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Purification of 2-oxo acid dehydrogenase multienzyme complexes from ox heart by a new method

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A new method is described that allows the parallel purification of the pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase multienzyme complexes from ox heart without the need for prior isolation of mitochondria. All the assayable activity of the 2-oxo acid dehydrogenase complexes in the disrupted tissue is made soluble by the inclusion of non-ionic detergents such as Triton X-100 or Tween-80 in the buffer used for the initial extraction of the enzyme complexes. The yields of the pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes are many times greater than those obtained by means of previous methods. In terms of specific catalytic activity, banding pattern on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, sedimentation properties and possession of the regulatory phosphokinase bound to the pyruvate dehydrogenase complex, the 2-oxo acid dehydrogenase complexes prepared by the new method closely resemble those described by previous workers. The greatly improved yield of 2-oxo acid dehydrogenase complexes occasioned by the use of Triton X-100 or Tween-80 as solubilizing agent supports the possibility that the bulk of the pyruvate dehydrogenase complex is associated in some way with the mitochondrial inner membrane and is not free in the mitochondrial matrix space.

The overall reaction of the 2-oxo acid dehydrogenase multienzyme complexes can be represented as:

$$R\text{–}CO\text{–}CO_2H + CoA + NAD^+ \rightarrow R\text{–}CO\text{–}SCoA + CO_2 + NADH + H^+$$

where $R = CH_3$ (pyruvic acid) or $[CH_2]_2\text{–}CO_2H$ (2-oxoglutaric acid).

The ox heart pyruvate dehydrogenase complex [for a review see Reed (1974)] consists of multiple copies of three consecutive enzymes: pyruvate decarboxylase (E1) (EC 1.2.4.1), lipoate acetyltransferase (E2) (EC 2.3.1.12) and lipoamide dehydrogenase (E3) (EC 1.6.4.3). Enzyme E1 is composed of two types of polypeptide chain (α and β) of relative molecular mass 42000 (α) and 37000 (β), arranged as a tetramer ($\alpha_2\beta_2$) when separated from the complex (Barrera *et al.*, 1972). Enzyme E3 is a dimer comprising two polypeptide chains of relative molecular mass 53000 (Barrera *et al.*, 1972). Enzyme E2 forms the structural core of the complex and contains a single type of polypeptide chain whose relative molecular mass was estimated as 74000 by means of sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and as 52000 by means of sedimentation equilibrium in 6M-guanidine hydrochloride (Barrera *et al.*, 1972). On adopting a relative molecular mass of 3.1×10^6 for the lipoate acetyltransferase, the lower value (52000) suggests the presence of 60 subunits in the intact E2 component, consistent with the eicosahedral symmetry predicted from electron microscopy (Reed & Oliver, 1968).

The 2-oxoglutarate dehydrogenase complexes from the mitochondria of ox kidney (Reed & Oliver, 1968) and pig heart (Koike & Koike, 1976) have also been studied. They comprise multiple copies of 2-oxoglutarate decarboxylase (E1) (EC 1.2.4.2), lipoate succinyltransferase (E2) (EC 2.3.1.61) and lipoamide dehydrogenase (E3). From pig heart, the E1 component is a dimer of identical subunits each with a relative molecular mass of 113000, and the E2 component, once again the structural core, contains a single type of polypeptide chain with a relative molecular mass of 48000 (Koike & Koike, 1976). The intact lipoate succinyltransferase has a relative molecular mass of about 1.0×10^6 and appears in the electron microscope to consist of 24 polypeptide chains arranged with octahedral symmetry (Koike & Koike, 1976).

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The mammalian pyruvate dehydrogenase complexes are regulated by phosphorylation in the presence of ATP (Reed, 1974; Denton *et al.*, 1975). The E1α component is phosphorylated by a kinase tightly bound to the lipoate acetyltransferase core (Reed, 1974) with concomitant loss of activity. In the pig heart complex (Sugden *et al.*, 1979) and ox heart and kidney complexes (Yeaman *et al.*, 1978), inactivation is correlated with the phosphorylation of the first of three specific serine residues in the E1α chains. An Mg^{2+}-requiring phosphatase exists to remove the phosphate groups and restore activity (Reed, 1974). No comparable phosphorylation of the 2-oxoglutarate dehydrogenase complexes has been reported.

The most widely used methods for purifying the mammalian pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes rely on a preliminary purification of mitochondria (Kerbey *et al.*, 1979; Roche & Cate, 1977; Koike & Koike, 1976; Linn *et al.*, 1972), in which organelle these enzymes appear to be localized (Denton *et al.*, 1975). In the present paper we describe a new method that allows the parallel purification of both complexes from ox heart in greatly improved yield. The time-consuming preparation of mitochondria is dispensed with and the enzyme complexes are purified from a whole-tissue extract solubilized in the non-ionic detergent Triton X-100. The implications of our work for the intramitochondrial location of the complexes are considered.

Materials and methods

Chemicals

Mops (4-morpholinepropanesulphonic acid), phenylmethanesulphonyl fluoride and dithiothreitol were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Poly(ethylene glycol) 6000, Triton X-100 and benzamidine were purchased from BDH Chemicals, Poole, Dorset, U.K. Tween-80 was bought from Koch–Light Laboratories, Colnbrook, Bucks., U.K., and silicone antifoam from Hopkin and Williams, Chadwell Heath, Essex, U.K. Sepharose CL-2B was purchased from Pharmacia, Uppsala, Sweden. Poly(ethylene glycol) was dissolved in glass-distilled water at a final concentration of 35% (w/v).

Enzyme assays

Pyruvate dehydrogenase complex, 2-oxoglutarate dehydrogenase complex and lipoamide dehydrogenase activities were assayed as described by Brown & Perham (1976). Pyruvate dehydrogenase kinase was assayed by incubation of a sample of the pyruvate dehydrogenase complex at 30°C with 1mM-ATP and 3mM-$MgCl_2$. The pH was kept at 7.0. Samples were removed and assayed for activity of pyruvate dehydrogenase complex or 2-oxoglutarate dehydrogenase complex. The loss of the former activity was first-order for more than 90% of the reaction.

Protein assay

Protein was determined by the method of Lowry *et al.* (1951), with sheep serum albumin as a standard. To prevent interference by Triton X-100 in the sample, a final concentration of 0.1% sodium dodecyl sulphate was used to dissolve the precipitate that Triton forms with the Folin reagent.

Sodium dodecyl sulphate / polyacrylamide - gel electrophoresis

Samples of protein prepared as described by Perham & Thomas (1971) were run in 7.5% (w/v) polyacrylamide gels in the phosphate system of Shapiro & Maizel (1969) or in 11.5% (w/v) slab gels in the Tris/glycine system of Laemmli (1970).

Results

All operations and centrifugation steps were carried out at 4°C unless stated otherwise. The pH values of all buffers were adjusted at 20°C.

Initial extraction of 2-oxo acid dehydrogenase complexes

Ox hearts were chilled in ice immediately after slaughter and trimmed of fat and connective tissue. The hearts were cut into 2cm cubes and frozen at −20°C. Total enzyme activities of the pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes were unchanged after storage for 1 year.

Frozen cubes of tissue (about 250g) at −20°C were suspended at room temperature in twice their volume of 50mM-Mops containing 2.7mM-EDTA, 0.1mM-dithiothreitol and 3% (v/v) Triton X-100 or Tween-80, adjusted to pH7.0 with 6M-NaOH. After addition of phenylmethanesulphonyl fluoride to 1mM, benzamidine hydrochloride to 1mM and silicone antifoam (0.5ml/l), the tissue was rapidly dispersed and blended in a small Waring blender for 5min at maximum speed. The final temperature was generally about 9°C. The homogenate was diluted with an equal volume of the same buffer adjusted to pH6.8 with 6M-NaOH, and the mixture was clarified by centrifugation at 10000*g* for 20min in a Sorvall GS-A rotor. The substantial pellet was washed with the same buffer as was used for blending, and the washings were added to the supernatant from the initial centrifugation. The remaining insoluble material was discarded. Over 90% of the activity of pyruvate dehydrogenase complex assayable in the blended tissue was extracted by this procedure.

Phosphate buffer (50mM, pH7) can be substituted for Mops in the initial extraction of the enzyme complexes, but during the subsequent fractionation by poly(ethylene glycol) precipitation Mops has been found to be preferable.

Fractionation with poly(ethylene glycol)

The pH of the supernatant from the previous step was adjusted to 6.45 at 4°C with 10% (v/v) acetic acid, and 0.12 vol. of 35% poly(ethylene glycol) was rapidly added. The mixture was stirred for 30min and the precipitate was collected by centrifugation at 25000*g* for 10min in a Sorvall GS-A rotor. The pellet was resuspended in about 400ml of 50mM-Mops containing 2.7mM-EDTA and 0.1mM-dithiothreitol, adjusted to pH6.8 at 20°C with 6M-NaOH. This was the minimum volume necessary to dissolve fully both enzyme activities. Triton was not included in this step, since there is sufficient in the pellet to make both complexes soluble. Full resuspension was achieved by brief sonication at 100W for 30s on 100ml batches of solution in a Dawes Soniprobe. The solution was clarified at 25000*g* for 40min at 20°C in a Sorvall GS-A rotor and the pellet was discarded. The supernatant was filtered through muslin to remove particles of fat. The protein concentration in the supernatant was now about 2mg/ml.

The supernatant was maintained at pH7.0 by addition of 0.5M-NaOH while the solution was made 13mM with respect to $MgCl_2$ and warmed to 30°C in a water bath. (The pH of the Mops buffer falls as the temperature is raised.) This procedure caused a rapid increase in activity of pyruvate dehydrogenase complex, which reached its maximum within 5min at 30°C, presumably owing to activation of the relevant phosphatase. If maximum phosphatase activity is desired, phenylmethanesulphonyl fluoride should be omitted from the initial blending buffer, since it is an inhibitor of the phosphatase (Hutson *et al.* 1978). The extent of activation varied from 15% to 200%. A similar increase (a constant 15%) in activity of 2-oxoglutarate dehydrogenase complex was noted at this stage.

The solution was cooled to below 10°C and Triton X-100 was added to a final concentration of 1%. The ionic strength was raised by adding 1M-sodium phosphate buffer, pH6.3, to a final concentration of 50mM. This prevented the formation of a substantial precipitate, which otherwise appeared at pH values below 6.70. The pH was then lowered to 6.45 with 10% (v/v) acetic acid, and 0.12 vol. of 35% poly(ethylene glycol) was rapidly added. After 30min the precipitate was collected by centrifugation at 25000*g* for 10min in a Sorvall GS-A rotor. The pellet was removed, care being taken to avoid contamination with fat particles, and resuspended by standing overnight at 4°C in about 100ml of 50mM-Mops containing 2.7mM-EDTA, 0.1mM-dithiothreitol and 1% Triton X-100, adjusted to pH6.8 at 20°C with 6M-NaOH. This was the minimum volume necessary to dissolve fully both enzyme activities.

Separation of complexes by isoelectric precipitation

After being kept overnight at 4°C, the pellet was briefly sonicated (100W for 20s on the whole sample in a Dawes Soniprobe) to complete the resuspension. The solution was clarified at 40000*g* for 60min in a Sorvall SS-34 rotor. The clear supernatant had a protein concentration of about 6mg/ml. Then 1M-sodium phosphate buffer, pH6.3, was added to a final concentration of 50mM to prevent a non-specific precipitate forming below pH6.7.

The pH was lowered to 5.7 by addition of 10% (v/v) acetic acid. The solution was kept at 4°C for 1h and a small sample was centrifuged. Most of the activity of the 2-oxoglutarate dehydrogenase complex was found to have been precipitated, but if necessary the pH was lowered by a further 0.1pH unit until less than 5% of the 2-oxoglutarate dehydrogenase activity was left in solution. Under these conditions, more than 95% of the pyruvate dehydrogenase complex remained soluble. The precipitate was collected at 10000*g* for 10min in a Sorvall SS-34 rotor and the pellet was suspended in 20ml of 50mM-sodium phosphate buffer, pH7.0, containing 2.7mM-EDTA and 1% Triton X-100. The supernatant containing the pyruvate dehydrogenase complex was concentrated by adjusting it to pH6.45 with 0.5M-NaOH and adding 0.12vol. of 35% poly(ethylene glycol). After 30min, the precipitate was collected at 25000*g* for 10min in a Sorvall SS-34 rotor and resuspended in 10ml of 50mM-sodium phosphate buffer, pH7.0, containing 2.7mM-EDTA and 1% Triton X-100.

Separation of the complexes by precipitation with poly(ethylene glycol)

An alternative method for separating the two complexes by means of precipitation with poly(ethylene glycol) was based on the work of Linn *et al.* (1972). We prefer this method because it avoids the need for accurate control of pH (±0.1pH unit), which is necessary for their separation by isoelectric precipitation.

The pellet from the second poly(ethylene glycol) precipitation was resuspended by standing overnight at 4°C in about 100ml of 50mM-Mops containing 2.7mM-EDTA, 0.1mM-dithiothreitol and 1% Triton X-100, adjusted to pH6.8 at 20°C with 6M-NaOH, as described above. Phenylmethanesulphonyl fluoride was added to a final concentration of 1mM and the solution was then allowed to warm to room temperature (18–22°C). After being

stirred gently for 1h, brief sonication as described above was used to complete the resuspension of the enzyme complexes. The solution was clarified by centrifugation at 40000*g* for 60min at 20°C in a Sorvall SS-34 rotor. The clear supernatant was gently stirred at room temperature for a further 1h.

The pH of the supernatant was lowered to pH6.4 by addition of 10% (v/v) acetic acid. The extensive precipitation observed earlier below pH6.7 at lower temperatures and ionic strengths (see above) did not now take place. Small amounts of a poly(ethylene glycol) solution (35%, w/v) were then added and samples (0.5ml) of the mixture were tested for precipitation of the 2-oxoglutarate dehydrogenase complex after centrifugation in a bench centrifuge at room temperature. After 0.08vol. of poly(ethylene glycol) solution had been added, the 2-oxoglutarate dehydrogenase activity remaining in the supernatant had been decreased to less than 1% of its starting value, whereas more than 95% of the pyruvate dehydrogenase activity remained in solution. At this point the precipitate was collected by centrifugation in a Sorvall SS-34 rotor at 25000*g* for 10min at 20°C. The pyruvate dehydrogenase complex was then precipitated by adding more (a further 0.14 vol.) of the poly(ethylene glycol) solution and centrifuging as above. The pellets of 2-oxoglutarate dehydrogenase complex and pyruvate dehydrogenase complex were resuspended in 20ml and 10ml, respectively, of 50mM-sodium phosphate buffer, pH7.0, containing 2.7mM-EDTA and 1% Triton X-100.

Gel filtration on Sepharose CL-2B

Samples of the separated complexes were gel-filtered on a column (110cm × 3cm) of Sepharose CL-2B in 50mM-sodium phosphate buffer, pH7.0, containing 2.7mM-EDTA and 1% Triton X-100. Dithiothreitol was omitted from the buffer, since it appeared to cause loss of FAD from the E3 component of the enzymes during gel filtration, with accompanying inactivation. The flavin was detected in the column effluent by means of its absorbance at 455nm. A typical separation of a mixture of pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes is shown in Fig. 1. The excluded material of high molecular weight was not investigated further, since it lacked FAD and 2-oxo acid dehydrogenase activity.

The peaks of enzyme activity were pooled and the complexes were precipitated from them by addition of 0.15vol. of 35% poly(ethylene glycol) at pH6.45. The precipitates were collected by centrifugation at 25000*g* for 10min in a Sorvall GS-A rotor and resuspended at a protein concentration of about 10mg/ml in 50mM-Mops containing 2.7mM-EDTA, 0.01% NaN_3, 30% (v/v) glycerol and 1% Triton X-100, adjusted to pH6.8 with 6M-NaOH at 20°C.

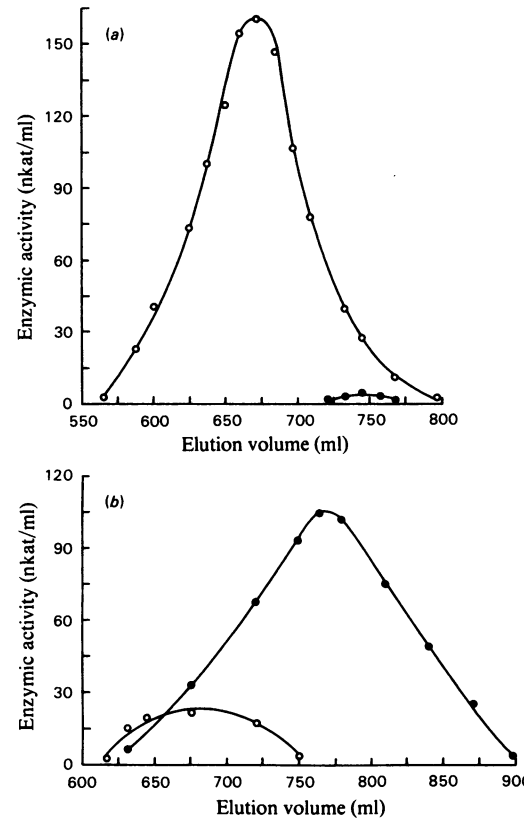

Fig. 1. *Gel filtration of (a) partly purified pyruvate dehydrogenase complex and (b) partly purified 2-oxoglutarate dehydrogenase complex from ox heart on a column (110cm × 3cm) of Sepharose CL-2B*

The buffer was 50mM-sodium phosphate, pH7.0, containing 2.7mM-EDTA and 1% (v/v) Triton X-100. For further experimental details see the text. ○, Pyruvate dehydrogenase activity; ●, 2-oxoglutarate dehydrogenase activity.

Both enzymes were stable for at least several weeks on storage at 4°C. Omission of Triton X-100 from the storage buffer caused appreciable loss of enzymic activity during this period. Freezing induced precipitation and was avoided.

Summary of the purification procedure

A summary of the purification procedure starting with about 250g of frozen ox heart is shown in Table 1.

Some properties of the purified enzyme complexes

As recorded in Table 1, the final specific activities of the pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes were about 185nkat/mg and 195nkat/mg respectively, when assayed at 30°C. These values compare favourably with typical

Table 1. *Purification of pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes from ox heart*
All values refer to a preparation of the enzyme complexes from about 250g of ox heart. For experimental details see the text.

Step	Volume (ml)	Total protein (mg)	Enzymic activity (nkat)		Specific activity (nkat/mg)		Yield (%)	
			Pyruvate dehydrogenase	2-Oxoglutarate dehydrogenase	Pyruvate dehydrogenase	2-Oxoglutarate dehydrogenase	Pyruvate dehydrogenase	2-Oxoglutarate dehydrogenase
First poly(ethylene glycol) precipitate	400	800	27000*	31800*	33.8	39.8	100†	100†
Second poly(ethylene glycol) precipitate	90	540	23500	28800	43.5	53.4	87	91
Isoelectric precipitation								
Supernatant after concentration	10	205	20500	816	100	4.0	76	—
Redissolved precipitate	20	280	1000	23300	3.6	83.2	—	72
Gel filtration on Sepharose CL-2B								
Pyruvate dehydrogenase complex	9	99	18300	267	185	2.7	68	—
2-Oxoglutarate dehydrogenase complex	7	90	200	17500	2.2	194	—	55

* Assays were carried out after incubation with $MgCl_2$ to activate the phosphatase.

† Accurate values for the activities of pyruvate dehydrogenase complex and 2-oxoglutarate dehydrogenase complex were difficult to obtain before poly(ethylene glycol) precipitation, because of interference by NADH oxidase and lactate dehydrogenase.

values of 214nkat/mg for the pyruvate dehydrogenase complex and 188nkat/mg for the 2-oxoglutarate dehydrogenase complex of pig liver (Roche & Cate, 1977) and 150nkat/mg for pyruvate dehydrogenase complex from pig heart (Cooper *et al.*, 1974). They also compare well with values of 158–280nkat/mg and 220nkat/mg for the pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes of ox heart purified by Linn *et al.* (1972). The differences are small enough to reflect slight differences in the assay conditions and methods of measuring protein.

When examined by means of sodium dodecyl sulphate/polyacrylamide-gel electrophoresis in the phosphate buffer system (Fig. 2), the pyruvate

Fig. 2. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of 2-oxo acid dehydrogenase multienzyme complexes from ox heart*
(*a*) Sodium dodecyl sulphate/polyacrylamide-gel (11.5%, w/v) electrophoresis of pyruvate dehydrogenase (P) and 2-oxoglutarate dehydrogenase (O) complexes performed in the Tris/glycine system of Laemmli (1970). (*b*) Sodium dodecyl sulphate/polyacrylamide-gel (7.5%, w/v) electrophoresis of pyruvate dehydrogenase complex performed in the phosphate system of Shapiro & Maizel (1969). For further experimental details see the text.

dehydrogenase and 2-oxoglutarate dehydrogenase complexes gave protein bands identical with those previously published for the ox heart enzymes (Linn *et al.*, 1972). An additional band migrating near lipoamide dehydrogenase (E3) was observed in the pyruvate dehydrogenase complex when the Tris/glycine buffer system was substituted (Fig. 2). We have not identified this band, but it was found only in the pyruvate dehydrogenase complex and could therefore be the phosphokinase specifically associated with that complex (Reed, 1974). Its polypeptide chain is reported to have a relative molecular mass of about 50000 (Reed, 1974). Densitometric scanning of the gels obtained from several different preparations of the complexes indicated that the polypeptide-chain ratios were constant within experimental error. By the same criterion, no change in polypeptide-chain ratios could be detected after gel filtration on Sepharose CL-2B.

We also examined our preparations of the ox heart complexes by means of sedimentation-velocity ultracentrifugation (Fig. 3). The pyruvate dehydrogenase complex showed a single large aggregate with an apparent $s_{20,w}$ of 68S, whereas the major component of the 2-oxoglutarate dehydrogenase complex sedimented with an apparent $s_{20,w}$ of 37S. These runs were carried out at single protein concentrations. The Schlieren traces were not completely symmetrical (Fig. 3), which might be due to partial dissociation into smaller aggregates. The raised baseline at the meniscus is probably due to the presence of micellar Triton X-100.

Regulatory enzymes

We monitored the distribution of the regulatory enzymes, phosphokinase and phosphatase, during the purification of the pyruvate dehydrogenase complex. The phosphatase was present and very active after the initial fractionation with poly(ethylene glycol), but was not detectable in later stages of the purification. On the other hand, the kinase was very active and was not lost during the preparation of the complex. Purified samples of the pyruvate dehydrogenase complex were rapidly inactivated at 30°C in 50mM-sodium phosphate buffer, pH7.0, in the presence of 1mM-ATP and 3mM-$MgCl_2$. The loss of activity was apparent first-order for more than 90% of the inactivation ($t_{\frac{1}{2}} = 5$min). This rate is similar to that described by Linn *et al.* (1969). We infer that our preparations of the ox heart pyruvate dehydrogenase complex are not deficient in the enzyme-bound phosphokinase.

Removal of Triton X-100

We have not made a detailed study of the removal of Triton X-100 from our samples of 2-oxo acid dehydrogenase complexes. However, the method described by Holloway (1973) has proved satisfactory, with the substitution of Amberlite XAD-2 resin for the recommended Bio-Beads SM-2. After removal of Triton, there was no immediate change in catalytic activity, but the complexes were less stable on storage at 4°C (see above).

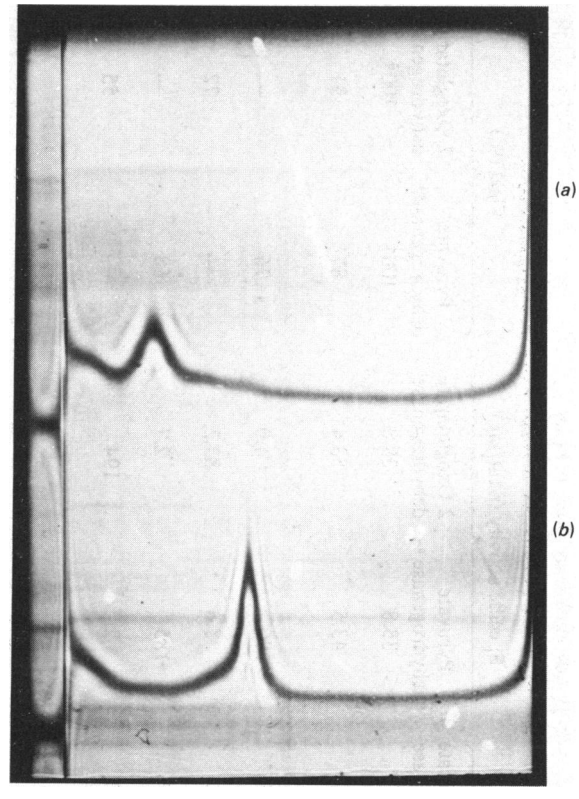

Fig. 3. *Sedimentation-velocity ultracentrifugation at 22°C of the 2-oxo acid dehydrogenase multienzyme complexes from ox heart*

(*a*) 2-Oxoglutarate dehydrogenase complex (initial concentration 2.2mg/ml); (*b*) pyruvate dehydrogenase complex (initial concentration 3.1mg/ml). The buffer was 50mM-Mops containing 2.7mM-EDTA, 0.1mM-dithiothreitol, 0.01% Triton X-100 and 0.1M-NaCl, adjusted to pH6.8 at 20°C with 6M-NaOH. For further experimental details see the text. The photograph was taken 24min after the rotor had attained its speed of 24630rev./min and sedimentation is from left to right.

Discussion

The method that we have described for the parallel purification of the pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes of ox heart allows the preparation of material that closely resembles the enzymes obtained by previous workers using different methods. Our attempt to avoid the

laborious preparation and stock-piling of mitochondria has been rewarded with a greatly enhanced yield of the 2-oxo acid dehydrogenase complexes. For example, the yield of pyruvate dehydrogenase-complex activity, of approx. 73μkat/kg of heart, represents more than 200-fold improvement over the protamine method of Linn *et al.* (1972) and 80-fold improvement over the poly(ethylene glycol) method of the same authors.

Much of the improvement is due to the inclusion of the non-ionic detergent Triton X-100 in the buffer used for the initial disruption of the heart tissue. Tween-80 was an effective substitute for Triton X-100 in this procedure. All the assayable activities of the pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes were found to be soluble under these conditions, compared with the modest proportion (much less than one-third) of the pyruvate dehydrogenase complex that could be purified by our method in the absence of Triton X-100. About the same proportion of the activity of the pyruvate dehydrogenase complex was released by freezing and thawing of isolated mitochondria, even after extensive incubation with phospholipase A or sonication (Kenney *et al.*, 1972). Although the location of the pyruvate dehydrogenase complex within the mitochondrial inner membrane appears beyond question, there have earlier been doubts that this complex is free in the mitochondrial matrix space (Kenney *et al.*, 1972; Denton *et al.*, 1975). The large increase in yield that accompanies the addition of non-ionic detergents to the extraction buffer is consistent with the idea that much if not all of the pyruvate dehydrogenase-complex activity is associated with the inner membrane, either intimately and directly or by means of an intermediary as yet unidentified. Further experiments will be needed to resolve this point.

Poly(ethylene glycol) is a mild precipitating agent for proteins, viruses etc. that can be used in low salt concentrations at pH values close to neutrality. Under these conditions, inadvertent loss of protein subunits from multienzyme complexes is likely to be minimized. Poly(ethylene glycol) has already found favour elsewhere for purifying the pyruvate dehydrogenase complex of ox heart and for separating mixtures of the pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes (Linn *et al.*, 1972; Roche & Cate, 1977). We experienced difficulty in separating the complexes by means of selective precipitation with poly(ethylene glycol) until the procedure that we describe was carried out at room temperature. The period of 3h at room temperature before adjustment of pH and addition of poly(ethylene glycol) was essential if clean separation were to be achieved. The alternative procedure of isoelectric precipitation has the advantage of being carried out at 4°C, but requires strict control of pH. No differences have been observed between the complexes purified by either procedure. Selective precipitation with poly(ethylene glycol) has proved to be simpler and more reliable.

The method that we have described for purifying the 2-oxo acid dehydrogenase complexes of ox heart is also rapid. However, the possibility of proteolysis in the early stages of the preparation prompted us to include the proteinase inhibitors phenylmethanesulphonyl fluoride and benzamidine in the extraction buffer [see, e.g., Lumsden & Coggins (1977) and Hutson *et al.* (1978)]. They may be superfluous, owing to the presence of serum proteinase inhibitors that are likely to occur in the blood in heart muscle. Indeed, rabbit serum has been used to protect pig liver pyruvate dehydrogenase complex during the early stages of a mitochondrial preparation (Roche & Cate, 1977). Phenylmethanesulphonyl fluoride is an inhibitor of the phosphatase activity in the pyruvate dehydrogenase complex of pig heart (Hutson *et al.*, 1978). However, its presence in the buffer that we used to extract the complexes from ox heart did not totally inhibit the phosphatase activity, although it did slow down the activation of the pyruvate dehydrogenase complex in the presence of Mg^{2+}. Our experiments indicate that phenylmethanesulphonyl fluoride can be omitted from the extraction buffer, but its inclusion may be a worthwhile precaution. Similarly, EDTA appeared to exercise a protective effect on both enzyme complexes during the initial extraction, and we therefore included it throughout the preparation.

Further tests will be required to compare the products of this new method of purification with the 2-oxo acid dehydrogenase complexes obtained by simple extraction of isolated mitochondria. There is every reason to hope that the method can be applied to tissues other than ox heart.

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