Cofactor Requirements of the L-Malate Dehydrogenase of *Pseudomonas ovalis* Chester

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1. The L-malate dehydrogenase of *Pseudomonas ovalis* Chester, which is independent of nicotinamide nucleotides and which is structurally and functionally bound to the cell-wall membrane, has been prepared in a soluble form and partially purified. 2. The purified dehydrogenase exhibits a triple cofactor requirement for FAD, quinone and phospholipid, and in the presence of these cofactors can utilize 2,6-dichlorophenol-indophenol as hydrogen acceptor. 3. The formation of reduced forms of FAD was not detected, but in the presence of both FAD and phospholipid the enzyme catalysed the reduction of quinone by L-malate at rates equivalent to those obtained with 2,6-dichlorophenol-indophenol as terminal acceptor. The L-malate dehydrogenase of *Ps. ovalis* Chester is therefore an L-malate-quinone oxidoreductase. 4. The quinone and the phospholipids present in the fragments of the cell-wall membrane from which the soluble dehydrogenase was prepared have been extracted and purified. The quinone was identified as coenzyme Q₉. At least eight phospholipids were detected, and the major component is an unsaturated phosphatidylethanolamine. 5. The nature of the phospholipid required to activate the enzyme depends on the nature of the quinone used in the assay system. When 2-methyl-1,4-naphthaquinone is used, a wide variety of phospholipids, including all those isolated from the organism, will activate the enzyme, but when coenzyme Q₉ is used the phospholipid specificity of the enzyme is much more restricted, and the most effective activator is the unsaturated phosphatidylethanolamine isolated from the organism. 6. Evidence is presented to support the view that the restricted phospholipid specificity exhibited by the enzyme in the presence of coenzyme Q₉, as opposed to the broad specificity exhibited when 2-methyl-1,4-naphthaquinone is used, is due to the fact that coenzyme Q₉ has a large substituent on position 3.

It was shown previously that in *Pseudomonas ovalis* Chester the oxidation of L-malate to oxaloacetate is catalysed by an enzyme system that is independent of nicotinamide nucleotides and that is firmly bound to the cell-wall membrane. The L-malate-oxidase system of this organism can be obtained in particulate form by centrifuging an ultrasonic extract of whole cells, and the particles so prepared are capable of the stoichiometric oxidation of L-malate to oxaloacetate by oxygen, in the absence of added cofactors, by a chain of carriers that includes flavine, ubiquinone and cytochromes (Kornberg & Phizackerley, 1961; Francis, Hughes, Kornberg & Phizackerley, 1963).

The present paper is concerned with the preparation, partial purification and properties of a soluble form of the L-malate dehydrogenase of this organism. A preliminary account of some of this work has been presented to The Biochemical Society (Francis & Phizackerley, 1965).

METHODS AND MATERIALS

Maintenance and growth of organisms. Cultures of *Ps. ovalis* Chester were maintained on agar slopes and grown in liquid media, as described by Francis *et al.* (1963).

Preparation and partial purification of soluble L-malate dehydrogenase. Stage 1: disruption of whole cells with ultrasound. Washed whole cells (100 mg. dry wt./ml.) were suspended in 50 mM-tris adjusted to pH 6.0 with $m\text{-H}_3\text{PO}_4$ and exposed to ultrasound in an MSE Ultrasonic Disintegrator operated at 1.5 A for 9 min. at 0°. Intact cells were removed by centrifuging at 12000g for 10 min. at 2°. The supernatant fluid was then centrifuged in a Spinco model L preparative ultracentrifuge at 100000g for 1 hr. at 0°. The supernatant was discarded and the particulate sediment suspended with the aid of a loose-fitting Potter homogenizer in 50 mM-tris-phosphate buffer, pH 7.0. The centrifugation was repeated and the supernatant again discarded. The particles obtained in this way were used for the next stage in the purification procedure; they can be stored for several months at -15° with little loss of activity.

Stage 2: disruption of particles with ultrasound. Par-

ticles (4.0 mg. of protein/ml.) were suspended in 50 mM-tris-phosphate buffer, pH 8.5, and exposed to ultrasound as described above, for 5 min. at 0°. The extract was then centrifuged at 100000g for 4 hr. at 0°, and the supernatant fluid was used for further purification.

Stage 3: $(\text{NH}_4)_2\text{SO}_4$ fractionation. The fluid from stage 2 was brought to 50% saturation with $(\text{NH}_4)_2\text{SO}_4$ by adding slowly, with stirring, a saturated solution of metal-free $(\text{NH}_4)_2\text{SO}_4$ at 0°. The suspension was kept for 30 min. at 0° and then centrifuged. The sediment was dissolved in 50 mM-tris-phosphate buffer, pH 8.5, and dialysed overnight in cellophan tubing against 20 mM-tris-phosphate buffer, pH 7.0, at 4°. The dialysis residue was centrifuged, the small precipitate was discarded and the concentrations of protein and of nucleic acid in the supernatant fluid were determined by the method of Warburg & Christian (1941).

Stage 4: column chromatography on DEAE-cellulose. Approx. 3.5 g. of DEAE-cellulose (Whatman DE50) that had been repeatedly washed with 2M-NaCl was used as a column (10.5 cm. $\times$ 2.8 cm.²). A layer of washed sand approx. 1 cm. deep was placed on top of the DEAE-cellulose. The column was washed with 20 mM-tris-phosphate buffer, pH 7.0, until the eluate ran constantly at pH 7.0. A volume of the dialysed fluid from stage 3 was then applied to the column, such that the load of protein was about 5 mg./g. of DEAE-cellulose and the load of nucleic acid was less than 2 mg./g. of DEAE-cellulose. The column was eluted stepwise at 4° with 20 mM-tris-phosphate buffer, pH 7.0, 50 mM-tris-phosphate buffer, pH 7.0, and then with 50 mM-tris-phosphate buffer containing successively greater concentrations of NaCl. In a typical preparation, NaCl concentrations of 0.10M, 0.25M, 0.50M and 2.0M were used, and the eluates are referred to as '0.10M-NaCl eluate' and so on. For some experiments a 0.05M-NaCl eluate was also prepared. The column was run at a constant flow rate of 60 ml./hr. maintained by a micro-pump (The Distillers Co. Ltd. Engineering Division, Great Burgh, Epsom, Surrey) and 10 ml. fractions were collected. The extinctions at 260 m μ and 280 m μ of each fraction were measured and elution under a particular condition was continued until no more u.v.-absorbing material was released.

Estimation of protein. Protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951), with bovine albumin (fraction V from bovine plasma; Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex) as standard, or by the method of Warburg & Christian (1941).

Spectrophotometric assays of L-malate dehydrogenase. All spectrophotometric assays were carried out in silica cells (1.5 ml. capacity, 1 cm. light-path) at $20 \pm 1^\circ$ with a Cary CF14 recording spectrophotometer. It is essential to clean the cuvettes with organic solvents after each assay, and the sequence water, ethanol, chloroform, ethanol and water was found satisfactory. The reagents were added in the order given, and the cuvette contents were mixed by inversion after each addition. Enzymic activity was calculated from the initial rate of bleaching of 2,6-dichlorophenol-indophenol measured at 600 m μ , by using a value of 19.1 for the millimolar extinction coefficient (Singer & Kearney, 1957).

(a) Assay with vitamin K₃ (2-methyl-1,4-naphthoquinone). The experimental cuvette contained water to 1.5 ml., 150 μ moles of tris-phosphate buffer, pH 7.0, 10 m μ g. atoms of phospholipid P in 0.01 ml. of ethanol, enzyme, 15 μ moles of potassium L-malate, 10 m μ moles of

FAD, 0.05 ml. of freshly prepared 2,6-dichlorophenol-indophenol (0.1%, w/v) and 1.0 μ mole of vitamin K₃ in 0.05 ml. of ethanol. L-Malate was omitted from the control cuvette. In this assay there was no lag period, and the initial reaction velocity was proportional to enzyme concentration over a 20-fold range.

(b) Assay with coenzyme Q₉. The experimental cuvette contained water to 1.5 ml., 150 μ moles of tris-phosphate buffer, pH 7.0, 10 m μ moles of phosphatidylethanolamine in 0.05 ml. of ethanol, 1.0 μ mole of coenzyme Q₉ in 0.05 ml. of ethanol, 10 m μ moles of FAD, enzyme, 15 μ moles of potassium L-malate and 0.05 ml. of freshly prepared 2,6-dichlorophenol-indophenol (0.1%, w/v). L-Malate was omitted from the control cuvette. In this assay, the reaction velocity became maximal after 4 min., and the rate of reaction measured in the fifth minute was proportional to enzyme concentration over a 20-fold range.

Extraction of lipids from Ps. ovalis Chester particles. Particles were prepared as described above and the lipids extracted by the method of Folch, Lees & Sloane-Stanley (1957).

Separation and purification of individual phospholipids. A 20 g. portion of silicic acid (100 mesh; Mallinckrodt Chemical Works), washed as described by Barron & Hanahan (1958), was heated at 110° for 14 hr. It was shaken in chloroform until it appeared purple by transmitted light and then packed under gravity in a column (8.5 cm. $\times$ 5.0 cm.²). Lipid extract containing 20 mg. of phospholipid P was applied to the column. The column was eluted successively with 370 ml. of chloroform, 180 ml. of chloroform-methanol (20:1, v/v), 270 ml. of chloroform-methanol (9:1, v/v), 180 ml. of chloroform-methanol (4:1, v/v), 180 ml. of chloroform-methanol (3:2, v/v) and 180 ml. of chloroform-methanol (1:4, v/v). The column was developed under N₂ at room temperature at a flow rate of 100 ml./hr. and 15 ml. fractions were collected. These were monitored by phosphorus estimation and by thin-layer chromatography. Of the phospholipid P applied to the column 105% was recovered in the eluates. The fraction eluted with chloroform, which did not contain phospholipid, was used for the preparation of coenzyme Q₉; the other fractions usually contained more than one phospholipid, and eluates rich in a particular component were combined, evaporated under N₂, dissolved in chloroform and re-chromatographed on small silicic acid columns. If necessary the process was repeated until each phospholipid ran as a single spot on thin-layer chromatography. In all, eight phospholipids were prepared; these were stored in chloroform, in ampoules sealed under N₂, at -15°.

Thin-layer chromatography. Quantitative thin-layer chromatography was carried out by the method of Skipski, Peterson & Barclay (1964). For qualitative thin-layer chromatography, 0.25 mm. plates were prepared with silica gel G, with CaSO₄ binder (Kieselgel G nach Stahl; E. Merck A.-G.). The plates were heated for 45 min. at 110° before use and were developed in chloroform-methanol-water (65:25:4, by vol.) (Wagner, Hörhammer & Wolff, 1961). Iodine vapour, bromothymol blue (0.04% in 0.01N-NaOH) and ninhydrin (0.2% in butan-1-ol) were used as locating reagents.

Estimation of phospholipid glycerol. Samples of phospholipid containing 0.2-1.0 μ mole of phospholipid glycerol were hydrolysed in sealed tubes with 1.0 ml. of 2N-HCl for 36 hr. at 120°. After neutralization, portions were taken for

glycerol estimation by the method of Wieland (1963). In control experiments it was found that 94% of a sample of glycerol subjected to this treatment was recovered, and so the observed values for phospholipid glycerol were multiplied by 1.06.

Estimation of phospholipid fatty acid. Samples of phospholipid containing 0.5–2.0 μ moles of phospholipid fatty acid were hydrolysed with 2.0 ml. of freshly prepared 1.0N-KOH in 95% (v/v) ethanol, under N_2 , for 4 hr. at 80°. The hydrolysate was evaporated to dryness at room temperature under N_2 at reduced pressure and the dry residue acidified with a slight excess of HCl. The fatty acids were extracted by shaking for 2 min. with 10 ml. of chloroform and were estimated by the method of Duncombe (1963) against a standard solution of palmitic acid (99% pure; Sigma Chemical Co.). A sample of palmitic acid was put through the hydrolysis procedure and 98% was recovered.

Estimation of phospholipid phosphorus. Samples of phospholipid containing 0.03–0.3 μ g. atom of phospholipid P were heated with 0.9 ml. of $HClO_4$ (sp.gr. 1.70) for 30 min. at 200°. Orthophosphate was estimated by the method of Bartlett (1959).

Estimation of phospholipid nitrogen. Samples of phospholipid containing 0.5–2.0 μ g. atoms of phospholipid N were digested with 0.2 ml. of a mixture containing 100 mg. of $CuSO_4$, 100 mg. of Se and 3 g. of Na_2SO_4 /100 ml. of conc. H_2SO_4 for 24 hr. Then 2.8 ml. of water was added and portions were taken for NH_3 estimation by the method of Fenton (1962).

Identification of hydrolysis products of the phosphatidylethanolamine prepared from *Ps. ovalis* Chester. Acid hydrolysis was carried out in 5.8N-HCl in sealed tubes at 115° for 18 hr. The hydrolysate was evaporated to dryness *in vacuo* and the residue extracted with propan-2-ol-water (1:9, w/v). Descending paper chromatography was carried out in butan-1-ol-acetic acid-water (12:3:5, by vol.), phenol saturated with water, and phenol saturated with water-aq. NH_3 (sp.gr. 0.88) (200:1, v/v); ethanolamine was used as marker. Mild alkaline hydrolysis, extraction of the hydrolysate and descending paper chromatography in phenol saturated with water-acetic acid-ethanol (50:5:6, by vol.) were carried out as described by Dawson, Hemington & Davenport (1962), with glycerophosphorylethanolamine as marker.

Preparation of derivatives of the phosphatidylethanolamine prepared from *Ps. ovalis* Chester. Lysophosphatidylethanolamine was prepared by using phospholipase A (phosphatide acyl-hydrolase, EC 3.1.1.4) by the method of Long & Penny (1957); 1,2-diglyceride was prepared by using phospholipase C (phosphatidylcholine phosphohydrolase, EC 3.1.4.3) by the method of Long & Maguire (1954), except that three times as much enzyme was used and the incubation was carried out at 30° for 48 hr. Saturated phosphatidylethanolamine was prepared by catalytic hydrogenation in the presence of 10% Pd on charcoal. The reaction was carried out in ethanol in a Warburg apparatus at 30°; H_2 uptake ceased after 18 hr. and approx. 2 moles of H_2 were taken up/g. atom of P. In each case the derivatives were purified by column chromatography on silicic acid until they ran as a single spot on thin-layer chromatography. Glycerophosphorylethanolamine and free fatty acids were prepared by mild alkaline hydrolysis of phosphatidylethanolamine by the method of Dawson *et al.* (1962).

Preparation of coenzyme Q_9 from *Ps. ovalis* Chester. The

chloroform eluate obtained by silicic acid column chromatography of the particle lipids was evaporated to dryness under N_2 and the residue dissolved in the minimum quantity of ethanol. A white precipitate that separated on standing overnight at room temperature was removed by centrifugation and the deep-yellow solution was evaporated to dryness under N_2 . The residue was dissolved in hexane, oxidized with $FeCl_3$ (1%, w/v, in ethanol) and, after washing several times with water, was applied to plates of silica gel G and developed in the solvent system of Brown & Johnston (1962). The quinone was eluted with warm acetone and, after evaporation of the solvent, dissolved in cyclohexane. A small residue was separated by centrifugation and the solution was evaporated to dryness. The residue was dissolved in the minimum quantity of warm ethanol and allowed to crystallize over several days at –15°. The ultraviolet and infrared spectra of this material agreed very well with those given by Lester, Hatefi, Widmer & Crane (1959) for ubiquinones; the m.p. 43° and the R_f 0.24 on chromatography by the method of Lester & Ramasarma (1959) indicated that it was coenzyme Q_9 .

Materials. FAD, 2-methyl-1,4-naphthaquinone, 2-methyl-3-phytyl-1,4-naphthaquinone, palmitic acid (99% pure), ox-liver catalase (EC 11.1.6) and phospholipase C (EC 3.1.4.3) were obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A.; phospholipase A (EC 3.1.1.4) was obtained from Burroughs Wellcome Ltd., Beckenham, Kent; L- α -dipalmitoyl-phosphatidylcholine and L- α -dipalmitoyl-phosphatidylethanolamine (Mann assayed grade) were obtained from Mann Research Laboratories Inc., New York, N.Y., U.S.A.; 10% Pd on charcoal, coenzyme Q_8 and L-malic acid were obtained from L. Light and Co. Ltd., Colnbrook, Bucks.; L-glycerol 1-phosphate-NAD oxidoreductase (EC 1.1.1.8) and ATP-glycerol phosphotransferase (EC 2.7.1.30) were obtained from C. F. Boehringer and Soehne G.m.b.H., Mannheim, Germany. Kieselgel G nach Stahl, with and without $CaSO_4$ binder, was obtained from E. Merck A.-G., Darmstadt, Germany. Silicic acid (100 mesh, $SiO_2 \times H_2O$) was obtained from Mallinckrodt Chemical Works, St Louis, Mo., U.S.A. 2,3-Dimethoxy-5-methyl-1,4-benzoquinone was a gift from Professor R. A. Morton. Organic solvents were redistilled before use.

RESULTS

Preparation and partial purification of L-malate dehydrogenase from *Ps. ovalis* Chester. Ultrasonic disruption was carried out in two stages. In stage 1 of the purification procedure the object was to disrupt whole cells in such a way that the maximum yield of enzyme was obtained in particulate form, and in stage 2 the object was to disrupt the particulate oxidase so as to give the maximum yield of the soluble dehydrogenase. About 85% of the L-malate-dehydrogenase activity is sedimented at stage 1 and about 60% of the dehydrogenase is released from the particles at stage 2. The main purpose of the ammonium sulphate fractionation was to remove much of the nucleic acid; attempts to achieve this by protamine sulphate precipitation led to large losses in enzyme activity. The fraction precipitated at 0–50% saturation with ammonium

Fig. 1. Chromatography of L-malate dehydrogenase on DEAE-cellulose (stage 4 in the purification procedure). The dialysed $(\text{NH}_4)_2\text{SO}_4$ preparation (from stage 3) containing 10 mg. of protein and capable of oxidizing 825 μmoles of L-malate/hr. was applied to the column. Tris buffer was adjusted to pH 7.0 with $\text{m-H}_3\text{PO}_4$, and the following eluents were used: A, 20 mM-tris buffer; B, 50 mM-tris buffer; C, 0.10 M-NaCl in 50 mM-tris buffer; D, 0.25 M-NaCl in 50 mM-tris buffer; E, 0.50 M-NaCl in 50 mM-tris buffer; F, 2.0 M-NaCl in 50 mM-tris buffer. (a) Elution pattern of u.v.-absorbing material: $E_{280\text{ m}\mu}$ ($\circ$); $E_{260\text{ m}\mu}$ ($\bullet$). (b) Elution pattern of L-malate dehydrogenase; the activity of the enzyme was determined spectrophotometrically by using the assay based on vitamin K_3 in the presence ($\bullet$) and absence ($\circ$) of added phosphatidylethanolamine.

Table 1. *Variation in specific activity and in cofactor requirements of L-malate dehydrogenase during purification*

The purification procedure is described in the text. L-Malate dehydrogenase was estimated spectrophotometrically by using the assay based on vitamin K₃, with cofactors omitted as shown. The concentration of protein in the 0.05 M-NaCl eluate was too low for accurate estimation.

Step in purification procedure	Enzyme	Relative L-malate-dehydrogenase activity					Specific activity of complete system (μ moles of L-malate oxidized/mg. of protein/min.)
		No cofactors	Vitamin K ₃ omitted	FAD omitted	Phosphatidylethanolamine omitted	Complete system	
1	Particles	7.0	8.0	44	92	(100)	0.43
2	Stage 2 supernatant	2.7	3.8	50	77	(100)	1.46
3	Dialysed (NH ₄) ₂ SO ₄ preparation	0.8	3.2	13	54	(100)	1.28
4	0.10 M-NaCl eluate	0.0	0.0	0.0	23	(100)	9.3
4	0.05 M-NaCl eluate	0.0	0.0	0.0	6	(100)	—

Fig. 2. Cofactor requirements of L-malate dehydrogenase (0.05 M-NaCl eluate). In all experiments cuvettes contained water to 1.5 ml., 150 μ moles of tris-phosphate buffer, pH 7.0, enzyme and 0.05 ml. of freshly prepared 2,6-dichlorophenol-indophenol (0.1%, w/v). Experimental cuvettes contained 15 μ moles of potassium L-malate. Experimental and control cuvettes contained: $\square$, 10 μ moles of FAD and 1.0 μ mole of vitamin K₃; $\circ$, 10 μ moles of FAD and 1.0 μ mole of coenzyme Q₆; $\blacksquare$, 10 μ moles of FAD, 1.0 μ mole of vitamin K₃ and 10 μ moles of phosphatidylethanolamine; $\bullet$, 10 μ moles of FAD, 1.0 μ mole of coenzyme Q₆ and 10 μ moles of phosphatidylethanolamine. Changes in extinction were measured at 600 m μ . No oxidation of L-malate was observed in cuvettes from which FAD was omitted.

0.1 M-sodium chloride and 0.25 M-sodium chloride in 50 mM-tris-phosphate buffer, pH 7.0. The effects of the various steps in the purification procedure on the specific activity of the enzyme are shown in Table 1.

Alteration in the properties and cofactor requirements of the L-malate dehydrogenase from Ps. ovalis Chester at various stages of purification. Francis *et al.* (1963) showed that the particulate enzyme prepared from whole cells by the first treatment with ultrasound rapidly oxidized L-malate to oxaloacetate, in the absence of added cofactors, with oxygen, phenazine methosulphate, 2,6-dichlorophenol-indophenol, ferricyanide or cytochrome c as hydrogen acceptor. The soluble enzyme released from the particles by the second treatment with ultrasound differed from the particulate enzyme in three main particulars. In the first place, it showed an increased dependence on both FAD and a quinone and as purification proceeded these requirements became absolute (Table 1 and Fig. 2). Secondly, of the electron acceptors listed above, the soluble enzyme will utilize only 2,6-dichlorophenol-indophenol and, provided that the quinone used to activate the enzyme is autoxidizable, oxygen. Thirdly, it can be shown that the second treatment with ultrasound breaks the link between L-malate dehydrogenase and its terminal oxidase. Thus, although the soluble enzyme (supplemented with FAD and vitamin K₃) resembled the unsupplemented particulate oxidase in that 0.5 μ mole of oxygen was consumed/ μ mole of L-malate oxidized, in the former case oxygen is reduced initially to hydrogen peroxide and not to water. Thus potassium cyanide (10 mM) inhibited oxygen uptake by the particulate oxidase by over 90%, but when added to the soluble enzyme supplemented as described above it increased the rate of oxygen

sulphate was used, after dialysis, for column chromatography, and a typical chromatogram is shown in Fig. 1. L-Malate dehydrogenase was eluted with 0.05 M-sodium chloride (not shown in Fig. 1),

Fig. 3. Effect of KCN (10mm) and of 0.05ml. of ethanol and 1mg. of catalase on O₂ uptake by L-malate dehydrogenase (stage 2 supernatant). All flasks contained 300 μmoles of tris-phosphate buffer, pH 7.0, 1.0 μmole of vitamin K₃ in 0.05 ml. of dimethylformamide, 10 μmoles of FAD and water to 3.0 ml. Centre wells contained 100 μmoles of KCN, or 100 μmoles of KOH if main compartments of flasks did not contain KCN. Side arms contained enzyme (3 mg. of protein) and 10 μmoles of potassium L-malate. ●, Main compartment of flask contained in addition 100 μmoles of KCN, with 1 mg. of catalase added at arrow; ○, main compartment of flask contained in addition 0.05 ml. of ethanol and 1 mg. of catalase; ▲, main compartment of flask contained 1 mg. of catalase, or 0.05 ml. of ethanol, or no addition. There was no O₂ uptake in the absence of L-malate. All experiments were carried out in air at 30°.

utilization and the amount of oxygen consumed approached 1.0 μmole/μmole of L-malate. However, if an excess of catalase was then added, there was a marked evolution of oxygen (Fig. 3). Similarly catalase and ethanol when added together cause a greater oxygen uptake than if either or both were omitted; this is due to oxidation of ethanol by hydrogen peroxide (Keilin & Hartree, 1945). These results suggest that the uptake of oxygen by the soluble enzyme, in the presence of FAD and vitamin K₃, is due to the non-enzymic autoxidation of the quinol, yielding hydrogen peroxide, which is broken down by catalase present in the enzyme preparation at this stage. This interpretation is confirmed by the finding that if vitamin K₃ is replaced by coenzyme Q₆ or coenzyme Q₉, which are not significantly autoxidizable, the enzyme can no longer use oxygen as hydrogen acceptor although it will still react rapidly with 2,6-dichlorophenol-indophenol.

No important change in the properties of the soluble enzyme occurred as a result of the ammonium sulphate fractionation and subsequent dialysis, but the enzyme eluted from the DEAE-cellulose column revealed an additional requirement

Table 2. Activation of L-malate-dehydrogenase activity of 0.10 M-sodium chloride eluate by 0.25 M-sodium chloride eluate

The preparation of the eluates is described in the text. L-Malate-dehydrogenase activity was determined by using the assay based on vitamin K₃, except that phospholipid was omitted from the assay mixture and NaCl was added to give a final concentration of 50 mm in all experiments.

Enzyme	L-Malate oxidized (μmoles/min.)
0.10 M-NaCl eluate	5.0
Boiled 0.10 M-NaCl eluate	0.0
0.25 M-NaCl eluate	5.5
Boiled 0.25 M-NaCl eluate	0.0
0.10 M-NaCl eluate + 0.25 M-NaCl eluate	13.5
0.10 M-NaCl eluate + boiled 0.25 M-NaCl eluate	12.0
Boiled 0.10 M-NaCl eluate + 0.25 M-NaCl eluate	5.5
0.10 M-NaCl eluate + ashed 0.25 M-NaCl eluate	5.0
0.10 M-NaCl eluate + 0.25 M-NaCl eluate preincubated with phospholipase C	5.0

for phospholipid. It can be seen from Fig. 1 that when the enzyme in the column eluates was assayed in the presence of FAD and 2-methyl-1,4-naphthoquinone the total activity recovered was small, but when phospholipid was included in the assay system about 85% of the enzymic activity applied to the column was recovered. The activating effect of phospholipid was greater in the fractions eluted with low concentrations of sodium chloride, and was in fact most marked in the fraction eluted with 0.05 M-sodium chloride (Fig. 2). This suggests that the fractions eluted at high sodium chloride concentrations contain more phospholipid than those eluted at low concentrations of sodium chloride. It is not possible to test this conclusion directly by analysis because of the low concentration of protein in the eluates and the very low apparent *K_m* for phospholipid, but indirect evidence in support of this conclusion has been obtained. As shown in Table 2, when samples of the 0.25 M-sodium chloride eluate and of the 0.10 M-sodium chloride eluate were mixed, enzyme activity (assayed in the absence of added phospholipid) was greater than the sum of the separate activities. Further, although boiling for 10 min. destroyed the activity of each fraction tested separately, it did not destroy the activating effect of the 0.25 M-sodium chloride eluate on the untreated 0.10 M-sodium chloride eluate. Table 2 also shows that the activating effect of the 0.25 M-sodium chloride eluate is destroyed by ashing or by prior incubation with phospholipase C.

These results suggest that enzymic activity in

the absence of added phospholipid (Fig. 2 and Table 1) is due to the presence of endogenous phospholipid. A preparation that is completely inert in the absence of added phospholipid has not been obtained but, as shown in Table 3, the degree of activation by added phospholipid is greatly increased if the enzyme is preincubated with phospholipase C.

It is concluded therefore that the L-malate dehydrogenase of *Ps. ovalis* Chester has a triple cofactor requirement for FAD, a quinone and a phospholipid.

Nature of the phospholipids present in particles prepared from Ps. ovalis Chester by ultrasonic disruption. The particles obtained at stage 1 contained 4.7 mg. of phospholipid P/g. of particle dry wt. A thin-layer chromatogram of these phospholipids is represented in Fig. 4. In some cases, additional components were revealed during the purification of individual phospholipids by column chromatography on silicic acid. Thus lipid 1A was purified from material that remained at the origin

on the thin-layer plate; lipid 4 was separated into two components, designated 4A and 4B, and lipid 6 into two components, designated 6A and 6B. Analytical data relating to these purified phospholipids are given in Table 4.

The major constituent (lipid 5) is phosphatidylethanolamine. This identification is supported by the data in Table 4, by the results of enzymic degradation (see the Methods and Materials section), by the finding that after hydrolysis in 5.8 N-hydrochloric acid at 115° for 18 hr. a single ninhydrin-

Table 3. *Effect of preincubation with phospholipase C on L-malate dehydrogenase (0.10 M-sodium chloride eluate)*

L-Malate dehydrogenase (0.10 M-NaCl eluate) was preincubated for 3 hr. at room temperature with and without 1 mg. of phospholipase C. The L-malate-dehydrogenase activity of each sample was then determined by using the assay based on coenzyme Q₉ in the presence and absence of 1 μ mole of phosphatidylethanolamine.

	Phosphatidylethanolamine	L-Malate oxidized (m μ moles/min.) [*]
Enzyme not preincubated	Omitted	4.0
with phospholipase C	Present	6.5
Enzyme preincubated with phospholipase C	Omitted	0.6
	Present	6.5

Fig. 4. Formal representation of thin-layer chromatographs on plates of silica gel G of lipids extracted from ultrasonic particles prepared from *Ps. ovalis* Chester. (a) Lipid extracted from particles; (b) individual phospholipids purified from this extract. In both cases the plates were developed in chloroform-methanol-water (65:25:4, by vol.) and lipids were located with I₂ vapour followed by 0.2% ninhydrin in butan-1-ol. Ninhydrin-positive lipids are shown hatched.

Table 4. *Composition of phospholipids purified from ultrasonically prepared particles obtained from Ps. ovalis Chester*

The preparation of the phospholipids and the analytical methods used are given in the text.

Lipid	Identification	Molar proportions			
		Phosphate	Glycerol	Fatty acid	Nitrogen
5	Phosphatidylethanolamine	1	0.92	1.8	1.0
2	Lysophosphatidylethanolamine	1	0.97	1.0	—
3	Phosphatidylglycerylglucosamine	1	1.84	1.8	1.0
4A	? Phosphatidylglycerol	1	1.72	1.8	—
4B		1	1.89	1.5	—
6A	Cardiolipin	1	1.36	1.8	0.0
6B	Polyglycerophosphatide	1	1.27	1.6	0.0
1A	? Cardiolipin derivative	1	1.49	1.6	—

Table 5. *Effect of phospholipids prepared from Ps. ovalis Chester on purified L-malate dehydrogenase (0.10 M-sodium chloride eluate)*

The methods of enzyme assay are described in the text. All lipids were tested at a final concentration of 1.5 μ M in terms of lipid P.

Lipid added	Quinone in assay system... ..	L-Malate-dehydrogenase activity			
		(m μ moles of L-malate oxidized/min.)		(% of rate in absence of added lipid)	
		Vitamin K ₃	Coenzyme Q ₉	Vitamin K ₃	Coenzyme Q ₉
None		3.9	1.4	(100)	(100)
1A		10.2	0.2	260	12
2 (lysophosphatidylethanolamine)		13.0	2.1	330	150
3		11.8	1.3	300	89
4A		9.5	1.2	240	82
4B		12.6	1.2	320	85
5 (phosphatidylethanolamine)		13.8	5.1	350	360
6A		12.6	1.4	320	100
6B		10.6	1.4	270	100

positive compound is liberated that co-chromatographs with ethanolamine hydrochloride in three solvent systems, and by the fact that after mild alkaline hydrolysis by the method of Dawson *et al.* (1962) a single water-soluble ninhydrin-positive derivative was formed that co-chromatographs with authentic glycerophosphorylethanolamine. On similar evidence lipid 2, which is a minor constituent, was identified as lysophosphatidylethanolamine.

The remaining phospholipids, which together account for about 24% of the total, fall into two groups. Lipids 3, 4A and 4B each have a P:glycerol ratio approaching 1:2, which suggests that they are derivatives of glycerophosphorylglycerol. Lipid 3 is ninhydrin-positive and evidence has been obtained that the ninhydrin chromophore is glucosamine (Phizackerley, MacDougall & Francis, 1966); lipids 4A and 4B are ninhydrin-negative. The second group, which consists of lipids 1A, 6A and 6B, are all ninhydrin-negative, and the phosphate:glycerol molar ratios indicate more complex structures. Lipid 1A is a minor deep-yellow constituent with a sharp absorption peak at 400 m μ ; the analytical data are compatible with it being a derivative of bis(glycerophosphoryl)glycerol (P:glycerol:fatty acid proportions 2.2.98:3.8). Lipid 6A has been tentatively identified as an acylated bis(glycerophosphoryl)glycerol, partly on the data given in Table 3 and partly because it is indistinguishable from ox-heart cardiolipin on thin-layer chromatography and on column chromatography on silicic acid. Lipid 6B, although it is indistinguishable from lipid 6A on thin-layer chromatography (Fig. 4), differs in that on column chromatography of the particle lipid extract it is eluted with chloroform-methanol (1:4, v/v) whereas

Table 6. *Effect of derivatives of phosphatidylethanolamine on L-malate dehydrogenase (0.05 M-sodium chloride eluate)*

Lipid added	L-Malate-dehydrogenase activity	
	(m μ moles of L-malate oxidized/min.)	(% of rate in absence of added lipid)
None	0.7	(100)
Phosphatidylethanolamine	4.3	650
Saturated phosphatidylethanolamine	1.0	140
L- α -Dipalmitoyl-phosphatidylethanolamine	0.7	100
Lysophosphatidylethanolamine	1.6	240
L- α -Palmitoylphosphatidylethanolamine	1.4	200
L- α -Dipalmitoyl-lecithin	1.2	180
Phosphatidic acid	1.0	140
Fatty acids	0.2	30
Diglyceride	0.2	30
Glycerophosphorylethanolamine	0.7	100
Glycerol 1-phosphate	0.2	30
Phosphoethanolamine	0.2	30
Ethanolamine	0.9	140

lipid 6A is eluted with chloroform-methanol (20:1, v/v). The analytical data show that lipid 6B is a polyglycerophosphatide and suggest that it has a structure more complex than that of cardiolipin.

Relationship between the phospholipid requirement and the quinone requirement of the L-malate dehydrogenase of Ps. ovalis Chester. As shown in Table 5,

each of the phospholipids that have been prepared from the particulate oxidase activated the purified dehydrogenase in the assay in which vitamin K₃ was used. At the concentration employed the activating effect of each lipid was similar, and hence it appears that in this assay system the phospholipid requirement of the enzyme is non-specific. In contrast, when coenzyme Q₉ was used in the assay, of the phospholipids present in the particulate oxidase only the unsaturated phosphatidylethanolamine markedly activated the enzyme (Table 5); lysophosphatidylethanolamine produced a much smaller activation, which was not maintained. The other lipids were either inert or inhibitory. The same results were obtained when coenzyme Q₉ was replaced by coenzyme Q₆. The naturally occurring unsaturated phosphatidylethanolamine is a much more potent activator than any of the derivatives prepared from it, and those derivatives that are not phospholipid in nature are, in general, inhibitors (Table 6).

Coenzyme Q₉ differs from vitamin K₃ in that it is a benzoquinone as opposed to a naphthaquinone and, in addition, it carries a bulky substituent at position 3, whereas vitamin K₃ is unsubstituted in the analogous position. The phospholipid specificity of the enzyme with a benzoquinone unsubstituted at position 3, and a naphthaquinone carrying a bulky substituent at the analogous position, has therefore been investigated. 2,3-Dimethoxy-5-methyl-1,4-benzoquinone (coenzyme Q₀) and 2-methyl-3-phytyl-1,4-naphthaquinone (vitamin K₁) fulfil these structural criteria. The phospholipids chosen for these experiments were unsaturated phosphatidylethanolamine and the polyglycerophosphatide (lipid 6B); the latter was chosen because it was the bulkiest of the available phospholipids. It can be seen from Table 7 that the enzyme system with coenzyme Q₀ resembles that with

vitamin K₃ in that it is activated both by phosphatidylethanolamine and by polyglycerophosphatide, whereas the enzyme system with vitamin K₁ resembles that with coenzyme Q₉ or coenzyme Q₆ in that it is activated by phosphatidylethanolamine but not by polyglycerophosphatide. These results suggest that it is the presence or absence of a large side chain rather than the nature of the nucleus that determines the behaviour of the quinone relative to phospholipid in the enzyme system.

Effect of variation in the sequence of addition of cofactors on the activity of the L-malate dehydrogenase of Ps. ovalis Chester. During investigations into factors that have to be taken into account to obtain reproducible enzyme assays, it became apparent that altering the order in which phospholipid and quinone were added to the enzyme had marked effects on its activity, and that the sequence that gave maximum rates depended on the nature of the quinone used in the assay. These results are shown in Table 8. There are similarities between the behaviour of vitamin K₁ and coenzyme Q₉ on the one hand, and between vitamin K₃ and coenzyme Q₀ on the other. Thus, with both vitamin K₁ and coenzyme Q₉, maximum rates were obtained by exposing the enzyme to phospholipid and quinone simultaneously, but, when phosphatidylethanolamine was added to the enzyme before either of these quinones, the rates were much lower. In contrast, both vitamin K₃ and coenzyme Q₀ gave maximum rates with this last sequence. In all cases the rate of reaction was suboptimum when quinone was added to the enzyme before phospholipid.

Apparent Michaelis constants for L-malate, FAD, coenzyme Q₉, vitamin K₃ and phosphatidylethanolamine of the L-malate dehydrogenase of Ps. ovalis Chester. These constants were determined by the

Table 7. *Phospholipid specificity of L-malate dehydrogenase (0.10 M-sodium chloride eluate) assayed in the presence of coenzyme Q₀ or vitamin K₁*

Each assay was carried out spectrophotometrically by using the sequence of reagent addition that gave maximum rates (Table 8). The final concentration of coenzyme Q₀ was 0.67 mM, that of vitamin K₁ was 33 μ M and that of phospholipid was 1.7 μ M.

Quinone	Phospholipid added	L-Malate-dehydrogenase activity	
		(m μ moles of L-malate oxidized/min.)	(% of rate in absence of added lipid)
Coenzyme Q ₀	None	2.5	(100)
	Phosphatidylethanolamine	6.7	270
	Polyglycerophosphatide (lipid 6B)	5.7	230
Vitamin K ₁	None	1.8	(100)
	Phosphatidylethanolamine	4.3	240
	Polyglycerophosphatide (lipid 6B)	1.7	95

Table 8. *Effect of variation in the sequence of addition of cofactors on L-malate dehydrogenase (0.10 M-sodium chloride eluate)*

The final concentrations of coenzyme Q₀ and of vitamin K₁ in the assay system are shown in the Table. Other reactants were used at the concentrations given in the Methods and Materials section, and in all experiments FAD, L-malate and 2,6-dichlorophenol-indophenol were added, in that order, after the sequences listed below.

Quinone used in assay system	Simplified order of addition of cofactors	L-Malate oxidized	
		(mμmoles/min.)	(% of optimum rate)
Vitamin K ₃	Vitamin K ₃ ; phosphatidylethanolamine; enzyme	11.6	(100)
	Phosphatidylethanolamine; enzyme; vitamin K ₃	11.0	95
	Vitamin K ₃ ; enzyme; phosphatidylethanolamine	6.1	52
Coenzyme Q ₀ (0.67 mM)	Coenzyme Q ₀ ; phosphatidylethanolamine; enzyme	6.9	65
	Phosphatidylethanolamine; enzyme; coenzyme Q ₀	9.4	(100)
	Coenzyme Q ₀ ; enzyme; phosphatidylethanolamine	4.7	50
Coenzyme Q ₉	Coenzyme Q ₉ ; phosphatidylethanolamine; enzyme	8.5	(100)
	Phosphatidylethanolamine; enzyme; coenzyme Q ₉	6.0	70
	Coenzyme Q ₉ ; enzyme; phosphatidylethanolamine	5.3	62
Vitamin K ₁ (33 μM)	Vitamin K ₁ ; phosphatidylethanolamine; enzyme	3.9	(100)
	Phosphatidylethanolamine; enzyme; vitamin K ₁	2.3	60
	Vitamin K ₁ ; enzyme; phosphatidylethanolamine	2.3	60

Table 9. *Apparent K_m values for L-malate, FAD, coenzyme Q₉, vitamin K₃ and phosphatidylethanolamine of L-malate dehydrogenase (0.10 M-sodium chloride eluate)*

L-Malate dehydrogenase was assayed spectrophotometrically as described in the text. The apparent K_m values were determined by the method of Lineweaver & Burk (1934). The concentrations of reactants are given in the text.

Reactant	Apparent K _m
L-Malate	0.45 mM
FAD	0.40 μM
Coenzyme Q ₉	2.4 μM
Vitamin K ₃	3.0 mM
Phosphatidylethanolamine	0.6 μM

method of Lineweaver & Burk (1934) with the enzyme eluted on DEAE-cellulose chromatography with 0.10 M-sodium chloride, and the results are given in Table 9. The concentration of each reactant was varied separately and the concentration of the other components was held constant at the values given in the Methods and Materials section. In each case the reciprocal of the initial reaction velocity varied linearly with the reciprocal of the concentration of the reactant whose concentration was varied. It should be noted that because of their low solubility the concentrations of vitamin K₃ and of coenzyme Q₉ are suboptimum, and it follows that the observed Michaelis constants of the other reactants may be higher than the true values for this reason. A second limitation arises from the fact that it is implicit in the determination

of Michaelis constants by this method that the specific concentration of the reactant (Strauss & Goldstein, 1943) is high relative to the concentration of the enzyme. In the present case this may not be so. Finally, there is a particular difficulty in relation to the apparent Michaelis constant for phosphatidylethanolamine in that the enzyme used in these studies contains endogenous phospholipid. The effect of this will be to make the observed Michaelis constant for phosphatidylethanolamine lower than the true value.

DISCUSSION

Several micro-organisms are now known to contain nicotinamide nucleotide-independent enzyme systems capable of oxidizing L-malate to oxaloacetate. In addition to *Ps. ovalis* Chester, these systems have been demonstrated in *Pseudomonas fluorescens* (Stanier, Gunsalus & Gunsalus, 1953), *Serratia marcescens* (Linnane & Still, 1955), *Azotobacter agilis* (Alexander & Wilson, 1956), *Micrococcus lysodeikticus* (Cohn, 1958), *Mycobacterium phlei* (Asano & Brodie, 1963), *Mycobacterium avium* (Kimura & Tobari, 1963) and *Acetobacter xylinum* (Benziman & Perez, 1965). In two instances L-malate dehydrogenase has been partially purified, and in both cases the enzyme resembles that prepared from *Ps. ovalis* Chester in exhibiting a triple cofactor requirement for FAD, quinone and phospholipid. The enzyme prepared from *Myco. avium* by Kimura & Tobari (1963) and Tobari (1964) utilized a number of benzoquinones and naphthaquinones and, although it was activated by several phospholipids, evidence was presented that the

natural phospholipid activator was cardiolipin. With the enzyme prepared from *Myco. phlei* by Asano & Brodie (1963) and Asano, Kaneshiro & Brodie (1965), which utilized naphthaquinones, it was necessary to supply the phospholipid in micellar form, but there was no evidence that a specific phospholipid was required.

The L-malate dehydrogenase of *Ps. ovalis* Chester can be released from the fragments of the cell-wall membrane to which it is structurally and functionally bound by exposure to ultrasound. At this stage the enzyme exhibits a requirement for both FAD and a quinone, but it is still associated with phospholipids, mainly phosphatidylethanolamine, and hence does not reveal a phospholipid requirement. This latter requirement is only revealed by depleting the enzyme of phospholipid by column chromatography on DEAE-cellulose. Although the L-malate dehydrogenase can in appropriate circumstances utilize a number of quinones and a number of phospholipids when coenzyme Q₉, which is the naturally occurring quinone in this organism, is used, the physiological phospholipid activator is probably an unsaturated phosphatidylethanolamine.

In view of the requirement of the soluble dehydrogenase for both FAD and a quinone, it is reasonable to suppose that the enzyme catalyses the reduction of FAD by L-malate and that reduced FAD is reoxidized by quinone, and in fact when L-malate is added to the particulate L-malate oxidase of *Ps. ovalis* Chester there is marked bleaching at 450 m μ , indicating flavine reduction (Francis *et al.* 1963). However, with the soluble L-malate dehydrogenase no spectroscopic changes were detected over the range 300–1000 m μ in the anaerobic steady state in the presence of L-malate that could be attributed to the formation of reduced forms of FAD. This negative result was obtained in the presence and absence of quinone, and with both the enzyme preparation purified by column chromatography and a more concentrated preparation obtained by dialysis of the ammonium sulphate precipitate (see the Methods and Material section); in the latter case the cuvettes contained 2.4 mg. of protein, capable of oxidizing 3 μ moles of L-malate/min. In contrast, and in accord with the finding that on addition of L-malate to the particulate oxidase preparation the proportion of the endogenous quinone present in the reduced form increased from 10% to about 60%, the formation of quinol by the soluble dehydrogenase in the presence of L-malate was readily demonstrated. It was also shown by direct comparison under different conditions that the rate of quinol formation in the absence of 2,6-dichlorophenol-indophenol agreed well with the activity measured by the rate of dye reduction. It is concluded therefore that L-malate dehydrogenase

of *Ps. ovalis* Chester is an L-malate–quinone oxidoreductase.

There are several possible explanations for the phospholipid requirement of this enzyme system. In the first place it may be that the function of the phospholipid is to provide micelles in which the quinone can dissolve. On this view, phospholipid acts in effect as a physical carrier, transporting quinone to the enzyme. The physical state of the phospholipid and of the quinone under the conditions of enzyme assay has not been investigated, and even though the concentration of phospholipid is extremely low (1.5 μ M) it may well be that micelles are present. However, two main arguments can be advanced against the view that their sole function is to render the quinone water-soluble. In the first place, although coenzyme Q₀ is water-soluble, the activating effect of phospholipid is readily demonstrable (Table 7). Secondly, this explanation of the role of phospholipid does nothing more than account for the dual requirement of the enzyme for phospholipid and quinone; it does not of itself explain the restriction that the nature of the quinone imposes on the nature of the phospholipid required for activation. This finding would require an additional hypothesis, to the effect that vitamin K₁, coenzyme Q₈ and coenzyme Q₉ form mixed micelles with phosphatidylethanolamine but not with the other phospholipids tested. This, however, is unlikely because the formation of mixed micelles between these quinones and a number of phospholipids, including lecithin and cardiolipin, has been demonstrated (Fleischer, Brierley, Klouwen & Slaughterback, 1962; Asano *et al.* 1965). It is therefore necessary to suppose that the enzyme possesses a phospholipid-binding site and a quinone-binding site.

Several deductions can be drawn about the nature of these sites. In the first place, both sites are relatively non-specific, since the enzyme can react, under appropriate circumstances, with different phospholipids and with different quinones. Secondly, since a variety of non-phospholipid derivatives of phosphatidylethanolamine, and mixtures thereof, fail to activate the enzyme, it seems probable that the phospholipid-binding site requires to be occupied by an amphipathic molecule, i.e. that it possesses a polar and a non-polar portion. Similarly, it is likely that the quinone-binding site possesses a sector that binds the quinone nucleus and a sector that binds the quinone side chain. The first part of this conclusion follows directly from the demonstration that quinone is reduced to quinol during the enzyme reaction; and the second part is made probable by the finding that the apparent K_m values for coenzyme Q₈ and coenzyme Q₉ are lower by about three orders of magnitude than that of coenzyme Q₀. The third deduction

that can be made is that there is interaction between the quinone-binding site and the phospholipid-binding site, and the question arises as to the nature of this interaction.

Two main possibilities can be envisaged. The first possibility is that the interaction is steric; this theory requires that the two sites are adjacent and supposes that, to take a particular example, when the quinone-binding site is occupied by a quinone with a large side chain such as coenzyme Q₉, the space available on the phospholipid-binding site is such that a large phospholipid such as polyglycerophosphatide cannot be accommodated, whereas occupation of the quinone-binding site by vitamin K₃ or coenzyme Q₀ does not restrict occupation of the phospholipid site in this way. The second possibility is that, when either the phospholipid-binding site or the quinone-binding site is occupied, a conformational change takes place in the structure of the enzyme such that the binding capacity of the unoccupied site is modified. On this theory, it is not necessary to suppose that the two sites are contiguous. Thus it can be supposed that occupation of the quinone-binding site by coenzyme Q₉ would induce a conformational change at the phospholipid-binding site such that it was capable of binding phosphatidylethanolamine but not polyglycerophosphatide, whereas if the quinone-binding site were occupied by vitamin K₃ or coenzyme Q₀ this restrictive conformational change would not occur. Either theory offers a reasonable explanation of the observed quinone-phospholipid relationship but the evidence available does not permit them to be distinguished.

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REFERENCES

- Alexander, M. & Wilson, P. W. (1956). *J. Bact.* **71**, 252.
 Asano, A. & Brodie, A. F. (1963). *Biochem. biophys. Res. Commun.* **13**, 423.
 Asano, A., Kaneshiro, T. & Brodie, A. F. (1965). *J. biol. Chem.* **240**, 895.
 Barron, E. J. & Hanahan, D. J. (1958). *J. biol. Chem.* **231**, 493.
 Bartlett, G. R. (1959). *J. biol. Chem.* **234**, 466.
 Benzman, M. & Perez, L. (1965). *Biochem. biophys. Res. Commun.* **19**, 127.
 Brown, J. L. & Johnston, J. M. (1962). *J. Lipid Res.* **3**, 480.
 Cohn, D. V. (1958). *J. biol. Chem.* **233**, 299.
 Dawson, R. M. C., Hemington, N. & Davenport, J. B. (1962). *Biochem. J.* **84**, 497.
 Duncombe, W. G. (1963). *Biochem. J.* **88**, 7.
 Fenton, J. C. B. (1962). *Clin. chim. Acta*, **7**, 163.
 Fleischer, S., Brierley, G. P., Klouwen, H. & Slautterback, D. B. (1962). *J. biol. Chem.* **237**, 3264.
 Folch, J., Lees, M. & Sloane-Stanley, G. H. (1957). *J. biol. Chem.* **226**, 497.
 Francis, M. J. O., Hughes, D. E., Kornberg, H. L. & Phizackerley, P. J. R. (1963). *Biochem. J.* **89**, 430.
 Francis, M. J. O. & Phizackerley, P. J. R. (1965). *Biochem. J.* **95**, 25 p.
 Keilin, D. & Hartree, E. F. (1945). *Biochem. J.* **33**, 293.
 Kimura, T. & Tobari, J. (1963). *Biochim. biophys. Acta*, **73**, 399.
 Kornberg, H. L. & Phizackerley, P. J. R. (1961). *Biochem. J.* **79**, 10 p.
 Lester, R. L., Hatefi, Y., Widmer, C. & Crane, F. L. (1959). *Biochim. biophys. Acta*, **33**, 169.
 Lester, R. L. & Ramasarma, T. (1959). *J. biol. Chem.* **234**, 672.
 Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
 Linnane, A. W. & Still, J. W. (1955). *Biochim. biophys. Acta*, **16**, 305.
 Long, C. & Penny, I. F. (1957). *Biochem. J.* **65**, 382.
 Long, C. & Maguire, M. F. (1954). *Biochem. J.* **57**, 223.
 Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
 Phizackerley, P. J. R., MacDougall, J. M. & Francis, M. J. O. (1966). *Biochem. J.* **99**, 21 c.
 Singer, T. P. & Kearney, E. B. (1957). *Meth. biochem. Anal.* **4**, 307.
 Skipski, V. P., Peterson, R. F. & Barclay, M. (1964). *Biochem. J.* **90**, 374.
 Stanier, R. Y., Gunsalus, I. C. & Gunsalus, C. F. (1953). *J. Bact.* **66**, 543.
 Strauss, O. H. & Goldstein, A. (1943). *J. gen. Physiol.* **2f**, 559.
 Tobari, J. (1964). *Biochem. biophys. Res. Commun.* **15**, 50.
 Wagner, H., Hörhammer, L. & Wolff, P. (1961). *Biochem. Z.* **334**, 175.
 Warburg, O. & Christian, W. (1941). *Biochem. Z.* **310**, 384.
 Wieland, O. (1963). In *Methods of Enzymatic Analysis*, p. 208. Ed. by Bergmeyer, H. U. London and New York: Academic Press Inc.