Finally, it is interesting to compare the amino acid analyses of the α - and γ -keratases prepared from virgin wool of 64s quality with those of the corresponding wool fractions obtained from wool tops of 56s quality. Reference to Table 1 shows that the agreement between the two sets of results is quite close, and it would appear that the two keratases can in fact be characterized by their amino acid compositions. Also, with α -keratase, neither a change in the degradation procedure adopted for its preparation nor a change in the quality of the wool used has a marked effect on its amino acid composition.

SUMMARY

1. An Australian Merino 64s quality wool has been oxidized with peracetic acid and its amino acid composition determined. The analyses is in excellent agreement with that of virgin wool (Corfield & Robson, 1955), except that cystine is converted into cysteic acid and that small losses of histidine, tyrosine and phenylalanine occurred during oxidation.

2. α -, β - and γ -Keratases were prepared from oxidized wool and their amino acid compositions determined.

3. Only 75% of the total sulphur of oxidized wool and of the three keratases could be accounted for by their respective cysteic acid contents.

4. The total nitrogen and total sulphur of the three keratases have been compared with the amounts determined by other workers. For total sulphur the agreement is not good.

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The Identification of Two Lipid Components in Liver which enable *Penicillium notatum* Extracts to Hydrolyse Lecithin

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Fairbairn (1948) reported that aqueous dialysed extracts of the mould *Penicillium notatum* possessed an active phospholipase B which attacked the lysophosphoglycerides but had no action on the intact phosphoglycerides. The same preparation has been used as the basis of an enzymic method for the preparation of glycerylphosphorylcholine (Uziel & Hanahan, 1956) and here again no activity was found towards intact lecithin. Dawson (1957) attempted to detect lysolecithin in the crude lipids extracted from liver with a similar enzyme preparation from *P. notatum*, but found that,

although the mould extract did not attack purified ovoidlecithin, there was a rapid and almost quantitative release of the fatty acids from the lecithin present in the crude lipids extracted from liver. This activity was not caused by some difference between the ovoidlecithin and its counterpart in the liver lipids, since when purified the latter lost its susceptibility to attack by the *P. notatum* extracts. It became apparent that there was an additional factor in the crude lipid extract which was responsible for the *P. notatum* phospholipase B preparation attacking the liver lecithin. This paper

describes the isolation and identification of two lipids from the crude liver lipids, both of which, when added at low concentrations to a system containing purified lecithin and *P. notatum* phospholipase B, will bring about a degradation of the phosphoglyceride into glycerylphosphorylcholine and free fatty acids.

EXPERIMENTAL

Preparation of *P. notatum* extracts. The method of mould culture and the extraction of enzyme closely followed that of Fairbairn (1948). The *P. notatum* used was strain 1249/B/21, and was maintained on a Czapek agar slope. It was surface cultured at 25° on a corn-steep-liquor medium, and the mat of green sporulating mycelium which grew in about 12 days was separated from the medium by tipping the entire culture through a Büchner funnel. The mycelium was then washed with a little water, and damp-dried by pressing between filter paper. It was then ground to a slurry in a mortar with water (5 ml./g. of wet mycelium) and autolysed for 24 hr. at 25° in the presence of a little toluene. The avoidance of drying the mycelium before autolysis gave a better final yield of the enzyme. The autolysate was filtered and the golden-brown filtrate was dialysed against water at 4° for 24 hr. The dialysis sac was immersed in glycerol and removed when the volume of the contents had decreased to about one-quarter. The glycerol extract obtained had potent phospholipase B activity, but contained little protein; it could be stored for over a year at 0° without noticeable loss of activity.

For some experiments the enzyme was partially purified by fractionation with $(\text{NH}_4)_2\text{SO}_4$. The aqueous dialysed extract of the mould was mixed with 0.05 vol. of acetate buffer, pH 4.5. The mixture was cooled to 0° and solid $(\text{NH}_4)_2\text{SO}_4$ was added (50 g./100 ml.). The precipitate of inactive material was centrifuged off and discarded, and the supernatant containing the enzyme was, after filtration, dialysed until free of $(\text{NH}_4)_2\text{SO}_4$.

Materials. The methods used for the preparation of lecithin, lysolecithin and glycerylphosphorylcholine are given by Dawson, Mann & White (1957).

Estimations

Inositol. This was assayed by the microbiological procedure of Campling & Nixon (1954), the *Kloeckera brevis* yeast being kindly supplied by Dr E. F. Hartree. Samples of lipid were hydrolysed before inositol assay by heating at 105° for 18 hr. in 5N-HCl: the hydrolysate was evaporated to dryness *in vacuo* and the residue dissolved in water and adjusted to pH 5 before analysis. Samples were assayed in triplicate and standard growth curves (0.5 µg. of inositol) were prepared for each set of determinations. After 3 days' growth, turbidities were read in an EEL colorimeter with an OR1 red filter.

Glycerol. The method of estimation was largely based on an unpublished method kindly supplied by Dr J. N. Hawthorne except for the periodate reaction and titration, which was a micro-modification of the method of Voris, Ellis & Maynard (1940). Samples were hydrolysed as for inositol assay, and then portions were run on paper chromatograms (acetic acid-washed Whatman no. 1) with

propan-2-ol-acetic acid-water (3:1:1, by vol.). In other lanes were run standard glycerol spots and blanks and, on either side of the chromatogram, glycerol markers which were located by the AgNO_3 -alkali method of Trevelyan, Procter & Harrison (1950). Areas corresponding to the standard and unknown glycerol spots were cut out and eluted with water. The combined aqueous extracts (12 ml.) of a particular spot were mixed with 1 ml. of a solution containing 0.125 g. of KIO_4 in 500 ml. of 0.02N- H_2SO_4 and kept for 1 hr. at room temp. A volume (0.1 ml.) of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ soln. (15%, w/v) was added and then dil. NaOH soln. until a very faint turbidity developed. This was just cleared by the addition of 0.02N- H_2SO_4 and the solution was mixed with 3 ml. of phosphate buffer (12 g. of $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ and 42 ml. of N- H_2SO_4 diluted to 100 ml.). Then 0.2 ml. of 5% (w/v) KI soln. was added and the liberated I_2 was titrated with freshly diluted 0.001N- $\text{Na}_2\text{S}_2\text{O}_3$ soln., recently prepared starch solution being used as indicator. Standard glycerol curves were set up for each determination and the unknown was read directly from them; they were straight lines cutting the axis at about 1.2 µg. of glycerol.

Other estimations. Glycerylphosphorylcholine and free fatty acids were determined as described by Dawson (1956), acyl fatty-ester bonds by the method of Stern & Shapiro (1953) and phosphorus by the method of Fiske & Subbarow (1925). Aldehyde assays were kindly performed by Dr D. N. Rhodes by the method of Wittenberg, Korey & Swenson (1956). Nitrogen was estimated by a Conway microdiffusion technique after digestion overnight with the reagent of Borsook & Dubnoff (1939).

Chromatographic detection of glycerylphosphoryl 'base' was carried out as described by Dawson (1955a).

Isolation of active lipids

The source of active lipids mostly used was rat or sheep liver; recently horse liver has provided a rich source of one of the active lipids (monophosphoinositide).

Separation of methanol-soluble lipids. Fresh liver tissue (20 g.) was blended in a high-speed blender with 160 ml. of CHCl_3 -methanol (1:1, v/v). The suspension was centrifuged and the supernatant evaporated to dryness *in vacuo*; the residue was extracted with 0.5 vol. of methanol for every volume of supernatant used. The precipitate of methanol-insoluble lipids was removed by centrifuging and discarded; it possessed little activity.

Chromatography on activated aluminium oxide. The solution of methanol-soluble lipids (50 ml.) was evaporated to dryness *in vacuo*. The residue was taken up in 25 ml. of CHCl_3 -methanol (1:1, v/v), any undissolved material being centrifuged off and discarded. The dissolved lipids were then applied equally on two activated alumina (Hopkin and Williams Co. Ltd.) columns, 1.8 cm. diam., 22 cm. long; CHCl_3 -methanol was passed through the columns until 150 ml. was collected from each (30 min.). This effluent contained the lecithin in the lipid and much neutral fat and cholesterol but virtually no activity. The activity was eluted from the column by CHCl_3 -ethanol-water (2.5:2, by vol.) (Rhodes & Lea, 1957). It can be seen (Fig. 1) that the phosphorus from the column appears a little before the activity and consequently an enrichment was obtained by discarding the first 125 ml. of solvent coming through each column and collecting the next 125 ml. No improvement of this separation was obtained

Fig. 1

Fig. 2

Fig. 1. Elution of activity from an alumina column with CHCl_3 -ethanol-water (2:5:2, by vol.). $\circ$, Glycerylphosphorylcholine formed on adding lipid contained in 1 ml. of fraction to test system (i.e. 3.5 μ moles of liver lecithin in 1 ml. of acetate buffer, pH 4, incubated with 0.2 ml. of *P. notatum* enzyme for 6 hr. at 37°); $\bullet$, phosphorus contained in 1 ml. of fraction.

Fig. 2. Fractionation of activity on a short silicic acid column (11 cm.) with CHCl_3 -methanol (4:1, v/v). $\circ$, Glycerylphosphorylcholine formed on adding lipid contained in 1 ml. of fraction to test system as in Fig. 1; $\bullet$, phosphorus contained in 1 ml. of fraction.

Fig. 3. Fractionation of activity on a long silicic acid column (28 cm.) with CHCl_3 -methanol (4:1, v/v). $\circ$, Glycerylphosphorylcholine formed on adding lipid contained in 1 ml. of fraction to test system as in Fig. 1, except that lecithin was 1.75 μ moles; $\bullet$, phosphorus contained in 1 ml. of fraction.

by lengthening the column, varying the solvent or increasing the time usually taken for the elution of activity with the second solvent (90 min.).

Chromatography on an activated silicic acid column. The active lipids were next chromatographed on a column of activated silicic acid (Mallinckrodt Ltd.) essentially as described by Lea, Rhodes & Stoll (1955). The combined active fractions from the alumina columns (250 ml.) were evaporated to dryness *in vacuo*. The residue was dissolved in 15 ml. of CHCl_3 -methanol (4:1, v/v) and centrifuged; the supernatant was applied to a silicic acid column (1.8 cm. diam.; 11 cm. long). The CHCl_3 -methanol solvent (4:1, v/v) was used to develop the column and 10 ml. fractions of the effluent were collected in an automatic fraction collector. Two main peaks of activity consistently

appeared (Fig. 2): the first, in fractions 2-5 and just before the main phosphorus peak which was identified as phosphatidylethanolamine, and the second a long peak between fractions 30 and 45, which was coincident with a phosphorus peak at the same position (I). In some preparations, activity was also observed between these two peaks (Fig. 2), but in others it was completely absent. Fractions 1-10 were now combined and run in the same solvent on a long silicic acid column (28 cm.); this enabled a clear separation to be made of a minute phosphorus peak (II) containing the bulk of the activity and the major phosphatidylethanolamine peak (Fig. 3). The phosphatidylethanolamine usually possessed some activity in the test system, but this was very much lower on a molar basis than the activity of the initial peak, and was not observed at all with low concentrations of the phosphoglyceride.

The fractions comprising silicic acid peak I were evaporated to dryness *in vacuo*, the lipid was dissolved in 2 ml. of hot ethanol and the solution cooled for 1 hr. at 0°. The lipid precipitate consisted of minute transparent spheroids which tended to aggregate in clusters (active lipid I). The minute phosphorus-containing peak II obtained from the long silicic acid column was dissolved in 1 ml. of ether; it was slightly yellow and showed fluorescence in ultraviolet light. Acetone (3 ml.) was added and the ether removed by evaporation under reduced pressure. A white precipitate formed which possessed most of the phosphorus and activity, the yellow fluorescence being left in solution. The precipitate was collected by centrifuging and was washed with acetone (active lipid II).

RESULTS

Breakdown of liver phospholipids by *P. notatum* extracts

In initial experiments emulsions of crude liver lipids were incubated with the *P. notatum* phospholipase B preparation and the liberation of glycerylphos-

Fig. 4. Breakdown of rat-liver phospholipids on incubation with a *P. notatum* phospholipase B preparation. Chloroform-methanol-soluble phospholipids from 110 mg. of fresh liver were incubated at 37° with 0.2 ml. of *P. notatum* extract in acetate buffer, pH 4.

phorylcholine was measured, with the expectation that this would indicate the level of lysolecithin present in the lipids. However, the release of glycerylphosphorylcholine, measured as water-soluble acid-labile choline, was far in excess of the amount of lysolecithin which could reasonably be expected to be present in the tissue. Furthermore, when a liver-lipid emulsion was incubated with *P. notatum* extract at pH 4, a distinct physical change occurred: the originally milky fluid coalesced and a layer of fatty substance separated, leaving a clear underlying layer of the suspending medium. A considerable portion of the phosphorus in the lipid became water-soluble and was no longer precipitable by perchloric acid (Fig. 4). Moreover, a considerable release of free fatty acids occurred in the mixture and its acyl-fatty acid ester content decreased. All of the water-soluble choline which appeared in the reaction mixture was combined in an acid-labile form and no free base was liberated. By paper chromatography, after removal of the glycerol from the enzyme preparation by dialysis, it was confirmed that not only glycerylphosphorylcholine but also glycerylphosphorylethanolamine had been released. These observations strongly suggested that the main reaction occurring in the mixture was the breakdown of the intact phosphoglycerides, lecithin and phosphatidylethanolamine into free fatty acids and the corresponding glycerylphosphoryl 'bases'.

The liver phosphoglycerides were not attacked because of any structural peculiarity, e.g. in their fatty acid composition. When, for example, egg lecithin, which by itself was not attacked by the enzyme preparation, was added to the liver lipids

Table 1. Breakdown of lecithin by *P. notatum* extracts

System contained 3.3 μ moles of ovolecithin (Expt. 1) or of liver lecithin (Expt. 2) in 1 ml. of acetate buffer, pH 4, and 0.2 ml. of *P. notatum* extract. Incubated for 340 min. at 37°.

Expt. no.	Active lipid added (μ mole P)	Liberation of glycerylphosphorylcholine (μ mole)
1	0	0
Active lipid I (monophosphoinositol)	0.042	0.096
	0.164	0.440
	0.290	0.955
2	0	0
Active lipid II (polyglycerol phospholipid)	0.052	0.27
	0.075	0.55
	0.090	0.75

there was a marked stimulation of the reaction. Furthermore, when liver lecithin was separated from the other phospholipids by an alumina-column technique (Hanahan, 1954), it was no longer susceptible to attack by the enzyme preparation. The simplest explanation, therefore, was that in the lipid extract itself there was an activating compound which in some way assisted the enzyme preparation to attack whole phosphoglyceride molecules. It is well known that crude lipid extracts impart a lipid-solvent solubility on to non-lipids which are therefore carried along with the lipids themselves. However, when a liver lipid extract was purified from such non-lipids by the procedure of Folch, Ascoli, Lees, Meath & LeBaron (1951), the breakdown of phosphoglycerides on adding *P. notatum* enzyme still occurred at the same rate. The active agent was precipitated from ethereal solution by acetone, which suggested that it might be associated with the phospholipid fraction. It was stable when suspended in an aqueous buffer (pH 4) and kept at 100° or even after a brief autoclaving at 120°, but 5 min. in 0.1N-HCl at 100° or in methanolic 0.2M-KOH at room temperature was sufficient to suppress virtually all activity.

Identification of active lipids

The active lipids, I and II, isolated as described in the Experimental section caused the liberation of glycerylphosphorylcholine when added to a system containing pure lecithin, *P. notatum* phospholipase B and acetate buffer at pH 4 (experiments reported in Table 1 are typical), effects still being demonstrable when the lipids were added individually at concentrations as low as 30 μ M, 1 phosphorus atom/molecule being assumed to be present. The molar release of glycerylphosphorylcholine was very many times

greater than the molar amount of active lipid which was added to the system.

Lipid I. The lipid contained phosphorus but no nitrogen. After acid hydrolysis in 5N-HCl at 100°, followed by paper chromatography in propan-2-ol-acetic acid, two reducing substances became visible on spraying with alkaline silver nitrate; they were identified as glycerol and inositol. The lipid gave no Molisch test for carbohydrate (Found: inositol, 22.7; P, 3.85%; molar ratio inositol: P:fatty acid:glycerol, 1.0:1.0:2.0:0.9. Calc. for dipalmitoylglycerolphosphorylinositol: inositol, 22.5; P, 3.8%; molar ratio, 1:1:2:1). The lipid was therefore a monophosphoinositide.

Lipid II. This lipid was isolated only in very small amounts; thus from each preparation from rat liver only about 40–60 µg. of P was obtained. The N:P molar ratio was <0.07:1. On acid hydrolysis, followed by paper chromatography of the hydrolysate in propan-2-ol-acetic acid, only a single reducing spot corresponding to glycerol became visible on spraying with alkaline silver nitrate. No inositol was detected either by paper chromatography or by the microbiological method, nor was carbohydrate found by the Molisch test. The molar ratio fatty acid:P:glycerol was 2.8:2.0:2.9. On methanolic alkaline hydrolysis followed by paper chromatography, no glycerophosphoric acid was detectable as would be expected if the lipid was a simple phosphatidic acid (Dawson, 1954). The alkaline hydrolysis product also contained a little aldehyde. McKibbin & Taylor (1952) reported the isolation from horse liver of a polyglycerol phospholipid which approximated to a molar ratio of fatty acid:P:glycerol of 3:2:3. The observations reported here suggest that active lipid II is a compound of this type, which possibly contains in addition a nitrogen-free lipid containing a long-chain fatty aldehyde.

DISCUSSION

Two phospholipids, monophosphoinositide and polyglycerol phospholipid, have been isolated from liver and when these are added at low concentrations to a system containing lecithin and *P. notatum* phospholipase B they enable the enzyme to attack the intact lecithin molecules. This is a novel type of activation of an enzyme system and, if it is assumed that the lecithin is degraded to glycerylphosphorylcholine by a successive enzymic removal of the two fatty acids (i.e. via lysolecithin), as the preparation is capable of attacking lysolecithin very rapidly the net effect of the activation is the development of phospholipase A activity in the system for removing the first fatty acid. If this is so, the effect could be considered either as the addition of an essential cofactor which activates a

latent phospholipase A in the enzyme preparation or a changing of the specificity of the phospholipase B. However, other explanations are possible and work is in progress with a view to ascertaining the precise mechanism of the activation.

It is by no means certain that these two lipids represent the only ones that are active and indeed some evidence has been obtained that phosphatidylethanolamine can in much higher concentrations cause a small breakdown of liver lecithin by the *P. notatum* extracts.

Monophosphoinositide was first isolated from animal tissues by Faure & Morelec-Coulon (1954), using heart muscle. McKibbin (1954), Hawthorne (1955) and Dawson (1955b) have described the presence of lipid inositol in liver, and McKibbin (1956) isolated monophosphoinositide contaminated with a little nitrogenous lipid from horse liver. The structure of the lipid is thought to be analogous to that of lecithin, except that the base is replaced by a molecule of inositol. The structure of the polyglycerol phospholipid of horse liver (McKibbin & Taylor, 1952) has not yet been ascertained with certainty, although it may exist as a polymer and may be related to the phosphatidic acid cardiolipin, isolated by Pangborn (1947) from ox-heart muscle.

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SUMMARY

1. Extracts of *Penicillium notatum* and the partially-purified phospholipase B it contains will attack lysolecithin, but are without action on pure lecithin.
2. Such extracts attack the lecithin and phosphatidylethanolamine contained in an emulsion of crude liver lipids suspended in a buffer at pH 4, producing free fatty acids and glycerylphosphorylcholine or glycerylphosphorylethanolamine.
3. Two active lipids have been isolated from liver which cause *P. notatum* extracts to break down intact lecithin when added in concentrations as low as 30 µM.
4. One of these lipids has been identified as monophosphoinositide; the other is most probably a polyglycerol phospholipid.

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The Reported Occurrence of Sphingosylphosphorylcholine in Animal Tissues

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Little is known about the intermediary metabolism of sphingomyelin. Rossi (1935) and Goebel & Seckfort (1948) reported an uncharacterized sphingomyelinase in extracts of spleen, brain and liver, and Thannhauser & Reichel (1940) and Fujino (1952) have produced some evidence that this acts by splitting the bond between lignoceryl-sphingosine and phosphorylcholine. Booth (1935) and Booth & Milroy (1935) described the isolation of a water-soluble choline precursor from various animal tissues, and identified this as the choline ester of sphingosinephosphoric acid. King & Small (1939) provisionally identified a similar substance from pig pancreas as the same compound. Aloisi (1947) also isolated the substance from pancreas and studied the rate at which it was oxidized by various tissues.

It was considered by the author that if sphingosylphosphorylcholine occurred in tissues some insight into the metabolism of sphingomyelin might be obtained by studying the relationship between the phosphodiester and the phospholipid after the injection of isotopes. Analogous studies have indicated the metabolic relationship between glycerylphosphorylcholine and lecithin and also between glycerylphosphorylethanolamine and phosphatidylethanolamine in the liver of the rat (Dawson, 1955*a*). However, from fractions obtained by methods equivalent to those described by Booth (1935) and King & Small (1939) no evidence could be obtained for the presence of

sphingosylphosphorylcholine, nor could the compound be detected chromatographically in various tissues of the rat or sheep. These negative results are reported because of the frequency with which the isolation of the phosphodiester is quoted in the literature, e.g. Celmer & Carter (1952), Artom (1952), Rouser, Berry, Marinetti & Stotz (1953).

Attempts were made to prepare sphingosylphosphorylcholine both by the acid and alkaline methanolysis of sphingomyelin. Whereas the results confirm the findings of Rennkamp (1949) that sphingosylphosphorylcholine is a minor component of the acid hydrolysate of sphingomyelin, none of the diester could be obtained from alkaline hydrolysates where its presence has been reported by Rouser *et al.* (1953).

EXPERIMENTAL

Materials. The sphingomyelin used was generously provided by Dr J. Olley and had been prepared from ox spinal cord (Found: N, 3.45; P, 3.8%; N:P molar ratio 2.01:1. Calc. for lignoceryl-sphingosylphosphorylcholine: N, 3.4; P, 3.7%; N:P molar ratio 2:1). No carbohydrate could be detected by the Molisch test. A small sample of sphingomyelin prepared by Dr D. N. Rhodes on a silicic acid column and having an N:P ratio of 2.08:1 gave the same hydrolysis products as the main sample. Phosphorylcholine and glycerylphosphorylcholine were prepared as described by Dawson (1955*b*, 1956). A solution containing sphingosylphosphorylcholine was prepared by hydrolysis of sphingomyelin (20 mg.) with 2 ml. of methanolic 1.4*N*-HCl for 2 hr. in a sealed ampoule at 100°. The