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Effect of Lipids and Organic Solvents on the Enzymic Formation of Zinc Protoporphyrin and Haem

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1. Differences observed in earlier work between the enzymic chelation with protoporphyrin of Zn^{2+} and Fe^{2+} ions respectively have now been explained as being caused by the presence of peroxides in the ether used in the enzyme assay. The inhibitory effect of peroxides is established by the reducing agent, which is present in the assay for chelation of iron but not in that for zinc. There are now no reasons for the belief that two different enzymes catalyse formation of complexes with zinc and iron respectively. 2. Removal of lipid from both chromatophores and mitochondria markedly reduced chelatase activity. Activity could be partially restored by the addition of lipid fractions. Phosphatidic acid, but not phosphatidylcholine or phosphatidylethanolamine, actively stimulated the formation of zinc protoporphyrin and haem by chromatophores and mitochondrial preparations. 3. Lipid-containing extracts of chromatophores, and fractions thereof obtained by silicic acid chromatography, partially restored chelatase activity of Tween extracts of mitochondria. Thus, although both enzymes are considered to be lipoproteins, the identity of the lipids concerned is still uncertain. 4. A great number of organic solvents such as esters, ethers, ketones and, to a lesser extent, alcohols, stimulate enzymic chelation of both metals with protoporphyrin. A number of explanations for these findings are considered and it is suggested that organic solvents interact in some way with the enzyme lipoprotein, changing either its conformation or allowing closer contact between the enzyme and its substrates.

Neuberger & Tait (1964) reported the presence of an enzyme which catalysed the formation of zinc protoporphyrin in chromatophores of *Rhodopseudomonas spheroides* and in mitochondria from a number of mammalian tissues. They discussed whether this enzyme might be identical with the ferrochelatase present in the same sources. Some of the properties of these chelatases were studied and, in particular, a marked stimulation was noted when the reactions were brought about in water–ether emulsion.

In this paper we report further properties of both non-enzymic and enzymic systems which form zinc protoporphyrin and haem. Other organic solvents, in addition to ether, have been found to stimulate the enzymic reactions with both chromatophores and mitochondria. It was found that phosphatidic acid stimulates the enzymic reaction. The chelatase in mitochondria from guinea-pig and pig liver has been brought into solution and, in this form, its activity is stimulated by a lipid extract obtained from chromatophores of *R. spheroides*. The evidence reported here also leads us to believe that both zinc–protoporphyrin-chelatase and ferrochelatase activities are catalysed by the same enzyme. Generally assays have been done with zinc, and not with iron, because assays with the former are easier to perform and are more sensitive, but all the more important results have been obtained with both metals.

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MATERIALS AND METHODS

Phosphatidylethanolamine and phosphatidylcholine were prepared from egg yolk by the method of Rhodes & Dawson (1960). Phosphatidic acid was prepared from phosphatidylcholine by hydrolysis with a partially purified phospholipase D (EC 3.1.4.4) from cabbage (Davidson & Long, 1958), and was purified by chromatography on silicic acid followed by precipitation as the sodium salt (Kates, 1955). Trypsin, chymotrypsin and bovine plasma albumin were obtained from Armour and Co. Ltd., Eastbourne, Sussex. Organic solvents used in the enzyme assays were dried over Na_2SO_4, since it was found that a number of them, if used without drying, caused zinc protoporphyrin formation in the absence of added $ZnSO_4$. This did not occur with solvents

which had been dried before being added to the enzyme assays. Peroxide-free ether was prepared by the method of Vogel (1954).

Enzyme sources. Chromatophores were prepared according to Gibson, Neuberger & Tait (1963) from *R. spheroides*. The organisms used were grown semi-anaerobically in the light in the malate medium of Lascelles (1959).

Mitochondria were prepared from guinea-pig or pig liver essentially by the method of Hogeboom (1955). After removal of nuclei from the homogenate by centrifuging at low speed, the supernatant was centrifuged at 15000*g* for 10min. The supernatant and the loosely packed pink pellet were removed and the tightly packed brown pellet was washed, by suspending in 0·25M-sucrose and recentrifuging, to give 'heavy' mitochondria. The 15000*g* supernatant, including the loosely packed pink pellet, was centrifuged at the same speed for 20min. to give a pink pellet which was washed by resuspending and recentrifuging to give 'light' mitochondria. The 'light' fraction is probably a mixture of lysosomes (cf. Appelmans, Wattiaux & de Duve, 1955) and mitochondria which have been damaged during the isolation (cf. Hatefi & Lester, 1958).

Estimations and chromatographic methods. Estimations of protein and porphyrins, and of zinc–protoporphyrin-chelatase and ferrochelatase activities, were performed as described by Neuberger & Tait (1964). Lipids were extracted from aqueous suspensions of fractions with chloroform–methanol mixtures (Bligh & Dyer, 1959). Samples of lipids were digested and their phosphorus content was estimated according to Bartlett (1959).

Column chromatography of lipids was performed on silicic acid (100mesh; Mallinckrodt Chemical Works, U.S.A.) activated by heating at 100° overnight before use.

Thin-layer chromatography of lipids was performed on 20cm. × 20cm. glass plates coated with Keiselgel G (E. Merck A.-G., Darmstadt, Germany) and developed with chloroform–methanol–water (65:25:4, by vol.; Wagner, Hörhammer & Wolff, 1961). Lipids were located by exposure of the dried plates to iodine vapour (Sims & Larose, 1962), or by spraying with Rhodamine 6 G (Marinetti, Erbland & Kochen, 1957), or by spraying with 10N-H_2SO_4 followed by heating at 160° (Wagner *et al.* 1961). Constituents containing free amino groups were detected with ninhydrin. The identity of the lipids in mixtures was established by comparison of their R_F values with those of pure lipids prepared from egg yolk (see above) and with R_F values given in the literature (cf. Lepage, 1964).

RESULTS

Non-enzymic formation of zinc protoporphyrin. Neuberger & Tait (1964) have shown that under the conditions used in the enzyme assays (0·2M-tris buffer, pH8·4, 0·08mM-protoporphyrin, 0·06mM-zinc chloride, incubated at 37°) non-enzymic formation of zinc protoporphyrin was slow as compared with enzymic formation, and slow compared with the non-enzymic formation of zinc complexes of deuteroporphyrin, mesoporphyrin, haematoporphyrin and coproporphyrin. It was also shown that, unlike the enzymic reaction, the non-enzymic reaction was not stimulated by ether. If the pH was raised above 10, the rate of formation of zinc protoporphyrin increased markedly (Fig. 1). This was found in tris or triethanolamine buffers, but not in carbonate–bicarbonate or glycine–sodium hydroxide buffers of similar pH values. In tris buffer with the addition of sodium lauryl sulphate (Fig. 1) the reaction proceeded more rapidly, and even at lower pH values. The rate at a given pH value was dependent on the concentration of sodium lauryl sulphate up to 0·5% (w/v); higher concentrations did not stimulate the reaction further. Several anionic, cationic and non-ionic detergents, such as sodium deoxycholate, cetyltrimethylammonium bromide, acetylpyridinium bromide, Tween 20 and Tween 80 had no effect on zinc protoporphyrin formation, when used at a concentration of 0·5% (w/v) in place of sodium lauryl sulphate. Different pH–activity curves were obtained in the presence of sodium lauryl sulphate when triethanolamine was used

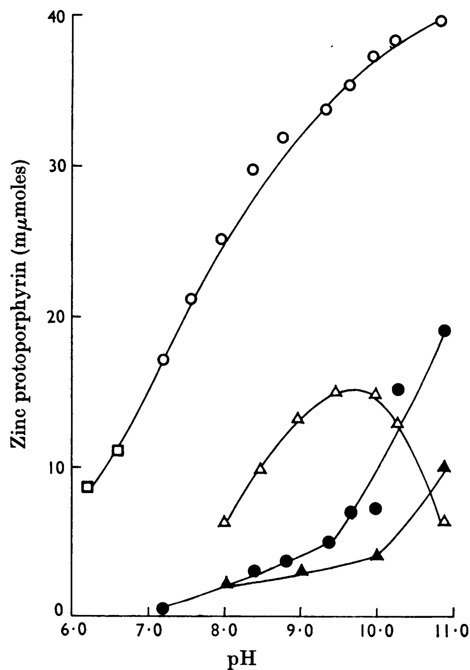

Fig. 1. Effect of pH in different buffers on the non-enzymic formation of zinc protoporphyrin in the presence and absence of sodium lauryl sulphate. Buffer (tris, ○; triethanolamine, △; maleate, □) of pH stated (100μmoles), protoporphyrin (45mμmoles) and $ZnSO_4$ (40mμmoles) were incubated in a total volume 0·5ml. at 37°. The incubation was for 45min. when sodium lauryl sulphate (2·5mg.) was present (○, △, □) and for 90min. when it was not used (●, ▲). The amounts of zinc protoporphyrin formed were measured as described by Neuberger & Tait (1964).

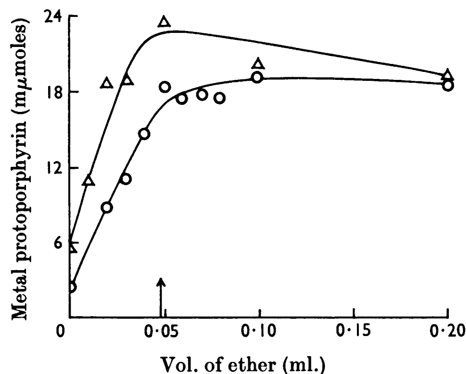

Fig. 2. Effect of diethyl ether on the formation of zinc protoporphyrin and haem catalysed by chromatophores. Tris, pH 8·4 (100 μmoles), protoporphyrin (42 mμmoles), chromatophores (1·1 mg. of protein) and either $ZnSO_4$ (40 mμmoles) or $FeSO_4$ (100 mμmoles) plus ascorbic acid (10 μmoles) were mixed in a volume 0·5 ml. The volume of ether stated was added, the tubes were tightly stoppered, shaken and incubated at 32° for 1 hr. Zinc protoporphyrin (○) and haem (△) were measured as described by Neuberger & Tait (1964). The arrow denotes the formation of a two-phase system.

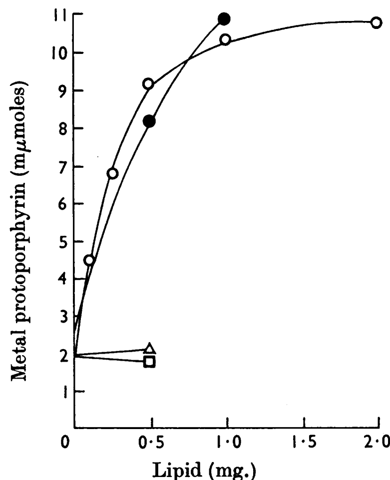

Fig. 3. Effect of lipids on zinc protoporphyrin and haem formation catalysed by chromatophores. Phosphatidic acid (○ and ●) or phosphatidylethanolamine (△) or phosphatidylcholine (□) were added in ether solution to the tubes, and the ether was evaporated by a stream of nitrogen. To the tubes were added tris, pH 8·4 (100 μmoles), protoporphyrin (40 mμmoles), either $ZnSO_4$ (40 mμmoles) or $FeSO_4$ (100 mμmoles) plus ascorbic acid (10 μmoles), chromatophores (0·9 mg. of protein) and water to 0·5 ml. The lipids were emulsified by vigorous manual shaking and the samples incubated at 37° for 1 hr. Zinc protoporphyrin (○, □, △) and haem (●) were measured as described by Neuberger & Tait (1964).

Table 1. *Effect of organic solvents on zinc–protoporphyrin-chelatase activity in chromatophores*

Tris, pH 8·4 (100 μmoles), protoporphyrin (42 mμmoles), $ZnSO_4$ (40 mμmoles), chromatophores (1·5 mg. of protein) and water were made to 0·5 ml. Organic solvent (0·02 ml., except where otherwise stated) was added and after thorough mixing incubation was carried out at 37° for 1 hr. The porphyrins were isolated and the amount of zinc protoporphyrin formed was estimated as described by Neuberger & Tait (1964).

Solvent	Zinc protoporphyrin (mμmoles/assay)	Solvent	Zinc protoporphyrin (mμmoles/assay)
None	3·2	Acetone (0·1 ml.)	12·0
Ether (0·2 ml.)	14·6	Ethyl methyl ketone (0·04 ml.)	13·0
Methyl acetate (0·07 ml.)	16·5	Ethylene glycol monoethyl ether	5·2
Ethyl acetate	18·1	Dioxan	8·7
Ethyl acetoacetate	16·5	Ethanol	5·9
Ethyl chloroacetate	14·1	Propanol	7·3
Ethyl formate	12·0	Butanol	6·1
Diethyl malonate	11·2	Hexane	4·1

instead of tris (Fig. 1). None of the organic solvents used in the enzymic work (see below) had any stimulatory effect in the absence of enzyme, as shown in experiments carried out in various buffers at high pH values and with the various detergents mentioned above.

Enzymic formation of zinc protoporphyrin and haem catalysed by chromatophores. Neuberger & Tait (1964) showed that the rate of formation of zinc protoporphyrin and haem, catalysed by chromatophores, was increased by a factor of about six when the assays were carried out in a water–ether emulsion. Fig. 2 shows that the stimulation was linearly proportional to the concentration of ether up to saturation; addition of more ether had no further effect. A similar increase in the rate of the reaction occurred when ether was replaced by ketones or esters, whereas alcohols and hydrocarbons had little effect (Table 1). The effect of a number of ketones and esters was studied in more detail and it was found that maximum stimulation was caused by addition of 0·1 ml. of acetone, 0·04 ml. of ethyl methyl ketone or 0·03 ml. of ethyl acetate to the 0·5 ml. of aqueous reaction mixture; at higher concentrations the rate of the reaction was reduced,

At optimum concentrations of each organic solvent the rate of the reaction was increased to about the same extent.

In the presence of organic solvents, as in their absence (Neuberger & Tait, 1964), the rate of the reaction was linear with increasing amounts of chromatophore protein per assay up to 1·2mg.

None of the above compounds had any effect on the rate of the non-enzymic formation of zinc protoporphyrin. The same detergents examined for an effect on the non-enzymic formation of zinc protoporphyrin were tested on the enzymic reaction. Tween 20 and 80 stimulated by a factor of two but the others were without effect. Any action of sodium lauryl sulphate on the enzymic reaction was obscured by its large effect on the non-enzymic reaction.

The effect of a number of phospholipids, prepared from egg yolk, was tested. Phosphatidic acid stimulated the production of zinc protoporphyrin and haem (Fig. 3), but phosphatidylcholine and phosphatidylethanolamine were inactive. The amount of phosphatidic acid required to give maximum stimulation increased with the amount of chromatophores used. When the reaction was performed in the presence of phosphatidic acid or phosphatidic acid plus ether, addition of excess of zinc sulphate did not inhibit the reaction as it did in the presence of ether only. Thus, when chromatophores (1·4mg. of protein) were assayed with 50mμmoles and 150mμmoles of zinc sulphate, the amounts of zinc protoporphyrin formed in 90min. were 14·6 and 6·3mμmoles respectively in the presence of ether (0·2ml.), 10·4 and 10·6mμmoles respectively in the presence of phosphatidic acid (1·5mg.) and 23·4 and 21·5mμmoles respectively in the presence of ether plus phosphatidic acid. From these results it can be seen that phosphatidic acid stimulates activity even in the presence of ether.

Attempts to extract the enzyme from chromatophores with a number of detergents were unsuccessful. Thus chromatophores were frozen and thawed in the presence of Tween 20 (10mg./ml.) and then centrifuged at 105000*g* for 1hr. Only low enzymic activity was detected in the supernatant, or the pellet (chromatophore-Tween pellet), or when the two fractions were combined.

Table 2. *Effect of extracting chromatophores with acetone on the zinc–protoporphyrin-chelatase activity*

Acetone-dried powder was prepared from chromatophores by the method of Morton (1955). It was suspended in 0·05M-tris, pH 7·4, before use. Chromatophores and acetone-dried powder were extracted with 90% (v/v) acetone at 0° as described by Lester & Fleischer (1961). The pellet was dried and suspended in 0·05M-tris, pH 7·4. The 90% (v/v) acetone extract was evaporated to dryness under N_2 and the residue dissolved in a small volume of dry ether. Chromatophore-Tween pellet was prepared as described in the text. Assay: tris, pH 8·4 (100μmoles), protoporphyrin (47·5mμmoles), $ZnSO_4$ (50mμmoles), preparations as described above, and water were made to 0·5ml. Ether (0·2ml.) or acetone (0·1ml.) was added and, after mixing, incubation was carried out for 60min. at 37°. The amount of zinc protoporphyrin formed was estimated as described by Neuberger & Tait (1964).

Preparation	Additions	Zinc protoporphyrin (mμmoles)	
		Ether	Acetone
Chromatophores (2·15mg. of protein)	None	31·8	—*
Acetone-dried powder (2·05mg. of protein)	None	12·8	17·9
Acetone-dried powder (2·05mg. of protein)	Chromatophore-Tween pellet (0·36mg. of protein)	22·9	16·4
Acetone-dried powder (1·56mg. of protein)	None	5·5	—
Acetone-dried powder (1·56mg. of protein)	Lipid extracted from chromatophores with $CHCl_3$–methanol (0·19μmole of lipid phosphate)	9·8	—
Powder prepared by extracting acetone-dried powder with 90% (v/v) acetone (1·71mg. of protein)	None	1·0	3·4
	Chromatophore-Tween pellet (0·55mg. of protein)	20·0	20·7
	Lipid extracted with 90% (v/v) acetone (0·55μmole of lipid phosphate)	9·0	—
None	Chromatophore-Tween pellet (0·55mg. of protein)	4·0	2·6
Powder prepared by extracting chromatophores with 90% (v/v) acetone (1·67mg. of protein)	None	6·0	13·4
	Chromatophore-Tween pellet (0·55mg. of protein)	18·7	19·2

* Not measured.

The activation of the chelatases by organic solvents, their inactivation by detergents, and the fact that chromatophores have a high content of lipid (cf. Newton & Newton, 1957) suggested that lipid played some role in these reactions. An acetone-dried powder of chromatophores had a lower zinc–protoporphyrin-chelatase activity than untreated chromatophores; further, the activity of the acetone-dried powder was increased by chromatophore-Tween pellet (prepared as described above) or by lipids extracted from chromatophores with chloroform–methanol mixture (Table 2). This acetone-dried powder was green, showing that all the bacteriochlorophyll had not been removed, and chromatography of an extract made with chloroform–methanol showed that a considerable amount of phospholipid had remained after acetone extraction. The acetone-dried powder and untreated chromatophores were extracted with 90% (v/v) acetone at 0°, a method reported by Lester & Fleischer (1961) to remove most of the phospholipid of mitochondria. Such preparations were colourless and had very low activity when assayed in the presence of ether or acetone; this activity was greatly increased by adding a chromatophore-Tween pellet or an ether solution of the lipid extracted with 90% (v/v) acetone (Table 2).

The ability of chromatophores to catalyse the formation of zinc protoporphyrin and haem was markedly reduced by preincubation with either trypsin or chymotrypsin. For example the zinc–protoporphyrin-chelatase activity of chromatophores (1·5mg. of protein) fell by about 50% on incubation at pH 8·4 and at 37° for 1hr. with trypsin (60μg.).

Effect of peroxides on chelatase activity in mitochondria. Ether and other organic solvents stimulated chelatase activity with mitochondria as they did with chromatophores. However, when mitochondria were assayed for zinc–protoporphyrin-chelatase activity in the presence of ether, reproducible results were not obtained; in addition the activity did not increase linearly with increasing amounts of mitochondria; the activity per mg. of protein was lower with small amounts of mitochondria and higher with large amounts of mitochondria.

Porra & Jones (1963*a*) and Labbe & Hubbard (1960) showed that the ferrochelatase activity appeared in the supernatant after centrifuging (36000*g* for 1hr.) a preparation of mitochondria previously frozen and thawed in Tween 20 (10mg./ml.). Such supernatants also showed zinc–protoporphyrin-chelatase activity (Neuberger & Tait, 1964). In the present work the mitochondria, after treatment with Tween 20, were centrifuged at 105000*g* for 1hr. and both activities were still present in the supernatant.

When this supernatant was assayed for zinc–protoporphyrin-chelatase activity in the presence of ether, results similar to those described above with whole mitochondria were obtained (Fig. 4). When chromatophore-Tween pellet (prepared as described above) was added, the activity was increased, particularly when small amounts of mitochondrial supernatant were used, and the enzyme–activity curve was now linear (Fig. 4). Stimulation of enzyme activity was also obtained by adding ascorbic acid to the assay. When assays were done in the presence of acetone reproducible results were always obtained and the addition of chromatophore-Tween pellet and ascorbic acid did not have marked effects on the activity. Reproducible results and linear enzyme–activity curves were usually obtained for

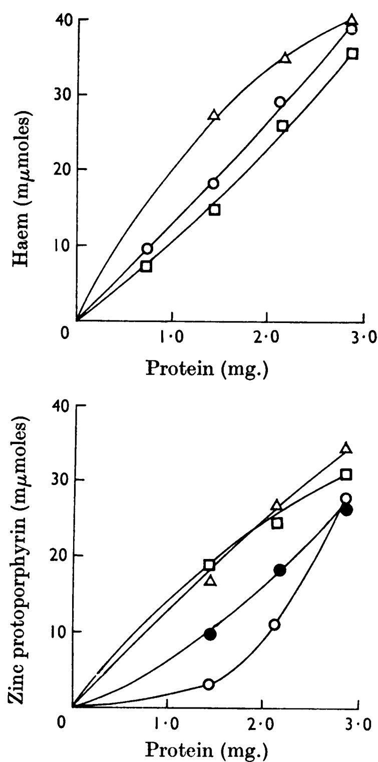

Fig. 4. Enzyme–activity curves for zinc protoporphyrin and haem formation catalysed by guinea-pig liver 'light'-mitochondrial-Tween supernatant. Tris, pH 8·4 (100μ-moles), protoporphyrin (47·5mμmoles), either $ZnSO_4$ (50mμmoles) or $FeSO_4$ (100mμmoles) plus ascorbic acid (10μmoles), 'light'-mitochondrial-Tween supernatant (as stated) and chromatophore pellet (when added, 0·36mg. of protein) were mixed in a volume 0·5ml. Ether (0·2ml.) or acetone (0·1ml.) was added and, after mixing, the samples were incubated at 37° for 1hr. Zinc protoporphyrin and haem were measured as described by Neuberger & Tait (1964). ○, Ether; △, ether+chromatophore pellet; □, acetone; ●, ether+ascorbic acid (10μmoles).

Table 3. *Activity of zinc–protoporphyrin chelatase in supernatant from Tween-treated guinea-pig-liver mitochondria when assayed in the presence of different compounds*

Supernatant from Tween-treated guinea-pig-liver mitochondria was prepared as described in the text. Assay: tris, pH 8·4 (100 μmoles), protoporphyrin (50 mμmoles), $ZnSO_4$ (50 mμmoles), supernatant from Tween-treated mitochondria (as stated) and, where added, ascorbic acid (10 μmoles) and chromatophore pellet (0·18 mg. of protein) were mixed in 0·5 ml. of water. Freshly dried ether (0·2 ml.) or peroxide-free ether (0·2 ml.) or acetone (0·1 ml.) was added and, after mixing, the tubes were incubated at 37° for 1hr. The amount of zinc protoporphyrin formed was estimated as described by Neuberger & Tait (1964).

	Zinc protoporphyrin (mμmoles)			
Additions to assay — Mitochondrial-Tween supernatant (mg. of protein/assay)	0·48	0·80	0·96	1·44
Ether	2·5	7·0	15·0	31·8
Ether + chromatophore pellet	13·9	25·5	26·4	36·3
Ether + ascorbic acid	7·5	21·8	20·5	34·8
Ether + ascorbic acid + chromatophore pellet	18·8	—*	36·3	39·3
Peroxide-free ether	12·1	19·0	23·1	35·0
Peroxide-free ether + ascorbic acid	—	20·0	—	—
Peroxide-free ether + ascorbic acid + chromatophore pellet	—	32·5	—	—
Acetone	—	—	23·5	—

* Not measured.

ferrochelatase activity, which is always assayed in the presence of ascorbic acid (Fig. 4). Chromatophore-Tween pellet stimulated ferrochelatase activity. These observations, and particularly those showing the effect of ascorbic acid on zinc–protoporphyrin-chelatase activity, suggested the possibility of an inhibitory effect by peroxides in the ether. This effect would be observed only when assays were done in the absence of a reducing agent such as ascorbic acid. The results also suggested that at least part of the effect of chromatophore pellet might be due to some component in it reacting with the peroxides and thus protecting the enzyme.

These suggestions were confirmed by comparing activities for zinc–protoporphyrin chelatase with different concentrations of mitochondrial supernatant in the presence of both ordinary dry ether and peroxide-free ether. The activities in the presence of the latter were higher than with the former and the enzyme–activity curve was linear (Table 3). With peroxide-free ether the activity was identical with that found by using acetone and with that observed when ascorbic acid was used together with ordinary dry ether.

All further work has been done with peroxide-free ether, except where stated otherwise, and as an additional precaution ascorbic acid has been added when assaying for zinc–protoporphyrin-chelatase activity.

Effect of chromatophore-Tween pellet and of lipids on chelatase activities. Although chromatophore pellet overcame the effect of peroxides in the ether, the results in Table 3 show that even with peroxide-free ether chromatophore pellet, which had negligible chelatase activity on its own, gave a marked stimulation of zinc–protoporphyrin-chelatase activity. Chromatophore pellet also stimulated ferrochelatase activity when ether or acetone was used (Table 4).

Table 4. *Effect of chromatophore pellet on the ferrochelatase activity in mitochondrial supernatant, assayed in the presence of ether and acetone*

Supernatant was prepared from 'heavy' and 'light' mitochondria of guinea-pig liver after treating with Tween 20, as described in the text. Assay: tris, pH 8·2 (100 μmoles), protoporphyrin (50 mμmoles), $FeSO_4$ (100 mμmoles), ascorbic acid (10 μmoles), 'heavy'-mitochondrial supernatant (0·65 mg. of protein) or 'light'-mitochondrial supernatant (0·42 mg. of protein), and, where added, chromatophore pellet (0·50 mg. of protein), were mixed in 0·5 ml. of water. Acetone (0·1 ml.) or ether (0·2 ml.) was added and, after shaking, the tubes were incubated at 37°. The amount of haem formed was estimated as described by Neuberger & Tait (1964).

Source of Tween supernatant		Haem (mμmoles)
Heavy mitochondria	Ether	15·0
Heavy mitochondria + chromatophore pellet	Ether	25·0
Heavy mitochondria	Acetone	15·5
Heavy mitochondria + chromatophore pellet	Acetone	21·4
Light mitochondria	Ether	0·0
Light mitochondria + chromatophore pellet	Ether	17·9
Light mitochondria	Acetone	0·6
Light mitochondria + chromatophore pellet	Acetone	10·9

Chromatophore pellet did not lose any of its ability to stimulate chelatase activity when boiled for 3 min. whereas mitochondrial supernatant was inactivated by this treatment. This finding suggested that the active factor was not a protein and this was confirmed when it was found that lipids extracted from chromatophores of chromatophore-Tween pellet and added to the assays in solution in ether stimulated chelatase activity in mitochondrial supernatant (Fig. 5). This experiment was done with ordinary ether, and while some of the effect of lipid in the zinc–protoporphyrin-chelatase assay might be to react with peroxides this cannot be the case in the ferrochelatase assay where ascorbic acid was present.

Fractionation of lipid from *R. spheroides* was performed on columns of silicic acid. Each fraction eluted from the column was evaporated to dryness and dissolved in peroxide-free ether. Ether solutions were tested for lipid phosphate, lipid composition by thin-layer chromatography, and ability to stimulate zinc–protoporphyrin-chelatase activity in mitochondrial supernatant (Table 5). It can be seen that the fraction from the first column that was active was the one eluted with methanol and which contained the bulk of the phospholipids together with the ornithine-containing lipid (Gorchein, 1964). On fractionation of these phospholipids on the second column the activity was mainly in the fraction eluted with methanol, which contained phosphatidylcholine as well as some phosphatidylethanolamine. It should be pointed out, however, that the amount of phospholipid which had to be added to obtain this stimulation was greatly in excess of the phospholipid contained in the amount of chromatophore pellet required to give the same stimulation.

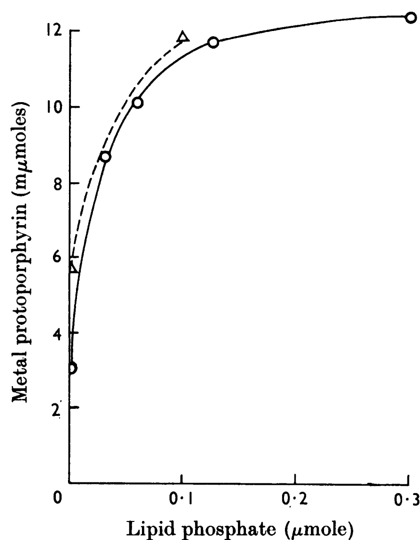

Fig. 5. Effect of chromatophore lipid on zinc–protoporphyrin-chelatase and ferrochelatase activities in mitochondrial-Tween supernatant. Tris, pH 8·4 (100 μmoles), protoporphyrin (40 mμmoles), either $ZnSO_4$ (50 mμmoles) or $FeSO_4$ (100 mμmoles) plus ascorbic acid (10 μmoles), and mitochondrial-Tween supernatant (1·17 mg. of protein) were mixed in a volume 0·5 ml. Crude lipid extract prepared from chromatophores by chloroform–methanol extraction (see the Materials and Methods section) was added to the assays in solution in ether and additional ether was added to 0·2 ml. After shaking, the samples were incubated at 37° for 90 min. and zinc protoporphyrin (○) and haem (△) were measured as described by Neuberger & Tait (1964).

Before the inhibitory effect of peroxides was appreciated a similar experiment to that reported in Table 5 was performed. In that experiment the active lipid fraction was the one containing phosphatidylglycerol and phosphatidic acid. Very little or no activity was observed in the fraction containing phosphatidylcholine.

Removal of lipid from mitochondrial-Tween supernatant by extraction at 0° with 90% (v/v) acetone reduced chelatase activities very markedly, as did similar treatment of chromatophores (cf. Table 2). Addition of chromatophore pellet or lipids extracted from chromatophores markedly stimulated both zinc–protoporphyrin-chelatase and ferrochelatase activities of these preparations.

Phosphatidic acid (prepared from egg-yolk phosphatidylcholine), but not phosphatidylcholine itself or phosphatidylethanolamine (both from egg yolk), stimulated both chelatase activities when assays were performed either in the presence or absence of ether in a manner similar to that found for chromatophores (cf. Fig. 3). Thus with mitochondrial-Tween supernatant (0·64 mg. of protein), assayed in the presence of ether, phosphatidic acid (1·0 mg.) caused an increase in zinc protoporphyrin formed from 10·0 mμmoles/hr. to 17·0 mμmoles/hr., and in the absence of ether the same amount of phosphatidic acid caused an increase from 0·0 to 4·9 mμmoles/hr. Ferrochelatase activity assayed in the presence of ether was increased from 5·1 to 7·5 mμmoles/hr. by the addition of phosphatidic acid (0·5 mg.).

Further properties of chelatases in mitochondrial extracts. A pH–activity curve for zinc–protoporphyrin chelatase obtained with mitochondrial supernatant and assaying in the presence of ether is shown in Fig. 6. There is a marked optimum at pH 8·8. This is similar to the curve found by Porra & Jones (1963*a*) for ferrochelatase in pig-liver mitochondria assayed in the absence of organic solvent.

Previous work by Mazanowska, Dancewicz & Kowalski (1962) showed that ferrochelatase activity

Table 5. *Effect of lipid fractions from* Rhodopseudomonas spheroides *on chelatase activity*

R. spheroides (2g. dry wt.), suspended in 25ml. of water, were extracted with chloroform–methanol by the technique of Bligh & Dyer (1959). This extract was evaporated to dryness and the lipid, in solution in chloroform, was applied to a column of 7g. of silicic acid. The column was eluted successively with 4vol. of chloroform, dry acetone and methanol. Each fraction was evaporated to dryness and the residue dissolved in 5ml. of peroxide-free ether. A portion (3ml.) of the fraction eluted with methanol was evaporated to dryness and applied in solution in chloroform to a column of 2g. of silicic acid. The column was eluted successively with 4 column vol. of chloroform, 5% methanol in chloroform, 15% methanol in chloroform and methanol. Each fraction was evaporated to dryness and dissolved in 3ml. of peroxide-free ether. Portions of the ether solutions were analysed for lipid phosphate and lipid composition as described in the Materials and Methods section. Samples were also tested for their ability to stimulate zinc–protoporphyrin-chelatase activity in the following assay system. In a volume of 0·5ml. of water, tris, pH 8·4 (100μmoles), protoporphyrin (50mμmoles), $ZnSO_4$ (50mμmoles), guinea-pig mitochondrial supernatant (0·64mg. of protein) and ascorbic acid neutralized to pH 8·4 (10μmoles) were mixed. Ether solutions of lipids and additional peroxide-free ether to 0·2ml. were added and, after thorough mixing, the tubes were incubated for 1hr. at 37°, after which the amount of zinc protoporphyrin formed was estimated.

Additions to assay	Lipid phosphate (μmoles/assay)	Zinc protoporphyrin (mμmoles/assay)	Main recognized components in lipid fraction
None	—	9·5	
Chromatophore pellet	0·03	14·3	
Lipid eluted from first column with chloroform	0·01	9·5	Bacteriochlorophyll, carotenoids
Acetone	0·05	9·5	Phosphatidic acid and traces of pigments
Methanol	1·15	11·5	Phosphatidic acid, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylcholine and ornithine lipid
Lipid eluted from second column with chloroform	0·03	9·4	None
5% methanol in chloroform	0·16	9·8	Phosphatidic acid, phosphatidylglycerol, ornithine lipid
15% methanol in chloroform	0·51	11·0	Phosphatidylglycerol, ornithine lipid, phosphatidylethanolamine
Methanol	0·62	14·1	Phosphatidylethanolamine, phosphatidylcholine

in mitochondria from a number of animal tissues was markedly inhibited by serum, and that the inhibitor appeared in fractions V, VI/2 and VI/3 of Cohn *et al.* (1946), which consist mainly of albumin and α-globulin. The assays of enzyme activity in that work were performed in the absence of organic solvent. When bovine plasma albumin was used instead of serum, zinc–protoporphyrin-chelatase and ferrochelatase activities were markedly inhibited, but only if assays were done in the absence of organic solvent. Thus 3mg. of albumin per assay in the presence of 1·5mg. of mitochondrial protein per assay inhibited the ferrochelatase by 100% and the zinc–protoporphyrin chelatase by 85%, whereas 1·5mg. of albumin under the same conditions gave about 50% inhibition of ferrochelatase. In the presence of an optimum amount of ether, 3mg. of albumin per assay under the above conditions gave no inhibition.

Attempts to purify the enzyme(s) responsible for the chelatase activities by treating mitochondrial supernatant with different amounts of ammonium sulphate were only partly successful, giving usually a twofold purification on a protein basis in the fraction precipitating between 20 and 40% saturation.

Mitochondrial supernatant, before or after dialysis or after fractionating with ammonium sulphate, was chromatographed on columns of carboxymethylcellulose and diethylaminoethylcellulose. Under a variety of conditions only two major protein peaks were eluted from each column, both of which contained chelatase activity, but the specific activity was not increased. Chromatography on columns of Sephadex G-50, G-100 and G-200 gave a protein peak which was eluted with the front and which contained all the chelatase activity.

DISCUSSION

Comparison of the non-enzymic and enzymic incorporation of zinc and iron. The reason for the increase in the rate of the non-enzymic formation of zinc protoporphyrin at high pH values is not known, but it may be related to a change in the

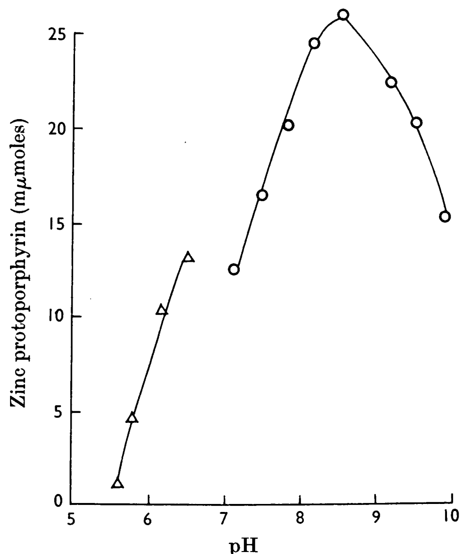

Fig. 6. pH–activity curve for mitochondrial zinc–protoporphyrin-chelatase activity. Maleate buffer (30 μmoles) (△) or tris buffer (100 μmoles) (○), pH stated, protoporphyrin (44 μmoles), $ZnSO_4$ (40 mμmoles) and guinea-pig-liver mitochondrial-Tween supernatant (1·33 mg. of protein) were mixed in a volume 0·5 ml. Ether (0·2 ml.) was added and, after thorough mixing, the samples were incubated at 37° for 90 min. Zinc protoporphyrin was measured as described by Neuberger & Tait (1964).

solubility or the state of ionization of the protoporphyrin or to a change in the state of the zinc. The stimulation by sodium lauryl sulphate of the formation of complexes between protoporphyrin dimethyl ester and a number of metal ions was described by Lowe & Phillips (1961), who considered that the detergent micelle reacted with protoporphyrin dimethyl ester, thereby rendering it soluble, and that metal ions were attracted to the anionic micelle–water interface, where reaction took place.

A comparison of the properties of these two systems forming zinc protoporphyrin with the reaction occurring in the presence of chromatophores and mitochondria shows that they are different in type and that the chromatophores and mitochondria cannot be considered simply as facilitating the reaction by raising the pH locally or by acting as anionic detergents. At high pH values and in the presence of sodium lauryl sulphate, organic solvents did not stimulate the rate of zinc protoporphyrin formation, whereas they did in the presence of chromatophores and mitochondria. In the non-enzymic reactions the rates increased up to pH 11 (Fig. 1), whereas with both chromatophores (Neuberger & Tait, 1964) and mitochondria (Fig. 6) the rates increased with pH up to 8·2 and 8·8 respectively and above these pH values the rates decreased.

Some evidence, such as values for the heat of activation of the reaction and the instability of the active factor to heat, indicated that the formation of zinc protoporphyrin and haem catalysed by chromatophores and mitochondria was enzymic in nature (Neuberger & Tait, 1964). Additional evidence, namely that of the reduction of activity by treatment with trypsin or chymotrypsin, is given in this paper. Although non-enzymic formation of haem was not detected in the present experiments, this was probably due to the insensitivity of the assay method used. Non-enzymic formation of haem was detected in previous work (cf. Granick & Mauzerall, 1958; Lowe & Phillips, 1961), but the rate of this reaction is very much lower than that catalysed by biological extracts (Labbe, 1959).

Probable identity of zinc–protoporphyrin-chelatase and ferrochelatase. In the experiments described in this and the previous paper (Neuberger & Tait, 1964) addition of ether caused an increase of zinc–protoporphyrin-chelatase and ferrochelatase activities of mitochondria and chromatophores. However, in the early experiments with mitochondria, when no special precautions were taken to free the ether of peroxides, differences between some of the properties of the two activities were noted. These differences were not observed when the assays were done in the presence of acetone, and were also not observed when ascorbic acid was added to the zinc–protoporphyrin-chelatase assay or when peroxide-free ether was used. Indeed it became apparent that peroxide-free ether and acetone had similar effects on both zinc–protoporphyrin-chelatase and ferrochelatase activities in both chromatophores and mitochondria. In view of the probable involvement of lipid in chelatase activity, evidence for which is discussed below, it is possible that peroxides exert their inhibitory effect by oxidizing this lipid, although they may also inhibit by oxidizing thiol groups in the protein. Conversely, reducing agents probably prevent the oxidation of the lipid as well as protect essential thiol groups and keep the ferrous iron reduced, as suggested by Labbe & Hubbard (1960) and Porra & Jones (1963*a*).

It appears that zinc–protoporphyrin chelatase and ferrochelatase behave very similarly under most of the conditions described in this paper, suggesting that one enzyme is responsible for both activities. Some of the earlier results reported by Neuberger & Tait (1964), which had suggested that different enzymes might be involved, are probably explained by the presence of a reducing agent, usually ascorbic acid, in the ferrochelatase assay but not in the zinc–protoporphyrin-chelatase assay: thus the effects of peroxides were eliminated in one assay but not in the other.

Involvement of lipid in chelatase action. A role of lipid in chelatase action was suggested by a number of findings. The chelatase in mitochondrial supernatant was stimulated in the presence of ether or acetone by chromatophore pellet, or a chloroform–methanol extract from chromatophores. Removal of lipid by preparation of acetone-dried powders or extraction of chromatophores or mitochondria with 90% (v/v) acetone reduced activity in the presence of ether and acetone. Activity could be partially restored by adding lipid or an extract containing lipid (e.g. chromatophore pellet). Phosphatidic acid, added as an aqueous emulsion or in ether solution, stimulated chelatase activity in chromatophores and in mitochondria. Albumin, or serum fractions containing albumin, reduced chelatase activity, but only in the absence of organic solvents. It may be that this action is due to the binding of lipids by the albumin. That the inhibitory effect is not due to the binding of metals by albumin was suggested by the fact that the inhibition was not reversed by excess of Fe^{2+} ions (Mazanowska *et al.* 1962).

While this work was in progress a similar finding of lipid involvement in ferrochelatase activity was reported by Yoshikawa & Yoneyama (1964). They showed that the activity of a soluble enzyme system from duck erythrocyte stroma was increased by the addition of an aqueous emulsion of phospholipid extracted from the same source.

Phospholipids, which have been shown to play a role in the function of a number of enzymes, particularly those bound to particles such as mitochondria (Asano, Kaneshiro & Brodie, 1965), could act in either of two ways: by aiding the binding of substrates to the enzyme, or by directly affecting the enzyme structure and therefore its function. Although the first explanation may apply to enzymes acting on compounds which are not water-soluble, it is not satisfactory for enzymes such as D-β-hydroxybutyrate dehydrogenase (EC 1.1.1.30; Jurtshuk, Sekuzu & Green, 1963) and malate–oxygen oxidoreductase (EC 1.1.3.3) using phenazine as electron acceptor (Tobari, 1964) whose substrates are water-soluble. In these enzymes it is suggested (Tobari, 1964) that phospholipid is an integral part of the enzyme complex, its presence being required for the formation of an 'active centre' in the enzyme molecule; such complexes are probably the natural form of these enzymes in intact subcellular particles. Very recently it has been shown by Pesch & Peterson (1965) that enzymic removal of phospholipid from mitochondrial $NADPH_2$–NAD oxidoreductase (EC 1.6.1.1) changes the substrate specificity of the system. It would seem likely that this is caused by a modification of the conformation of the protein, perhaps especially at its active site.

The active lipid extracted from *R. spheroides* appears to be phosphatidylcholine, but, as can be seen from Table 5, the amounts of lipid required to show stimulation of activity were much greater than the lipid contained in the amount of chromatophore pellet which gave the same degree of stimulation. This may mean that the lipid is unstable to the manipulations involved in extraction and fractionation or that when added in ether solution its ability to stimulate activity is much poorer than when present in its native state. Phosphatidylcholine prepared from egg yolk had no effect on chelatase activity, whereas phosphatidic acid prepared by enzymic hydrolysis of this phosphatidylcholine was active both in the presence and absence of ether. These observations might suggest that the two phospholipids act in different ways. Ward & Fantl (1963) reported that a number of hydrophilic cations could be transferred from an aqueous to a lipophilic phase by shaking a solution containing the cation with an ether solution of phosphatidic acid. This observation might suggest that phosphatidic acid, which is present in small amounts in chromatophores and also in mitochondria (Wajtczak, Wlodawer & Zborowski, 1963), binds Zn^{2+} and Fe^{2+} ions, and in this way facilitates chelatase reactions. Phosphatidic acid, as an aqueous emulsion, or in ether solution, protected the chromatophore system from inhibition by excess of Zn^{2+} ions, which might also be explained by its capacity to form a zinc salt. The action of phosphatidylcholine, on the other hand, might be due to its ability to react with the enzyme and alter its activity. For this the fatty acid composition may be important. In this connexion, Jurtshuk *et al.* (1963) found that only phosphatidylcholine which contained unsaturated fatty acids acted as cofactor for D-β-hydroxybutyrate dehydrogenase.

Action of organic solvents. After removal of lipid from chromatophores or mitochondria the activity dropped markedly when assayed in the presence of organic solvents, but could be restored by adding back lipid or an extract containing lipid (e.g. chromatophore-Tween pellet). It is therefore reasonable to assume that the organic solvents act in some way in conjunction with the lipid present in the chromatophores or mitochondria so as to alter the rate of the enzyme reaction. At present there is no evidence on the exact way in which this occurs.

Organic solvents may facilitate binding between the enzyme and the substrate, as has been suggested by Kates (1953, 1956, 1957) to account for their stimulatory effect on the phospholipase D (EC 3.1.4.4) of chloroplasts. The effect of ether on phospholipases A (EC 3.1.1.4) and B (EC 3.1.1.5), whose action produces fatty acids, has been noted by Hanahan (1952), Hanahan, Rodbell & Turner

(1954), Magee & Thompson (1960) and Dawson (1963). These workers considered the action of ether to involve first the breakdown of larger micelles of phosphatidylcholine to ones of smaller size, thereby increasing the availability of the substrate to the enzyme, and secondly the removal from the micelle–enzyme interphase of the fatty acids, whose accumulation would inhibit the reaction. Although protoporphyrin in water buffered at pH 8·4 is not in true solution (cf. Porra & Jones, 1963*b*), it is unlikely that the organic solvents act by changing the physical state of protoporphyrin since they have no effect on the non-enzymic formation of zinc protoporphyrin. Yoshikawa & Yoneyama (1964) reported that incubation of δ-aminolaevulic acid with haemolysates of duck erythrocytes yielded protoporphyrin, the bulk of which appeared in some way to be attached to the phospholipid of the stroma. They also showed that the 'lipid-bound' protoporphyrin was more readily converted into haem than was free protoporphyrin. Thus organic solvents may facilitate the combination of protoporphyrin with the phospholipid of chromatophores or of mitochondria and so aid the formation of metal complexes.

The effect of phosphatidic acid on chelatase activity and the possible mechanism by which this occurs was discussed above. It might be suggested that the ability of phosphatidic acid to form salts with zinc and iron is facilitated in the presence of organic solvents and in this way the rate of production of zinc protoporphyrin and haem is increased.

In conclusion it would appear that the chromatophore and mitochondrial enzymes responsible for chelation of zinc and iron with protoporphyrin are present in the native state in close association with phospholipid. Whether the phospholipid acts by combining with the enzyme in such a way as to produce favourable enzymic conformation or whether it acts by facilitating combination of the two substrates with the enzyme is still unknown. The effect of organic solvents will probably be understood when the exact mechanism of action of phospholipids is explained.

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