such compounds are present in considerably different proportions from those found in haddock-flesh extracts.

5. Hydrocarbons found in both cod- and haddock-lipid extracts are probably contaminants derived from rubber.

6. Complex acidic lipids occur in the cod extracts, as in those from haddock.

The work described was carried out as part of the programme of the Food Investigation Organization of the Department of Scientific and Industrial Research.

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The Lipids of Fish

7. PHOSPHATE ESTERS IN THE LIPIDS OF HADDOCK AND COD FLESH

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(Received 6 May 1955)

In the course of investigations of fish lipids in this laboratory, it became evident that in certain cases the phosphate esters present in the aqueous phase from an alkaline hydrolysate could not be accounted for as glycerophosphate. The solubility, chromatographic and hydrolytic properties were not compatible with those of the latter ester. Several methods other than the classical separations of the heavy-metal salts have been used in an attempt to identify these other phosphate esters. They are as follows: (1) Paper chromatography. (2) Solubility in ethanol; Fleury & Le Dizet (1950) found that sodium glycerophosphate has a negligible solubility in absolute ethanol even at the boiling point. (3) Susceptibility to hydrolysis at pH 4. Fleury (1948b) used this method to distinguish between glycerophosphoric acid and certain of its derivatives; the latter require a preliminary hydrolysis with N-HCl at 100° to convert them into glycerophosphoric acid which is then hydrolysed at pH 4 to yield inorganic phosphate and glycerol. (4) Estimation of the glycerol:phosphorus ratio of esters susceptible to hydrolysis at pH 4 and also of those hydrolysed at pH 4 only after a preliminary hydrolysis with N-HCl. (5) Separation of phosphate esters according to the solubility of their barium salts in water. 10% of

the phosphate esters from the ethanolic-KOH hydrolysates of the total acetone-insoluble phospholipids of the acetone extract of haddock flesh, which reacted with periodate, had been found to have water-insoluble barium salts (Olley & Lovern, 1953). Barium glycerophosphate is water-soluble.

Schmidt, Greenbaum, Fallot, Walker & Thannhauser (1955) have very recently published a method for distinguishing the glycerylphosphoryl esters by their complete resistance to prostatic and intestinal phosphatases.

EXPERIMENTAL

Two fractions from an acetone extract of cod muscle were examined. These have been referred to (Garcia, Lovern & Olley, 1956; Table 5) as A.S. 1-3 and A.I. 10-17, i.e. the first three most polar fractions in the counter-current distribution of the acetone-soluble (A.S.) lipids of the acetone extract between light petroleum: ethanol-water (85:15, by vol.), and the less polar fractions following the main 'lecithin' peak from the counter-current distribution of the acetone-insoluble (A.I.) lipids of the acetone-soluble fraction, respectively.

These fractions were hydrolysed by refluxing for 2 hr. with 0.5N-KOH in 95% (v/v) ethanol. They were acidified and the fatty acids and unsaponifiable matter were extracted with ether. The aqueous hydrolysate was taken to dryness and excess HCl was removed under vacuum. The

dried residue was then extracted twice with boiling absolute ethanol. The ethanol-soluble and ethanol-insoluble fractions were analysed for total phosphorus. Other fractions examined were a sample of the main acetone-insoluble 'lecithin' fraction from haddock muscle (shown in Fig. 1 of Olley & Lovern, 1953) and a sample of synthetic distearoyl-DL- α -lecithin, a gift from Dr T. H. Bevan, Bristol University. After hydrolysis, acidification and removal of fatty acids and unsaponifiable matter, the hydrolysates were neutralized before being taken to dryness for extraction with ethanol. This was found to be necessary as glycerophosphate is unstable in the pH range 3-5 and it was found that when the pH of a solution of 60 mg. of sodium glycerophosphate in 100 ml. of water was adjusted to 4 and taken to dryness *in vacuo*, 2% of the total phosphorus was hydrolysed to inorganic phosphate.

Phosphorus estimations. The method of LePage & Umbreit (1949) for estimation of total P by ashing with H_2SO_4 and H_2O_2 has always been found satisfactory for glycerophosphate. It was of interest to note, however, that resistance of the fractions from the cod extracts gave significantly low results by this method. King (1932) has pointed out that HClO_4 has four times the oxidative capacity of H_2SO_4 and therefore Allen's (1940) method of digestion with HClO_4 was generally adopted. This treatment appeared to give complete breakdown, since the value obtained agreed with that found after hydrolysis with N-HCl at 100° followed by autoclaving at pH 4. The resistance of certain water-soluble phosphate esters to digestion with H_2SO_4 probably explains the loss of P on alkaline hydrolysis of certain lipid fractions commented on in an earlier paper (Olley & Lovern, 1953).

Estimation of phosphate esters. α - + β -Glycerophosphate has been estimated by the periodate oxidation of the vicinal hydroxyl groups with simultaneous acid hydrolysis of the resulting glycollic aldehyde phosphate and measurement of the orthophosphate liberated, as described by Olley & Blewett (1950). This treatment would also produce orthophosphate from alkyl esters produced by mild alkaline hydrolysis of phospholipids (Fleury, 1948*a*), the polyglycerylphosphates of McKibbin & Taylor (1952), glycerylphosphoryl-choline, -serine and -ethanolamine, and from the many unidentified phosphate esters found together with glycerophosphate in rat liver (Olley & Blewett, 1950). This method was compared with that of Fleury (1948*b*), slightly modified. Fleury heated a solution adjusted to pH 4 at 120° for 24 hr. to obtain orthophosphate from glycerophosphate. The glycerophosphate derivatives were only hydrolysed to a very small extent at this pH. Another sample was first heated for 1 hr. at 100° with N-HCl to convert glycerophosphate derivatives into glycerophosphate, then brought to pH 4 and heated at 120° for 24 hr. Autoclaving for 7 hr. at 15 lb. pressure was found more reliable than Fleury's method, which gave slightly low results. Autoclaving caused no increase in the breakdown of esters resistant to hydrolysis at pH 4. All hydrolyses were carried out in Pyrex vessels. Soda glass was found to cause considerable changes in pH. The glycerol liberated by these hydrolyses was also estimated.

Estimation of glycerol. Glycerol was estimated chromatographically by the method of Fisher, Parsons & Morrison (1948), who found that the area of a chromatographic spot was proportional to the logarithm of the concentration. The chromatograms were run in *n*-propanol-water (80:20, by

vol.) on Whatman no. 1 papers to the specifications of Datta, Dent & Harris (1950). A set of standard glycerol spots was run on every sheet of paper. The papers were dipped in the AgNO_3 - NaOH reagent of Trevelyan, Proctor & Harrison (1950) and washed as described by them with 6*N*- NH_4OH ; this treatment was necessary as substances with the same R_F as glycerol were present in extracts which had not been hydrolysed with N-HCl . These latter spots were washed away with ammonia. The method was reliable for 6-20 μg . of glycerol. Glycerol spots eluted and run in two different solvents showed only a single spot. A further guide to the specificity of the method lay in the fact that glycerol reacts fairly slowly with AgNO_3 - NaOH , whereas other substances, e.g. dihydroxyacetone, which had the same R_F as glycerol in propanol-water, reduced AgNO_3 instantaneously when the paper was dipped in NaOH . In none of the fish extracts did a spot appear immediately. However, an unidentified spot which intensified on washing with NH_4OH appeared in all the ethanol-soluble fish extracts. This substance gave no reaction with the aniline hydrogen phthalate sugar spray or with the fuchsin spray for aldehydes. The R_F values of glycerol and the unknown were estimated in three solvent systems. They were: propanol-water (80:20, by vol.), 0.69, 0.49; phenol- NH_4OH , 0.68, 0.78; butanol-acetic acid-water (5:1:2, by vol.), 0.49, 0.49, respectively.

Barium hydroxide precipitation. Samples of the ethanol-soluble and ethanol-insoluble phosphate esters from hydrolysates of fractions A.S. 1-3 and A.I. 10-17 were shaken with finely-ground $\text{Ba}(\text{OH})_2 \cdot 8\text{H}_2\text{O}$ until alkaline to phenolphthalein. Inorganic P liberated by periodate oxidation and acid hydrolysis was estimated on the water-soluble and water-insoluble barium salts.

Removal of salts with ion-exchange resins. For chromatography of glycerol and phosphate esters it was sometimes necessary to remove inorganic salts. Dawson (1954) and Ansell & Norman (1955) used the weak carboxylic acid resin Amberlite IRC-50 to remove alkali from methanolic- NaOH hydrolysates of lipids. This method is effective, provided that it is done rapidly enough. Thus 0.25*N*- KOH in 50% (v/v) aqueous ethanol containing 5 mg. of Na β -glycerophosphate/ml., passed through a column (1 $\times$ 7.5 cm.) of IRC-50 at the rate of 0.5 ml./min., was found to be hydrolysed to the extent of 1.6% to orthophosphate; however, 12 mg. of Na β -glycerophosphate in 20 ml. of water shaken with 5 g. of IRC-50 was hydrolysed to orthophosphate to the extent of 13% in 1.5 hr. with no $\beta \rightarrow \alpha$ conversion; on the other hand, the same quantity of β -glycerophosphate shaken overnight with 5 g. of a strongly cross-linked sulphonated polystyrene resin (Partridge & Brimley, 1952) was unhydrolysed, but 3.4% was converted from the β into the α form. The same quantity of Na β -glycerophosphate shaken overnight at room temperature with H_2SO_4 equivalent to the acid capacity of 5 g. of polystyrene resin showed 37% $\beta \rightarrow \alpha$ conversion and no hydrolysis. This hydrolysis of glycerophosphate with the weaker resin is possibly explained by the fact that the environmental pH during desalting with IRC-50 is in the region of 4-5 (Kunin & Barry, 1949), a pH range at which glycerophosphate is particularly susceptible to hydrolysis. Thudichum (1884) was aware of the hydrolysis of glycerophosphate under mildly acid conditions. The stronger sulphonic acid resin could not, however, be used, as this appeared to cause hydrolysis of phosphate esters of the

type split by *N*-HCl. Fleury & Le Dizet (1952) do, in fact, comment that the properties of the phosphate esters obtained by the mild alkaline hydrolysis of egg lecithin were altered by ion-exchange resins. Some solutions were desalted by the electrolytic method of Consden, Gordon & Martin (1947).

Chromatography of phosphate esters. Chromatograms were run by the ascending technique in the apparatus of Datta *et al.* (1950). The solvent systems used were phenol- NH_3 and *n*-propanol-water (80:20, by vol.).

RESULTS

Table 1 shows the amounts of phosphorus in an ethanol-soluble and an ethanol-insoluble form present in various preparations. The negligible solubility of sodium and potassium glycerophosphates in absolute ethanol was confirmed (cf. Fleury & Le Dizet, 1950). Distearoyl-DL- α -lecithin, hydrolysed with ethanolic KOH, gave slightly

Table 2 shows that in the present work five types of phosphate ester could be distinguished:

(1) Phosphate esters with ethanol-soluble salts susceptible to hydrolysis at pH 4 after 7 hr. at 15 lb. pressure in an autoclave, producing more than one molecule of glycerol per molecule of phosphoric acid.

(2) Phosphate esters with ethanol-insoluble salts susceptible to hydrolysis at pH 4 after 7 hr. at 15 lb. pressure, producing one molecule of glycerol per molecule of phosphoric acid. This is presumably true glycerophosphate.

(3) A compound not based on glycerol, which after hydrolysis at pH 4 liberated small quantities of phosphoric acid and reduced the glycerol:P ratio in the glycerophosphate fraction to slightly less than 1.

(4) Phosphate esters with ethanol-soluble salts susceptible to hydrolysis at pH 4 only after

Table 1. *Solubility of the Na and K salts of the phosphate esters produced on alkaline hydrolysis of lipids*

Lipids hydrolysed by refluxing with 0.5*N*-KOH in 95% (v/v) ethanol for 2 hr. (~25 ml./g. of lipid). The lipid hydrolysates were acidified and extracted with ether to remove fatty acids and unsaponifiable matter and then neutralized before evaporation to dryness and extraction with ethanol, with the exception of A.S. 1-3 and A.I. 10-17 where excess HCl was removed *in vacuo*.

Fraction	Vol. of ethanol for extraction of phosphate esters (ml.)	Ethanol-soluble P (mg.)	Ethanol-insoluble P (mg.)
Cod muscle A.S. 1-3 (5 g.)	~300	58.4	81.4
Cod muscle A.I. 10-17 (5 g.)	~300	25.6	145.5
Haddock muscle (main 'lecithin' fraction from acetone extract) (3 g.)	200	6.45	84.4
Synthetic distearoyl-DL- α -lecithin (110 mg.)	100	0.29	4.2
Na β -glycerophosphate (100 mg.)	100	0.07	Not determined
K glycerophosphate (365 mg.)	100	0.07	Not determined
1 ml. 20% glycerophosphoric acid refluxed for 2 hr. with 0.5 <i>N</i> -KOH in 95% ethanol	100	0.06	11.8

more ethanol-soluble phosphorus than did glycerophosphoric acid, but this increased solubility may have been due to the presence of choline and traces of fatty acids in the hydrolysate. The 'lecithin' fraction from haddock muscle gave most of the phosphorus in the form of ethanol-insoluble salts, i.e. as true glycerophosphate after refluxing with 0.5*N*-KOH in 95% ethanol for 2 hr. The two cod fractions, on the other hand, in which after counter-current distribution appreciable quantities of unidentified lipids were concentrated, had far higher percentages of ethanol-soluble phosphate ester salts. Ovolecithin, a gift from Dr C. Long, Aberdeen University, behaved similarly to fish-muscle lecithin and appeared to be split to true glycerophosphate.

previous hydrolysis with *N*-HCl for 1 hr. at 100°. The unidentified ethanol-soluble phosphate esters of cod muscle from fractions A.S. 1-3 and A.I. 10-17 liberated after hydrolysis with *N*-HCl and at pH 4 had a glycerol:P ratio of approximately 1:1. The ratio for esters from an acetone-insoluble phospholipid fraction from an acetone extract of haddock muscle was 1.6:1. This difference can be explained by the fact that all of the acetone-insoluble unidentified lipids of the haddock extract were included in this fraction, whereas a fraction comparable to cod A.I. 10-17 would only have represented some 20% of the total unidentified lipids.

(5) Phosphate esters stable to hydrolysis at pH 4 and to *N*-HCl followed by hydrolysis at pH 4.

Table 2. *Periodate oxidation and acid hydrolysis of phosphate esters after alkaline hydrolysis of lipids*

All results related to % of wt. of total phosphorus. Thus, glycerophosphate would give 100% P liberated by periodate method, 100% P liberated at pH 4, and 298 % of glycerol. The difference in glycerol and phosphorus liberated between a preliminary hydrolysis with *n*-HCl at 100° followed by hydrolysis at pH 4 and hydrolysis at pH 4 alone would in this case be 0. Conditions of alkaline hydrolysis as in Table 1.

Fraction	Cod-muscle lipids				Haddock-muscle lipids		
	A.S. 1-3 (ethanol- soluble)*	A.S. 1-3 (ethanol- insoluble)	A.T. 10-17 (ethanol- soluble)	A.T. 10-17 (ethanol- insoluble)	Acetone-insoluble lipids from acetone extract (ethanol- soluble)	Acetone-insoluble lipids from acetone extract (ethanol-soluble) (ethanol dried over Na)	Synthetic distearoyl- lecithin (ethanol- insoluble)
Original alkaline hydrolysate							
Total P	100	100	100	100	100	100	100
Free glycerol	170	87.5	410	13.4	204	205	0
Orthophosphate P	3.5	43.4	2.4	9.3	0	0	0
P liberated by periodate	65.0	43.5	56.5	61.0	68.5	84.0	100
P liberated by <i>n</i> -HCl hydrolysis at 100°	0	0	0	0	0	0	0
Increase in constituents after hydrolysis at pH 4							
Orthophosphate P	66.5	51.0	14.6	70.0	58.5	48.5	100
Glycerol	326	122	102.5	220	230	242	316
Moles glycerol/atom orthophosphate P	1.64	0.80	2.35	1.05	1.32	1.68	1.06
Increase in constituents after hydrolysis with <i>n</i> -HCl at 100° for 1 hr. followed by hydrolysis at pH 4 over constituents present after pH 4 hydrolysis alone							
Orthophosphate P	22.5	0	67.2	9.5	20.8	45.0	0
Glycerol	62.0	1.7	167.0	24.4	99.0	206.0	0
Moles glycerol/atom orthophosphate P	0.92	—	0.84	0.86	1.6	1.54	—
P resistant to hydrolysis with <i>n</i> -HCl and at pH 4	7.5	5.6	15.8	11.2	20.7	6.5	0

* The terms ethanol-soluble or -insoluble refer to the water-soluble phosphate esters from the alkaline hydrolysis of the lipids and not to the lipids themselves.

The nature of these compounds is completely obscure, and it is possible that more prolonged hydrolysis with *N*-HCl might have placed them in the group hydrolysed at pH 4 after preliminary hydrolysis with *N*-HCl.

The fact that periodate oxidation and subsequent estimation of the phosphate liberated from the supposed resulting glycollic aldehyde phosphate is a very non-specific method of estimating glycerophosphate in biological material is particularly well illustrated by the ethanol-soluble fraction of A.I. 10-17 (Table 2). The periodate oxidation method gave 56.5 % of the P as glycerophosphate, while the method of hydrolysis at pH 4 showed only a possible 14.6 % of glycerophosphate and even then the glycerol:P ratio was 2:1 not 1:1.

reacted with periodate to liberate inorganic phosphate had water-insoluble barium salts. The ethanol-soluble phosphate esters all gave water-soluble barium salts and were not, therefore, of the type found by McKibbin & Taylor (1952) in hydrolysates of a polyglycerolphosphatide of dog liver.

Table 4 shows the R_F values of the ethanol-soluble phosphate ester salts from cod-muscle-fractions A.S. 1-3 and A.I. 10-17. In phenol/ NH_3 these salts had the same R_F as sodium glycerophosphate, but in propanol-water they showed a greater mobility than glycerophosphate and the different stability to hydrolysis at pH 4 of these two fractions, shown in Table 2, is reflected in the different R_F values of their two stronger phosphorus spots.

Table 3. *Phosphate esters which react with periodate, fractionated by solubility of their barium salts in water*

Fraction*	P liberated by periodate oxidation (mg.)	
	Water-soluble Ba salts	Water-insoluble Ba salts
Cod muscle		
A.S. 1-3 (ethanol-soluble)	38.0	0
A.S. 1-3 (ethanol-insoluble)	35.0	2.45
A.I. 10-17 (ethanol-soluble)	14.4	0
A.I. 10-17 (ethanol-insoluble)	88.5	1.4

* See footnote to Table 2.

Table 4. *Paper chromatography of ethanol-soluble phosphate esters after alkaline hydrolysis of cod-muscle lipids*

Conditions of alkaline hydrolysis as in Table 1. Chromatograms were run on papers washed with 2*N* acetic acid and sprayed with the reagent of Hanes & Isherwood (1949) for phosphorus compounds. The spots were developed with ultra-violet light (Bandurski & Axelrod, 1951).

Fraction*	Solvent	R_F	Solvent	R_F
Na glycerophosphate	<i>n</i> -Propanol-water	0.08	Phenol/ NH_3	0.27
Cod muscle				
A.S. 1-3 (ethanol-soluble)	<i>n</i> -Propanol-water	0.1 (faint), 0.2	Phenol/ NH_3	0.28
A.I. 10-17 (ethanol-soluble)	<i>n</i> -Propanol-water	0.12 (faint), 0.4	Phenol/ NH_3	0.27

* See footnote to Table 2.

Table 2 also shows that the periodate method is not quantitative for phosphate esters of glycerol other than glycerophosphate. Theoretically, since α and β esters would be estimated, it might have been expected that the inorganic P obtained by simultaneous periodate and acid hydrolysis would correspond to the phosphoric acid liberated by hydrolysis at pH 4 plus that liberated by *N*-HCl hydrolysis followed by hydrolysis at pH 4. Some 70-80 % of this P was estimated by acid periodate in most fractions.

Table 3 shows that only a negligible proportion of the ethanol-insoluble phosphate esters which

DISCUSSION

The classical methods of recovery of glycerophosphate from lecithins and kephalins have involved shaking with aqueous baryta solutions at room temperature, or refluxing with acid, both of which would cause hydrolysis of phosphate esters of the type discussed by Fleury (1948*b*) and Fleury & Le Dizet (1954), and of the cardiolipin of Pangborn (1947). Bailly (1916), who hydrolysed egg lecithin with NaOH in 50 % aqueous ethanol on a hot-water bath, did not use a reflux and, therefore, completed the hydrolysis in aqueous solution,

a medium which has been shown by Fleury & Le Dizet (1953) to produce less of the ethanol-soluble phosphate ester salts. Thus the complex phosphate esters found in fish muscle may be general constituents of tissues which have been overlooked owing to the methods of hydrolysis.

The mild hydrolysis of a lipid may give greater insight into its structure than drastic hydrolysis, or on the other hand may be more prone to produce artifacts. Fleury & Le Dizet (1953) claimed that the hydrolysis of ovoidlecithin with alcoholic alkali at 37° gave polyglycerophosphate chains containing 2–10 atoms of phosphorus depending on the alcohol and the alkali used. Products with supposed chains of 2–3 atoms of phosphorus were soluble in methanol and water, but insoluble in ethanol; those with chains of four or more atoms of phosphorus were soluble in methanol, ethanol and water (Fleury & Le Dizet, 1952). Kahane in discussion (see Fleury, 1948c) pointed out that methanolic-HCl hydrolysis of choline esters resulted in transesterification and thought that this could also occur in Fleury's work. Baer & Kates (1950) were unable to obtain insoluble lead salts of glycerophosphoric acid from synthetic dipalmitoyl-L- α -lecithin hydrolysed with methanolic NaOH at 37°; methanolysis had presumably occurred in this case also. Baer (1952) pointed out that a re-investigation was desirable of the tentative structure for cardiolipin of Pangborn (1947) which was based on ethanolic-KOH hydrolysis at room temperature. Fleury & Le Dizet (1954), in their later work, now claim that the ethanol-soluble fraction from the mild hydrolysis of ovoidlecithin with methanolic NaOH is a mixture of α - and β -methyl glycerophosphates. The methanol-soluble esters have yet to be investigated.

The results described in this paper would not appear to be attributable to artifacts of hydrolysis, although this possibility should be borne in mind. Hydrolyses of ovoidlecithin and synthetic distearoyl-DL- α -lecithin with 0.5N-KOH in 95% ethanol at 37° for 15 min. gave almost complete liberation of choline. Ethanolysis would not, therefore, have time to occur at 37° and even less under reflux. As only methanolysis occurs when choline is liberated from lecithin under mild hydrolytic conditions, it has been assumed that transesterification is a slow reaction; where choline is liberated rapidly from a lipid, free glycerophosphoric acid is formed. Table 1 shows that no esterification of glycerophosphoric acid occurs after refluxing for 2 hr. with 0.5N-KOH in 95% ethanol. This rapid liberation of choline with ethanolic KOH is in contrast to the action of 0.2N-NaOH in methanol at 37° for 15 min. which Dawson (1954) used to produce glycerylphosphorylcholine from lecithin. Baer & Kates (1950), in fact, found that only 80%

of the choline was liberated in 32 hr. under these conditions.

Table 2 showed that five types of phosphate ester could be distinguished from fish-muscle lipids. The differentiation of these types according to ethanol solubility and hydrolytic properties has been summarized in the 'Results' section of the paper. The susceptibility of a phosphate ester to hydrolysis at a particular pH provides a useful guide to its identity.

Desjobert, Fleury & Courtois (1948) found that, if the percentage liberation of inorganic phosphate from a phosphate ester at constant time was plotted against acid normality and pH, two different types of phosphate ester could be distinguished: those with no aldehydic or ketonic groups, such as ethyl phosphate, β -hydroxyethyl phosphate and glycerophosphate, which are hydrolysed at a maximal rate at pH 4, but at minimal rates at pH 2 and in more alkaline solutions, and the 'ose' phosphates, which have a minimal rate of hydrolysis at pH 4. They also found that, whereas the former group of phosphate esters were hydrolysed more readily by halogen acids than by sulphuric acid, the latter group were hydrolysed at similar rates by both acids. Fleury, Courtois & Desjobert (1948) also found that ethyl and β -hydroxyethyl phosphates had very different rates of hydrolysis with hydriodic, hydrobromic and hydrochloric acids. (Hydrochloric acid has always been used in this work for all hydrolyses and neutralizations. Trichloroacetic acid, which could have been removed with ether, thus facilitating subsequent chromatography, might have been employed with advantage. Its hydrolytic properties for phosphate esters would, however, have to be studied.) Other phosphate esters with a hydrolysis maximum at pH 4 are inositol phosphates (Fleury, 1946), *O*-carboxynaphthyl phosphates (Chanley & Gindler, 1953) and salicyl phosphates (Chanley, Gindler & Sabotka, 1952).

The hydrolysis of glycerophosphate has been most extensively studied (Malengreau & Prigent, 1911; Plimmer, 1913; Bailly, 1942; Swoboda & Crook, 1955). The last-named determined the rate constants, activation energies and Arrhenius frequency factors for glycerol 1-phosphate, glycerol 2-phosphate and ethanolamine phosphate. The rate of hydrolysis, which is negligible at pH 8, rises to a broad maximum at pH 3–4, is minimal at pH 0.5 and increases again in more acid solutions. The hydrolysis peak at pH 3–4 is attributed to the $C_3H_5(OH)_2.OPO_3H^-$ ion; therefore substituted derivatives, e.g. methyl glycerophosphate (Fleury & Le Dizet, 1954) and glycerylphosphorylcholine are hydrolysed only to free phosphoric acid and glycerol at pH 4 after a preliminary hydrolysis with N-HCl. Theoretically, cardiolipin-type com-

pounds and *bis*-phosphatidic acids should fall into the latter category. It is therefore difficult to explain the occurrence of phosphate esters with more than one glycerol residue per atom of phosphorus in both the group hydrolysed at pH 4 directly and at pH 4 only after a preliminary hydrolysis with *N*-HCl. The ethanol-soluble phosphate ester hydrolysed at pH 4 only after *N*-HCl hydrolysis, giving one molecule of glycerol to one atom of phosphorus, could not be glycerylphosphorylcholine in the present work owing to the latter's rapid hydrolysis with ethanolic KOH. Similarly, the presence of glycerylphosphoryl-ethanolamine and -serine is unlikely. Fractions A.S. 1-3 from cod muscle contained neither of these substances. Compounds of the type 'glycerylphosphoryl X' with a slower rate of hydrolysis in ethanolic KOH must, therefore, be envisaged. No increase in compounds reacting with ninhydrin or the phosphomolybdate spray of Levine & Chargaff (1951) were detected in the chromatograms of the fraction hydrolysed with *N*-HCl followed by hydrolysis at pH 4.

Garcia *et al.* (1956) have pointed out that their unidentified lipids from fish muscle had a fatty acid: 'glycerophosphate P' ratio of approximately 4.7:1. As discussed in the 'Results' section, only 70-80 % of the phosphates of glycerol were estimated by the periodate method and the corrected ratio should therefore be reduced to the more probable figure of 4:1. Structures of the bisphosphatidic acid type (Baer, 1952) could be envisaged:

These compounds were accompanied in ethanolic-KOH hydrolysates by free glycerol and could have been produced by the hydrolysis of cardiolipin-type compounds which according to Pangborn (1947) gave with ethanolic KOH, glycerophosphate, free glycerol, glycerylphosphatidate esters and glyceryltriglycerophosphate. The possibility of free glycerol deriving from the hydrolysis of mono-glycerides and inositol lipids has been discussed previously (Lovern & Olley, 1953; Olley & Lovren, 1954). Fractions A.S. 1-3, however, contained no inositol.

It is of possible relevant interest that Olley & Blewett (1950) found a series of phosphate esters reacting with periodate in a trichloroacetic acid extract of rat liver. Trichloroacetic acid has been shown to hydrolyse phospholipids (Etienne-Petitfils & Kahane, 1954), and it is possible that these phosphate esters were of lipid origin.

SUMMARY

1. Phospholipid fractions from haddock and cod muscle, containing appreciable quantities of unidentified lipids, were hydrolysed with 0.5*N*-KOH in 95 % ethanol for 2 hr. under reflux. Potassium salts of phosphate esters from these hydrolysates were fractionated on the basis of their solubility in ethanol, by paper chromatography, by stability to hydrolysis at pH 4 for 7 hr. in an autoclave at 15 lb. pressure and by stability to hydrolysis at pH 4 after a preliminary hydrolysis with *N*-HCl.

2. These methods showed the presence of a series of phosphate esters based on glycerol and small quantities of phosphoric acid in some other combination.

3. A micro-chromatographic method for the estimation of glycerol has been developed, whereby the amounts of glycerol and phosphoric acid liberated under different conditions of hydrolysis could be correlated. Phosphate esters with more than one glycerol residue per atom of phosphorus were found in the ethanol-soluble fractions, giving evidence of *bis*-phosphatidic acid-type structures. These could be divided into two classes according to stability at pH 4.

4. The method of acid-periodate oxidation has been found completely unspecific for glycerophosphate esters. In some fractions acid-periodate oxidation liberated more phosphoric acid than was formed by hydrolysis at pH 4, which is known to yield quantitative hydrolysis of glycerophosphoric acid. In other fractions, hydrolysed with *N*-HCl and then at pH 4, the glycerol and phosphoric acid liberated were more than could be accounted for by the results obtained from acid-periodate oxidation.

5. The dangers of using even weakly acid ion-exchange resins for the separation of phosphate esters have been discussed.

The work described above was carried out as part of the programme of the Food Investigation Organization of the Department of Scientific and Industrial Research.

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The Oxidative Activity of Particulate Fractions from Germinating Castor Beans

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(Received 6 April 1955)

During an investigation of the catabolic changes in germinating castor beans (*Ricinus communis*) highly active preparations were separated from the endosperm by methods similar to those employed by Laties (1953a, b) and by other workers with a variety of plant materials. The general properties of these particulate fractions are similar to those which have been described earlier, but some interesting distinguishing features were noted. Preparations from other tissues frequently show appreciable endogenous oxygen uptake and may be capable of rapid oxidation of Krebs-cycle acids without added cofactors or upon the single addition of adenosine triphosphate (ATP) or diphosphopyridine nucleotide (DPN) (e.g. Millerd, Bonner, Axelrod & Bandurski, 1951; Davies, 1953). The *Ricinus* preparations, on the other hand, are characterized by an extremely low endogenous uptake, and, with the sole exception of succinate, the unassisted oxidation of acids is slow. However,

high rates of oxidation are obtained in all cases after the addition of a full complement of cofactors, and these features make the preparations especially suitable for the study of substrate-cofactor interrelationships. Such an investigation has led to the demonstration of distinct requirements, not only for adenosine triphosphate (ATP) and diphosphopyridine nucleotide (DPN) but also for coenzyme A (CoA) and cocarboxylase.

MATERIALS AND METHODS

Substrates. All the substrate acids were obtained commercially; *cis*-aconitic acid and oxaloacetic acid of 99±1% purity were obtained from the California Foundation for Biochemical Research, Los Angeles, California. Sodium DL-isocitrate, sodium pyruvate and α-oxoglutaric acid were supplied by the H.M. Chemical Co., Santa Monica, California. L-Malic acid was obtained from Eastman Kodak Co. Ltd., Rochester, New York. For use, all the substrates were dissolved in water and carefully adjusted to pH 7, using a Beckman Model H2 pH meter.

Cofactors. DPN '90' and disodium ATP were products of the Sigma Chemical Co., St Louis, Missouri, and crystal-

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