

# The Activation of Surface Films of Lecithin by Amphipathic Molecules

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In the preceding paper it was shown that a phospholipase prepared from *Penicillium notatum* will hydrolyse lecithin only after the addition of a minimum proportion of anionic amphipathic molecules, e.g. dicetylphosphoric acid, cardiolipin, sodium dodecyl sulphate (Bangham & Dawson, 1959). The onset of hydrolysis occurred only when the emulsion particles carried a minimum net negative charge, independent of the species of anion added.

The question arose whether the negative charge on the surface of the emulsion particle was directly concerned with the reaction between enzyme and substrate at the interface, or whether it merely dispersed the substrate more efficiently. This paper attempts to answer this question by a study of the action of the phospholipase on surface films of lecithin and of lecithin containing dicetylphosphoric acid. The technique developed for this study was to float films of [ $^{32}\text{P}$ ]lecithin and to follow the loss of radioactivity from the surface film as the water-soluble glycerylphosphorylcholine produced by the enzyme diffused into the aqueous phase.

## EXPERIMENTAL

*Preparation of [ $^{32}\text{P}$ ]lecithin.* Baker's yeast (*Saccharomyces cerevisiae*) was grown under the conditions described by Williams & Dawson (1952), the medium being based on one described by Olson & Johnson (1949). It contained the following (per l.): glucose, 20 g.; ammonium acetate, 5.0 g.; potassium dihydrogen phosphate, 0.2 g.;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.25 g.; sodium citrate, 1.0 g.; L-asparagine, 2.5 g.; biotin, 20  $\mu\text{g}$ .; calcium pantothenate, 0.5 mg.; inositol, 10.0 mg.; thiamine, 4.0 mg.; pyridoxine, 1.0 mg.;  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 1.8 mg.;  $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ , 1.05 mg.;  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 0.1 mg.

A volume (2 l.) of the medium was adjusted to pH 5.0 with *n*-sulphuric acid and 2.4 mc of carrier-free  $^{32}\text{PO}_4^{3-}$  ions was added. The medium was not sterilized but it was heavily inoculated with fresh yeast. Growth was allowed to continue for 21 hr. at 30° with vigorous aeration, by which time 90–95% of the inorganic phosphorus had been incorporated into the yeast.

The suspension of yeast cells was centrifuged and the supernatant discarded. The yeast sediment was washed with 200 ml. of water and the residue extracted by refluxing with 100 ml. of ethanol for 30 min., with continuous stirring to avoid violent bumping. On cooling, the ethanolic extract was decanted and filtered, the residue was extracted by further refluxing for 30 min. with 150 ml. of

methanol-chloroform (1:1, v/v) and the suspension filtered. The filtrates were pooled and evaporated to dryness *in vacuo*. The lipid residue was then dissolved in 50 ml. of ethanol and the ethanol removed *in vacuo* to complete dehydration. The residue was next thoroughly extracted with 100 ml. of diethyl ether; insoluble matter was removed by filtration and the filtrate evaporated to dryness. The residue was dissolved in 4 ml. of ether and 40 ml. of acetone was added; after allowing the solution to stand for 1 hr. at 0°, the precipitate of phospholipids was recovered by centrifuging. The acetone-damp precipitate was extracted, first with 8 ml. of chloroform-methanol (1:1, v/v), and then once more with 4 ml. of the same solvent, any insoluble material being removed by centrifuging. The phospholipids were then passed through an activated alumina column (15 cm. long  $\times$  0.8 cm. diameter; Hopkins and Williams Ltd.) in chloroform-methanol (1:1, v/v). The lecithin fraction was detected by monitoring the effluent with a Geiger counter and came through in the first 25 ml. emerging from the column. This fraction was evaporated to dryness and chromatographed on a silicic acid column (10 cm.  $\times$  0.8 cm.; Mallinckrodt Ltd.) in chloroform-methanol (68:32, v/v). Fractions of 5 ml. were collected and the lecithin emerged in tubes 18–33 with some tailing to tube 40. The resulting lecithin counted at  $1.5\text{--}3.0 \times 10^6$  counts/min./ $\mu\text{mole}$  under a mica end-window  $\beta$ -counter and was stored at  $-15^\circ$  in chloroform solution. Analysis by mild alkaline hydrolysis (Dawson, 1954) showed glycerylphosphorylcholine as the sole phosphorus-containing degradation product.

*Apparatus for detection of enzyme action on [ $^{32}\text{P}$ ]lecithin films.* (See Fig. 1.) The [ $^{32}\text{P}$ ]lecithin was dissolved in light petroleum (b.p. 40–60°) and carefully delivered onto the

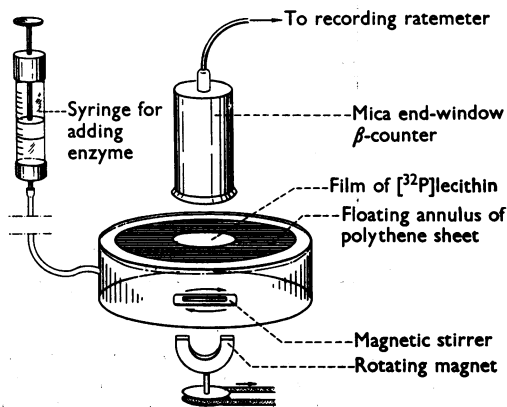


Fig. 1. Diagram of apparatus used for measuring enzymic hydrolysis of a radioactive film of lecithin.

cleaned surface of a 0.02M-sodium formate-formic acid buffer, pH 3.3 (surface cleaning by the method of Adam, 1941). The film of lecithin was confined in a floating annulus of polythene sheet, the buffer (19 ml.) being contained in a cylindrical glass vessel 4.2 cm. wide and 1.5 cm. deep. The buffer could be stirred magnetically from the underside of the glass vessel and fluid could be added or withdrawn through a polythene tube connected through the side wall. A mica end-window  $\beta$ -counter was mounted coaxially and immediately above (0.25 cm.) the lecithin film. The window of the counter was protected from radioactive contamination by a thin polythene membrane. The apparatus was arranged in an incubator so that measurements could be made at 37°. The counter was connected to a recording ratemeter (Labgear Ltd., Cambridge) and a continuous record of the surface counting rate obtained, an integration time of 10 sec. being used.

### RESULTS

When solutions of lecithin or lecithin plus dicetylphosphoric acid in light petroleum were added to the clean surface of the buffer, the lipids were seen to float in the form of 'rafts' or crystals, which are presumably separated from one another by a monolayer of lipid (Adam, 1941).

In the experiments to be described, sufficient [ $^{32}$ P]lecithin (approx. 0.02 ml. of 0.02M-solution) was added to give an initial counting rate of about 4000 counts/min. In preliminary experiments it was observed that there was a slow but steady loss of counts from the surface due to the floating microcrystals moving towards the sides of the glass vessel. This was prevented by the use of a floating annulus of polythene sheet, which retained them under the window of the counter.

Fig. 2 (A) shows that after the addition of [ $^{32}$ P]lecithin to the surface there was no loss of counts for a period of 20 min. If at this point 3 ml. of buffer was withdrawn and replaced by 3 ml. of enzyme preparation (Dawson, 1958*a*), the counting rate remained constant for a period of at least 40 min. The small deflexion in the tracing was due to lowering of the surface film as the buffer was withdrawn before addition of enzyme.

Fig. 2 (B) shows the same experiment repeated except that the [ $^{32}$ P]lecithin was mixed with sufficient dicetylphosphoric acid to activate the system (Bangham & Dawson, 1959). Here again the counting rate remained stable until the enzyme

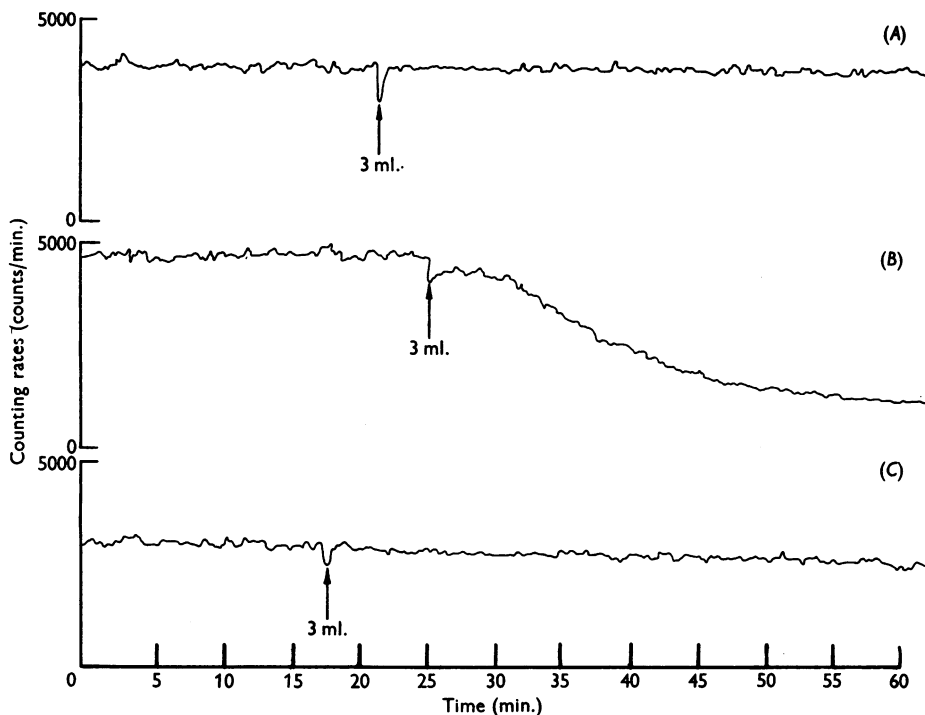


Fig. 2. Counting rates from a [ $^{32}$ P]lecithin film before and after the addition of enzyme. All experiments were carried out at 37°. Enzyme was added as indicated by the vertical arrows after removal of an equal quantity of buffer. (A) Film of [ $^{32}$ P]lecithin on 0.02M-sodium formate-formic acid buffer, pH 3.3; (B) film of [ $^{32}$ P]lecithin plus dicetylphosphoric acid (100:24 molar mixture) on 0.02M-sodium formate-formic acid buffer, pH 3.3; (C) as for (B) except that the buffer was 0.02M-sodium acetate-acetic acid, pH 4.4.

was added and then over a period of 35 min. there was a continual loss amounting to 75% of the original surface radioactivity.

Fig. 2 (C) shows the effect of adding enzyme under a mixed film of [ $^{32}$ P]lecithin plus dicetylphosphoric acid floating on 0.02M-sodium acetate-acetic acid buffer, pH 4.4. The ionic strength of the sodium in this buffer was the same as that of the formate buffer used in the Expts. 2 (A) and 2 (B). The loss of counts was very small, which agrees with previous observations that the enzymic hydrolysis is negligible at this pH (Dawson, 1958b).

## DISCUSSION

The primary object of these experiments was to determine whether the activation of the lecithinase produced by the anionic amphipathic molecules was intimately concerned with the reaction between enzyme and substrate at an interface.

Earlier evidence had, in fact, suggested that the activation was not due to a more efficient emulsification of the substrate resulting merely in an increase in the available surface area. Thus when lecithin emulsions were centrifuged for 1 hr. at 77 000–155 000 g it was observed that a small percentage (5.2, 5.6, 4.7%) remained in the water-clear supernatant. This non-sedimenting lecithin must consist of very small micelles representing a very large surface area, nevertheless there was no evidence of any enzymic activity; this result suggests that activity is not merely a function of available surface area.

The present results obtained with surface films provide direct evidence that the enzyme specifically requires negative groups for its action at an interface. The regular loss of radioactivity observed on adding the enzyme under lecithin plus dicetylphosphoric acid films has been taken as meaning that direct enzymic hydrolysis of the film was occurring. This is substantiated by the observation that the loss of radioactivity is prevented by altering the pH to 4.4, a manoeuvre known to prevent the enzyme from hydrolysing lecithin (Dawson, 1958b). The conclusion reached is that the enzyme is reacting with the film without prior emulsification of the substrate and that this requires negative groups at the solid-liquid interface.

At this stage it is not possible to see clearly why the negative groups on the substrate surface are essential for the enzymic attack. One possibility is that they orientate the enzyme with respect to the substrate by attracting positive groups on the enzyme molecule. The observation that a certain critical density of negative grouping is necessary before activity commences would suggest that such an attraction must occur at more than

one charged site. This attachment by electrostatic forces would ensure that the enzyme was in the immediate vicinity of its substrate and might eventually assist the enzyme molecule to penetrate into the film between the molecules of orientated lecithin. Penetration in this sense would be equivalent, on a molecular level, to the coalescence observed by Kates (1957) of lecithin micelles and plant plastids in the presence of ether.

An alternative hypothesis is that the activator molecules, by electrostatic interaction with the charged choline groups of the lecithin, cause a reorientation of the substrate's surface. This could either assist penetration of the enzyme into the lecithin film, or alternatively expose the acyl-ester bonds so that these became more accessible for enzymic hydrolysis at the interface. This hypothesis would not need to assume a specific attraction between the enzyme and the surface of the substrate film.

Presumably, with low activator concentrations little denaturation of the enzyme on the surface occurs, although this might happen as excess of negative groups is added to the surface and explain why the activity falls away with excess of activator (Dawson, 1958b). A somewhat equivalent picture of the adsorption of an enzyme at a surface has been described by Fraser, Kaplan & Schulman (1955). These workers found that a lecithin surface had little attraction for catalase, but that it was possible to obtain adsorption of the enzyme with only partial denaturation on an oil surface possessing certain polar groups.

It is hoped to gain further information about the actual mechanism of activation by the study of the action of the lecithinase on monomolecular films.

## SUMMARY

1. [ $^{32}$ P]Lecithin of high specific activity has been prepared biosynthetically with baker's yeast.
2. The [ $^{32}$ P]lecithin has been used as a surface film to study the action of a *Penicillium notatum* phospholipase preparation. Loss of counts from the surface film into the bulk aqueous phase was used as a measure of enzymic activity.
3. The enzyme preparation was without action on a film of lecithin but readily lowered the counting rate from a surface film of lecithin plus dicetylphosphoric acid.
4. The loss of counts from the surface film did not occur on altering the pH of the aqueous phase from 3.3 to 4.4, which is known to inhibit the enzyme system.
5. It is concluded that the presence of a net surplus of negative groups is a direct requirement for the initiation of the enzymic attack on a lecithin surface.

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## Studies on the Metabolism of Hydroxypyruvate in Animal Tissues

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The preparation of pure hydroxypyruvate (Dickens & Williamson, 1958*a*) has made possible the use of this substance in metabolic experiments, e.g. with tissue slices, and in this paper we describe experiments on the utilization of hydroxypyruvate by the whole rat and by rat-liver slices. By the use of specifically labelled hydroxy[<sup>14</sup>C]pyruvate an incorporation of radioactivity into the molecules of glycogen and glucose has been observed in both series of experiments. By stepwise degradation of the glucose molecule the pattern of radioactivity has been determined and evidence has thus been obtained about the probable overall character of the route of incorporation. A brief preliminary account of this work was given by Dickens (1958).

### EXPERIMENTAL

#### Materials

**Radioactive substances.** These were obtained from The Radiochemical Centre, Amersham, Bucks, with the following approximate specific activities: generally labelled [G-<sup>14</sup>C]glucose, 10 mc/m-mole; [1-<sup>14</sup>C]glucose and [6-<sup>14</sup>C]glucose, 2 mc/m-mole; L-[3-<sup>14</sup>C]serine, 3 mc/m-mole; sodium [3-<sup>14</sup>C]pyruvate, [2-<sup>14</sup>C]pyruvate and [1-<sup>14</sup>C]pyruvate, 1 mc/m-mole. They were diluted for use as required (see tables) with non-isotopic material.

**Lithium hydroxypyruvate.** This was prepared and crystallized as already described (Dickens & Williamson, 1958*a*). The [1-<sup>14</sup>C]-, [2-<sup>14</sup>C]- and [3-<sup>14</sup>C]-hydroxypyruvate were also prepared in this way for use in most experiments, but in some, particularly where a high specific activity was needed, the corresponding bromo[<sup>14</sup>C]pyruvate was used directly after conversion into a solution of hydroxypyruvate by neutralization with 2 equivalents of alkali (Sprinson & Chargaff, 1946). Although this avoided the considerable loss of radioactive material accompanying crystallization, such solutions contain traces of bromopyruvate as well as the NaBr formed on neutralization. They were found, however, to be suitable for injection into the whole animal,

when the results were checked by the use of the purified material.

In the preparation of bromo[<sup>14</sup>C]pyruvate by bromination of sodium [<sup>14</sup>C]pyruvate and added unlabelled pyruvic acid (Dickens & Williamson, 1958*a*), very thorough mixing of these substances is essential before bromination, otherwise undissolved particles of sodium salt may escape

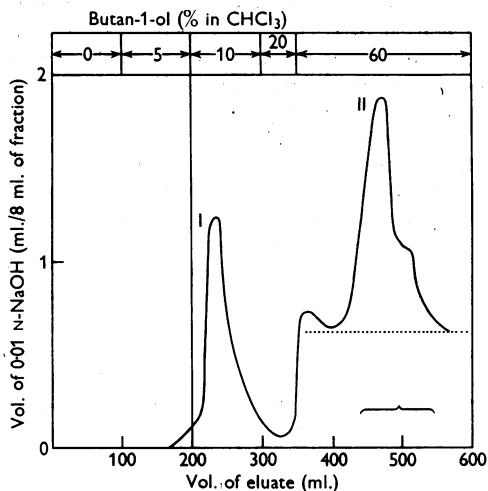


Fig. 1. Column separation of pyruvate and hydroxypyruvate. An acidified aqueous solution containing non-isotopic pyruvate (50  $\mu$ moles) and hydroxy[3-<sup>14</sup>C]-pyruvate (90  $\mu$ moles) was adsorbed on Celite and the column was eluted successively with chloroform and then with chloroform containing the stated percentage (v/v) of butan-1-ol. Titration of 8 ml. fractions is shown by the curve; the dotted line indicates level of H<sub>2</sub>SO<sub>4</sub> in eluates and the solid curve total acidity (keto acid plus H<sub>2</sub>SO<sub>4</sub>). Peak I, pyruvate (39.5  $\mu$ moles, 79% recovery). Peak II, hydroxypyruvate (70  $\mu$ moles, 78%; after vacuum-concentration and removal of indicator, overall recovery 60%). For details see text.