fluon, Imperial Chemical Industries Ltd.) of 75 ml. capacity, 20 cm. long, 4·5 cm. wide and 0·83 cm. deep. This was filled to the brim with water adjusted to the pH optimum of the enzyme (3·3) with HCl. The surface was cleaned with a waxed glass sweep. The solution was stirred from below by using a reciprocating magnet moving a glass-sheathed mumetal-wire stirrer. Sufficient [^{32}P]lecithin (approx. 10^{16} molecules) dissolved in light petroleum (b.p. 60–80°) to form a monolayer was added to the surface by means of an Agla microsyringe.

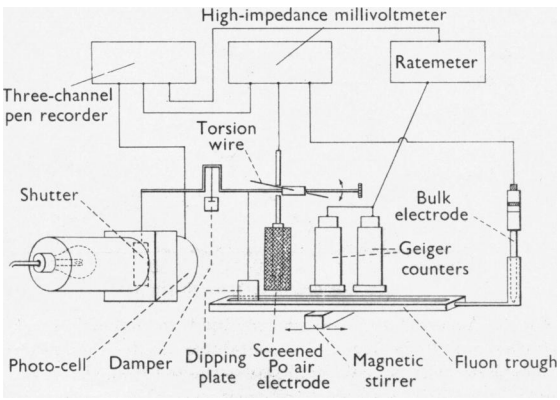

Fig. 2. Diagram of the apparatus for the simultaneous measurement of the radioactivity, surface pressure and potential of a monolayer of [^{32}P]lecithin.

The pressure of the surface was adjusted by moving a waxed glass sweep along the trough and was recorded by means of a Wilhelmy (1863) hydrophilic dipping plate (microscope coverslip fused to a platinum wire); this was suspended from an arm of a torsion balance. An extension of the arm operated a shutter in front of a photoelectric cell and the current arising therefrom was recorded continuously by a pen recorder. The torsion balance was calibrated in dynes/cm. of surface pressure by means of a hanging pan and weights. Care was taken to ensure that the contact angle of the liquid on the plate approached zero by careful washing of the dipping plate with ethanolic KOH and concentrated HNO_3 followed by multiple rinses with dilute HCl. The plate was allowed to drain before the torsion balance was calibrated in dynes/cm. or any surface-pressure measurements were made.

The radioactivity of the surface film was measured by two mica end-window β-counter tubes (Mullard MX 123) in parallel and adjusted so that their windows were at a distance of 0·5 cm. from the film. The mica windows were protected from accidental contamination with ^{32}P by a thin polythene sheet. The pulses were integrated (over 20 sec.) in an Ecko ratemeter and the output voltage was fed into a pen recorder.

The surface potential Δv (i.e. the change observed in the potential which exists between an electrode in air and the aqueous phase on spreading a monolayer) was measured with a 12 mc ^{210}Po air-ionizing electrode (Ministry of Supply, The Radiochemical Centre) held 0·5 cm. from the film. The electrode was screened with an earthed cylinder of copper gauze: the aqueous electrode was a standard calomel electrode seated in a polythene extension to the Fluon trough, a measure designed to prevent contamination of the aqueous phase in the trough with KCl from the electrode. The potential from the polonium electrode was fed by low-microphony cable for amplification by a standard Pye Universal pH/millivoltmeter which had an input impedance of 500 MΩ. The output from the millivoltmeter, the ratemeter and the surface-pressure photoelectric cell were plotted continuously with a three-channel Honeywell–Brown DC pen recorder.

The trough, dipping plate, ^{210}Po air electrode and β-counter tubes were contained in a copper-lined incubator so that observations could be made at temperatures above those in the laboratory. It was observed that temperature fluctuation markedly affected the stability of the surface potential, and it was found better to eliminate the thermostatic control on the incubator and feed its heater with a constant low voltage supplied by a variable transformer.

Paper chromatography of the ^{32}P-containing reaction products. A major part of the aqueous phase (50 ml.) under the unimolecular film was withdrawn by suction and cooled in ice. To the solution was added, as carriers, 88 μg. of glycerylphosphorylcholine P (Dawson, 1956) and 52 μg. of purified ovolecithin P. After vigorous shaking a sample (10 ml.) was taken for assay of the total radioactivity. The

remaining 40 ml. was passed down a column of De-Acidite E (15 cm. long × 0·8 cm. diam.) to remove the HCl which had been used to adjust the solution to pH 3·3 (the enzyme optimum). The column had been previously treated with aq. 5 N-NH_3 soln. and was well washed with water. The weakly basic arylamine grouping removed the HCl but not the glycerylphosphorylcholine. The effluent plus 10 ml. of washings was evaporated to dryness in a rotary drier *in vacuo* below 50°. The residue was dissolved in 0·6 ml. of ethanol and 0·4 ml. was applied to a paper chromatogram which was subsequently developed in ethanol–aq. NH_3 soln. (sp.gr. 0·88)–water (6:3:1, by vol.). After spraying with acid molybdate reagent (Hanes & Isherwood, 1949) the paper was irradiated with u.v. light to locate the spots containing phosphorus and 1 cm. strips were scanned for radioactivity.

RESULTS

Hydrolysis of low-pressure monolayers of lecithin. When the purified enzyme solution was introduced into the aqueous phase below a pure [^{32}P]lecithin monolayer, at pressure below 30 dynes/cm. a loss of surface radioactivity was observed to occur. That this loss of surface counts coming from the film did truly represent hydrolysis of the lecithin was shown by taking the aqueous phase and subjecting it to paper chromatography. The radioactivity which appeared in the solution was exactly associated with an inactive glycerylphosphorylcholine marker which had been added to the solution (Fig. 3).

In films at a starting pressure of below about 16 dynes/cm. the loss of surface counts, representing the enzymic hydrolysis, was largely preceded by a progressive increase in both the surface potential and surface pressure (Fig. 4A). As the initial pressure of the film was increased above 16 dynes/cm. but below about 30 dynes/cm. the loss of radioactivity again followed changes in the surface pressure and potential which were the reverse of those occurring at lower pressures, i.e. both the

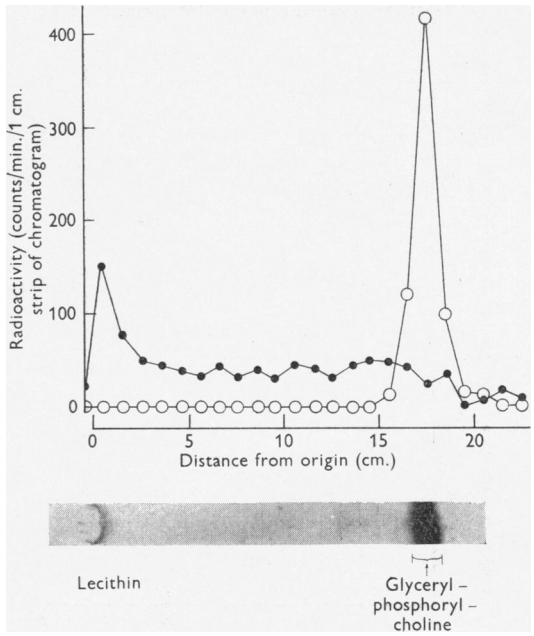

Fig. 3. Paper chromatography of the components containing ^{32}P in the aqueous phase (HCl at pH 3·3). ○, After enzymic hydrolysis of a low-pressure [^{32}P]lecithin film; ●, after an equivalent amount of [^{32}P]lecithin had been emulsified with HCl at pH 3·3 (no enzymic digestion). Lower chromatogram shows marker spots sprayed with reagent, etc., to reveal phosphate esters.

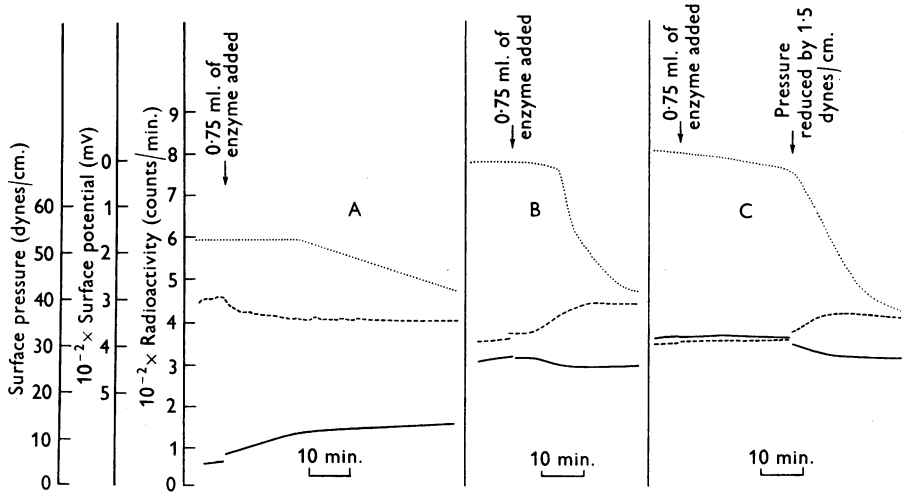

Fig. 4. A, B and C are separate experiments at different initial pressures showing the surface radioactivity, pressure and potential of a [^{32}P]lecithin monolayer undergoing enzymic hydrolysis. —, Surface pressure; - - - -, surface potential; . . . ., surface radioactivity (line evaluated from observations made every 18 sec.).

pressure and the potential decreased (Fig. 4B). If the starting pressure was increased to a level of about 30 dynes/cm. or above there were no changes in surface pressure or potential and no hydrolysis occurred. The sharpness of this pressure threshold is illustrated by the experiment shown in Fig. 4C, in which the reduction of the pressure of a pure lecithin film from 32·5 to 31 dynes/cm. is sufficient to cause the enzymic hydrolysis to begin. The threshold pressure at which hydrolysis ceased varied with individual enzyme preparations but generally lay in the range 28–33 dynes/cm.

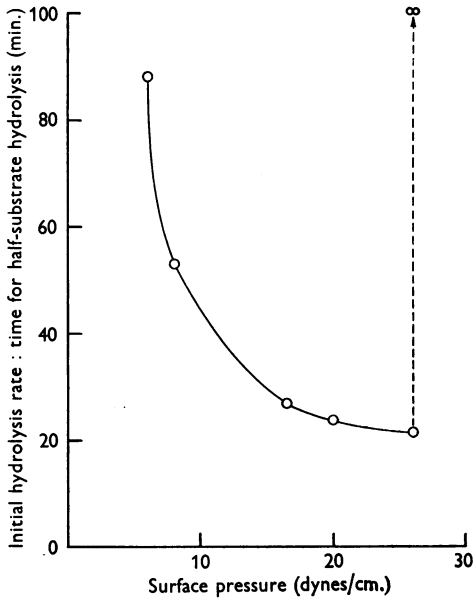

Fig. 5. Relationship between the surface pressure of a pure lecithin monolayer and its initial rate of enzymic hydrolysis.

It was observed (Figs. 4A, B) that the rate at which the film was hydrolysed increased with increasing initial starting pressures of the film up to a maximum just below the critical-pressure threshold. In Fig. 5 the time in minutes required for half the lecithin in the film to disappear has been plotted against the initial starting pressures of the lecithin films.

Hydrolysis of high-pressure mixed monolayers (dicetylphosphoric acid–lecithin). With a high-pressure film of lecithin, that is above about 30 dynes/cm., no change in hydrolysis or surface pressure or potential was observed (Fig. 6A). However, if progressive amounts of dicetylphosphoric acid were added to the lecithin monolayer the pressure threshold at which hydrolysis ceased could be raised. This was not observed if a positively charged amphipathic molecule was used instead of the dicetylphosphoric acid, e.g. heptadecylamine. Fig. 7 shows that there is a linear relationship between the molar percentage of dicetylphosphoric acid added and the pressure threshold at which hydrolysis ceased. With as much as 20–25% of dicetylphosphoric acid added, hydrolysis occurred at the collapse pressure of the monolayer (45–50 dynes/cm.; Fig. 6B).

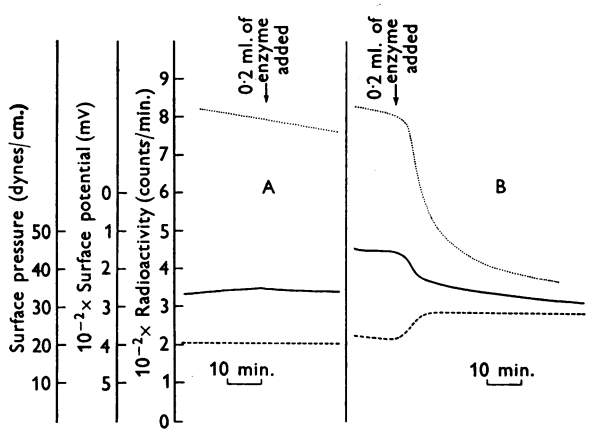

Fig. 6

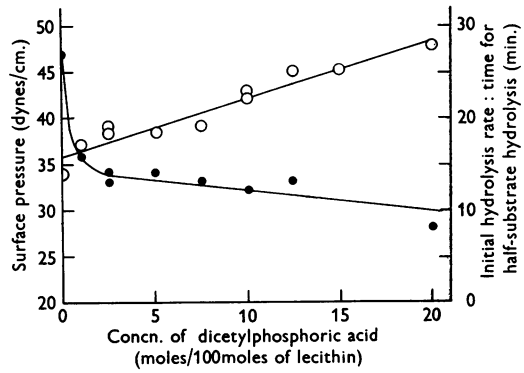

Fig. 7

Fig. 6. Recordings showing the surface radioactivity, pressure and potential of a [^{32}P]lecithin monolayer (A) and a [^{32}P]lecithin–25% dicetylphosphoric acid mixed monolayer (B) at pressures above 30 dynes/cm. and in the presence of enzyme. —, Surface pressure; - - - -, surface potential; ....., surface radioactivity (line evaluated from observations made every 18 sec.).

Fig. 7. Relationship between the molar percentage of dicetylphosphoric acid in a lecithin monolayer and ○, the maximum pressure of the monolayer which allowed enzymic hydrolysis; or ●, the initial rate of hydrolysis when the initial pressure of the monolayer was the same as ○.

The reaction rate with films containing small amounts of dicetylphosphoric acid (1–2%) was appreciably faster than that of the lecithin film alone; however, as the percentage of dicetylphosphoric acid was increased there was only a small further increase in this reaction rate (Fig. 7). With the technique of surface-radiation counting it would be expected that the rate be inversely proportional to the area per lecithin molecule. The resolution of the method, however, does not obviously reveal the hyperbolic nature of this relationship (Fig. 8).

Action of bivalent cations. If Ca^{2+} ions (3·6 mM) were added to the bulk phase below a monolayer of pure lecithin, enzymic hydrolysis occurred normally below pressures of about 30 dynes/cm. If a similar concentration of Ca^{2+} ions was added below a mixed monolayer of lecithin and dicetylphosphoric acid (20%) at 45 dynes/cm. the surface potential increased, presumably due to counter-ion binding of the anion sites; at the same time no hydrolysis occurred when enzyme was added. If, however, the pressure of the mixed film was reduced, hydrolysis commenced at some 32 dynes/cm., a pressure which is near the threshold for hydrolysis of pure lecithin with or without Ca^{2+} ions present in the bulk phase.

Uranyl ions (UO_2^{2+}), which are noted for their high affinity for charged phosphate groups (Kruyt, 1949), were shown to inhibit the action of the enzyme on a lecithin–20% dicetylphosphoric acid film (45 dynes/cm.) at a concentration of 1 mM. When the pressure of this film was reduced to 32 dynes/cm. or less the hydrolysis commenced.

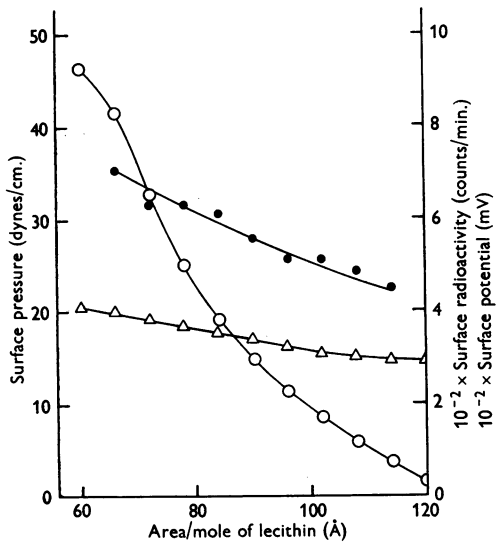

Fig. 8. Graphs showing the relationship between the area of a lecithin molecule in a monolayer and ○, surface pressure; ●, surface radioactivity; △, surface potential.

DISCUSSION

Previous experiments with collapsed films of [^{32}P]lecithin (Dawson & Bangham, 1959) had demonstrated that the presence of a net surplus of negative groups was a prerequisite for the enzymic attack of lecithin at its oil–water interface. With the methods used it was not possible to follow the hydrolysis of films other than those at their collapse pressure. With the present method, by the use of true monolayers it was possible to vary the surface pressure and to record both it and the surface potential continuously.

The results correlate with those obtained during earlier investigations on the relationship between the activity of the enzyme and the zeta potential of the lecithin particles of an emulsion (Bangham & Dawson, 1959*a*). It can be assumed that the surface of an emulsion particle would be equivalent to a monolayer of the same material at its collapse pressure. Thus in both cases a pure lecithin substrate was not attacked whereas lecithin containing a sufficient quantity of an anionic amphipathic molecule (dicetylphosphoric acid) was freely hydrolysed. It is pertinent that the enzymic hydrolysis of lecithin particles required the presence of a minimum percentage of dicetylphosphoric acid which was very similar in value to the amount required to initiate the hydrolysis of a monolayer at its collapse pressure.

With pure lecithin films which were hydrolysed only below a surface pressure of some 30 dynes/cm. it was not possible to demonstrate any change in surface potential by the addition of uni-univalent electrolyte (potassium chloride) to the aqueous phase. This indicates that at this pH (3·3) the lecithin is approximately isoelectric, and that, unlike monolayers above pressures of about 30 dynes/cm. excess of negative groups was not required for enzyme–substrate interaction. This is confirmed by the observation that hydrolysis of low-pressure lecithin or lecithin plus dicetylphosphoric acid films was not inhibited by the addition of bivalent cations or even by the addition of positive groups, e.g. heptadecylamine.

The increase of surface pressure observed when enzyme is introduced under a pure lecithin film at a pressure below 16 dynes/cm. would suggest that penetration or solution, or both, of proteins in the monolayer had occurred (Matalon & Schulman, 1949). However, it must be remembered that the enzyme preparation is not pure and starch–gel electrophoresis showed it to contain at least three components, one of which was electropositive at the pH (3·3) used. This means that the pressure rise observed is not necessarily due to penetration by enzyme.

It is also clear from Fig. 4B that when enzyme is introduced below pure lecithin films above 16 dynes/

cm. the surface pressure and potential both fall. It is possible that these changes may be associated with the release of the first fatty acid from the lecithin molecule forming lysolecithin. This phospholipid would then be subsequently hydrolysed with the release of water-soluble glycerylphosphorylcholine and a further fatty acid. From the present results, which make use of an impure enzyme preparation, it is not possible to explain in what way the enzyme is reaching the fatty acid–ester bonds of the lecithin since the film is above the collapse pressure of most proteins and the conditions for electrostatic adsorption (Matalon & Schulman, 1949) are not evident.

With high-pressure films of pure lecithin it is apparent from all measurements that no interaction takes place between the added enzyme and the surface film. This is in accord with the observations of Fraser, Kaplan & Schulman (1955) on the adsorption of catalase by lecithin and other particles.

When negative anionic molecules are added with lecithin to form a mixed monolayer it is apparent that the negative groups encourage adsorption of the enzyme at pressures in excess of 30 dynes/cm. One possible explanation is that the negatively charged films would attract a positively charged region of the large enzyme molecule and thus assist its adsorption and penetration into the film (Bangham & Dawson, 1959*b*). This would be analogous to the adsorption of positively charged haemoglobin to a long-chain alkyl sulphate monolayer described by Matalon & Schulman (1949). Thus the greater the negative charge on the film, the greater would be its affinity for the enzyme, allowing hydrolysis to occur at ever-increasing surface pressures. The results suggest that the increase in the maximum pressure at which hydrolysis occurs is directly proportional to the percentage of dicetylphosphoric acid added.

The radioactivity of the lecithin–dicetylphosphoric acid mixed films suggested that, at the pressure at which hydrolysis commenced, the area per lecithin molecule was constant, and this could indicate that the crucial effect of the dicetylphosphoric acid molecules was one of surface dilution. However, if an equivalent surface dilution was obtained by using a positively charged long-chain alkylamine no hydrolysis occurred. This indicated that electrostatic conditions at the interface were of prime importance, a conclusion confirmed by the inhibitory action of bivalent cations at constant area per lecithin molecule.

SUMMARY

1. The enzymic hydrolysis of unimolecular films of yeast [^{32}P]lecithin by a purified preparation of a phospholipase B from *Penicillium notatum* has been studied.

2. The rate of the hydrolysis was measured continuously by recording the loss of surface radiation as the water-soluble [^{32}P]glycerylphosphorylcholine was stirred into the bulk aqueous phase. Simultaneously, continuous determinations were made of the surface pressure of the film and the potential between the air above the film and the aqueous phase.

3. The enzyme preparation attacked low-pressure films of lecithin (< approx. 30 dynes/cm.) and it was shown by paper chromatography that labelled glycerylphosphorylcholine was present in the aqueous phase. The rate of hydrolysis increased as the pressure of the lecithin film was increased up to a critical pressure, above which hydrolysis no longer occurred.

4. Addition of anionic amphipathic molecules (dicetylphosphoric acid) to the film allowed the lecithin to be hydrolysed at pressures above those at which a pure lecithin film was attacked.

5. There was a linear correlation between the threshold pressure above which the hydrolysis did not occur and the amount of dicetylphosphoric acid added to the lecithin: 20–25 molar % produced hydrolysis at the collapse pressure of the film.

6. The addition of calcium or uranyl counter ions to the aqueous phase inhibited the hydrolysis of high-pressure lecithin–dicetylphosphoric acid mixed films, but not that of low-pressure lecithin films.

7. It is concluded, tentatively, that the enzyme can penetrate into low-pressure films of lecithin, whereas with high-pressure films a negatively charged surface is required to assist penetration or adsorption or both.

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