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Pyruvate Dehydrogenase Complex from Higher Plant Mitochondria and Proplastids[1]

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ABSTRACT

The pyruvate dehydrogenase complex from pea (*Pisum sativum* L.) mitochondria was purified 23-fold by high speed centrifugation and glycerol gradient fractionation. The complex had a $s_{20,w}$ of 47.5S but this is a minimal value since the complex is unstable. The complex is specific for NAD^+ and pyruvate; $NADP^+$ and other keto acids give no reaction. Mg^{2+}, thiamine pyrophosphate, and cysteine are also required for maximal activity. The pH optimum for the complex was between 6.5 and 7.5.

Continuous sucrose density gradients were used to separate castor bean (*Ricinus communis* L.) endosperm proplastids from mitochondria. Pyruvate dehydrogenase complex activity was found to be coincident with the proplastid peak on all of the gradients. Some separation of proplastids and mitochondria could be achieved by differential centrifugation and the ratios of the activities of the pyruvate dehydrogenase complex to succinic dehydrogenase and acetyl-CoA carboxylase to succinic dehydrogenase were consistent with both the pyruvate dehydrogenase complex and acetyl-CoA carboxylase being present in the proplastid. The proplastid fraction has to be treated with a detergent, Triton X-100, before maximal activity of the pyruvate dehydrogenase complex activity is expressed, indicating that it is bound in the organelle. The complex had a sharp pH optimum of 7.5. The complex required added Mg^{2+}, cysteine, and thiamine pyrophosphate for maximal activity but thiamine pyrophosphate was inhibitory at higher concentrations.

The pyruvate dehydrogenase complex catalyzes the oxidative decarboxylation of pyruvate with the formation of acetyl-CoA and the reduction of NAD^+. In addition to the three substrates (pyruvate, CoA, and NAD^+) Mg^{2+} ions, TPP,[3] lipoic acid, and FAD are involved in the reaction sequence. The complex consists of three enzyme components. The first enzyme is usually referred to as pyruvate dehydrogenase (pyruvate lipoate oxidoreductase, EC 1.2.4.1.) and catalyzes reactions 1 and 2 below. The second enzyme is lipoate transacetylase (acetyl-CoA dihydrolipoate S-acetyl transferse, EC 2.3.1.12) and catalyzes reaction 3. The third enzyme is dihydrolipoamide dehydrogenase (NADH lipoamide oxidoreductase EC 1.6.4.3) and catalyzes reactions 4 and 5.

The sequence of reactions catalyzed by these enzymes is:

$$\text{Pyruvate} + \text{TPP} - E_1 \rightarrow CO_2 + \text{hydroxyethyl} - \text{TPP} - E_1 \quad (1)$$

$$\text{Hydroxyethyl} - \text{TTP} - E_1 + \text{lipoyl} - E_2 \rightarrow \text{S-Acetyldihydrolipoyl} - E_2 + \text{TPP} - E_2 \quad (2)$$

$$\text{S-Acetyldihydrolipoyl} - E_2 + \text{CoA} \rightarrow \text{Acetyl-CoA} + \text{dihydrolipoyl} - E_2 \quad (3)$$

$$\text{Dihydrolipoyl} - E_2 + \text{FAD} - E_3 \rightarrow \text{Lipoyl} - E_2 + \text{reduced FAD} - E_3 \quad (4)$$

$$\text{Reduced FAD} - E_3 + NAD^+ \rightarrow NADH + H^+ + \text{FAD} - E_3 \quad (5)$$

Sum:

$$\text{Pyruvate} + NAD^+ + \text{CoA} \rightarrow \text{Acetyl-CoA} + CO_2 + NADH + H^+ \quad (6)$$

The *Escherichia coli* complex consists of 24 molecules of pyruvate dehydrogenase, 24 molecules of lipoate transacetylase, and 12 molecules of dihydrolipoamide dehydrogenase (6) and has a total mol wt of 4.8 million (6, 10). The complex isolated from *Azotobacter vinelandii* has a mol wt of 1.1 million (2). The bovine kidney and heart complexes are larger than the *E. coli* complex and consist of 60 pyruvate dehydrogenase molecules, made up of α and β subunits, 60 molecules of lipoate transacetylase, and 10 to 12 dihydrolipoamide molecules (1). A $s_{20,w}$ of approximately 85S has been determined for the complex from *Neurospora* (7) which indicates that this complex is similar to the mammalian complex. Very little data are available for the complex from higher plants. There is one report of the isolation of the complex from potato tubers (3) but the enzyme was not purified or characterized to any extent. Since the reaction catalyzed by this complex is at a very important branch point in metabolism, further characterization of the enzyme from plants would seem to be essential.

Acetyl-CoA generated from pyruvate is required for long chain fatty acid biosynthesis which is associated with the proplastid fraction from developing castor bean endosperm (5, 15, 24-26). The pyruvate dehydrogenase complex is found both in the mitochondrial and proplastid fractions in this tissue (17) so that proplastids can generate the acetyl-CoA from pyruvate. A 10,000*g* pellet incorporates radioactivity from sucrose into fatty acids which indicates tht the entire pathway from sucrose to fatty acids is particulate (24). Subsequently, it has been shown that phosphofructokinase and pyruvate kinase are present in purified proplastids from this fraction suggesting that this pathway is present in this organelle (4).

The pyruvate dehydrogenase complex from mitochondria may be the same complex as that found in proplastids or different complexes may be present in the two organelles. In order to distinguish between these two possibilities, the properties of the complexes from these two sources must be clearly defined. The kinetics and regulation of the proplastid and mitochondrial complexes are described in the accompanying reports (21, 22).

MATERIALS AND METHODS

Preparation of the Mitochondrial Pyruvate Dehydrogenase Complex. Pea (*Pisum sativum* L.) seeds were soaked in water overnight and grown in vermiculite at room temperature in the

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[3] Abbreviations: TPP: thiamine pyrophosphate; PMS: phenazine methosulfate; DCIP: 2,6-dichlorophenolindophenol.

dark until the shoots were 20 to 30 cm in height. The shoots (300 g) were harvested and ground, using a mortar and pestle, in 200 ml of 50 mM tris-HCl buffer (pH 7.8) containing 0.7 M sucrose, 2 mM EDTA, 57 mM 2-mercaptoethanol, and 0.5% (w/v) BSA (fraction V, Sigma). The homogenate was filtered through four layers of cheesecloth and centrifuged at 2,000*g* for 10 min. The supernatant was centrifuged at 27,000*g* for 45 min, the resulting pellet was resuspended in 180 ml of grinding medium and centrifuged again at 27,000*g* for 45 min. The surface of the pellet was washed with ice water, then resuspended in acetone at −20 C, centrifuged at 12,000*g* for 10 min, and resuspended and centrifuged an additional three times in cold acetone. The final pellet was dried with a stream of air and stored dessicated at −20 C. The activity of the complex in the acetone powder was stable under these conditions for at least 6 months.

The acetone powder was resuspended at 0 C in 25 mM TES buffer (pH 7.3) containing 1 mM TPP, 5 mM dithioerythritol, and 2 mM $MgSO_4$ at a concentration of 20 mg/ml using a Teflon homogenizer. The resuspended powder was centrifuged at 27,000*g* for 15 min and the supernatant used as the starting material for the purification. No pyruvate dehydrogenase complex activity was found in the pellet which contained 90% of the protein.

Nucleic acids were precipitated from the supernatant using 0.01 volume of a 0.2% (w/v) solution of protamine sulfate. The mixture was stirred at 0 C for 10 min and then centrifuged at 29,000*g* for 15 min. The pyruvate dehydrogenase complex was removed from the supernatant by centrifugation at 26,000 rpm (maximum g = 136,000) in a SW 27 rotor for 9 hr using a Beckman L-65 ultracentrifuge. The pellets were covered with a minimum volume of the resuspending buffer and allowed to stand 9 hr at 4 C before resuspending.

Linear glycerol gradients were prepared using a Buchler gradient former and a Buchler polystatic pump. The glycerol solutions were made up in 25 mM TES buffer (pH 7.3) on a w/w basis. In a typical gradient, 1.9 ml of resuspended pellet containing approximately 10 mg of protein was layered onto 34 ml of a 10 to 50% linear gradient. This was centrifuged for 6 hr at 26,000 rpm in an SW 27 rotor. The gradients were collected using an ISCO fractionator by inserting 80% glycerol containing methyl green into the base of the centrifuge tube and collecting approximately 1-ml fractions from the top. The concentration of glycerol in the gradient was measured using an Abbé refractometer.

Isolation of Proplastids. Proplastids were isolated from developing castor bean (*Ricinus communis* L.) endosperm by the procedures described earlier (17) except 1 mM $MgCl_2$ was included in the grinding medium. Both linear and discontinuous gradients were used, although for routine work discontinuous gradients were preferred. All of the sucrose solutions were buffered with 0.01 M TES (pH 7.5). The continuous gradients were 30 ml of 35 to 55% sucrose cushioned on 4 ml of 60% sucrose. After layering on a 1-ml aliquot of the resuspended 500 to 10,000*g* particles, the gradients were centrifuged for 3 hr at 104,000*g* using an SW 27 rotor in a Spinco L-2, type D ultracentrifuge. The discontinuous gradients contained 1 mM $MgCl_2$ and were composed of 10 ml of 35%, 14 ml of 45%, and 10 ml of 60% sucrose. Two ml of sample were layered on and the discontinuous gradients were centrifuged as above with the time reduced to 10 min. All gradients were collected dropwise from the bottom, the continuous gradient in 1.2-ml fractions and the discontinuous in 1.1-ml fractions. The fractions were numbered in the order they were collected. In some cases, in order to obtain sufficient material, the resuspended pellet was divided into two parts and each was centrifuged on separate identical gradients in the same rotor. Usually the protein profiles were identical but occasionally they differed by one fraction.

Assay for the Pyruvate Dehydrogenase Complexes. All spectrophotometric assays were performed in a Gilford modified Beckman DU spectrophotometer, using 1-cm path length cells, at 25 C. Activity is expressed as change in A/min or as μmol NADH formed/min·mg protein.

Before the pyruvate dehydrogenase complex from proplastids could be assayed, it had to be released from the proplastids. This was achieved by incubating the proplastids with a medium consisting of 20 mM $MgCl_2$, 50 mM tris-HCl buffer (pH 7.5), and 0.1% Triton X-100 for 30 min at 4 C. A high concentration of $MgCl_2$ was used since it activates the complex (22). The preparation was then centrifuged at 27,000*g* for 20 min to remove particulate material. The assay for both complexes was based on that described by Scharwtz *et al.* (18). The reaction mixture for the proplastid complex contained 0.5 mM TPP, 2.6 mM NAD^+, 2.4 mM cysteine·HCl, 0.09 mM CoA, 20 mM $MgCl_2$, 3 mM pyruvate, and 50 mM tris-HCl (pH 7.5) in a total volume of 3 ml. The reaction mixture for the pyruvate dehydrogenase complex from mitochondria was similar to that for the proplastid complex and contained 0.5 mM TPP, 1 mM $MgCl_2$, 1 mM NAD^+, 0.13 mM CoA, 1 mM cysteine·HCl, 1 mM sodium pyruvate, and 25 mM TES (pH 7.3) in a total volume of 3 ml. The reduction of NAD^+ was determined by measuring the change in A at 340 nm. All assay constituents, excluding pyruvate but including enzyme, were incubated for 1 min at 25 C when the reaction was initiated by the addition of pyruvate. In all cases, initial velocities were proportional to protein concentration over the range used and were linear with time.

Other Assays. The assay mixture for dihydrolipoamide dehydrogenase component of the complex contained 1 mM dihydrolipoamide, 1 mM NAD^+, 1 mM cysteine·HCl, and 25 mM TES (pH 7.3) in a volume of 3 ml. The reduction of NAD^+ was determined by measuring the change in A at 340 nm. The assay mixture plus enzyme was incubated for 30 sec in the absence of either NAD^+ or dihydrolipoamide and the reaction initiated by addition of the missing constituent.

Lipoate transacetylase was assayed by measuring the transfer of a [^{14}C]acetyl group from [1-^{14}C]acetyl-CoA to dihydrolipoamide. The reaction was performed in a 1.5-ml sealed plastic centrifuge tube, and the reaction mixture, in a final volume of 0.5 ml, contained 1.2 mM dihydrolipoamide, 1 mM acetyl-CoA containing 0.0488 μCi of ^{14}C and 25 mM TES (pH 7.3). The reaction was initiated by the addition of dihydrolipoamide. After 15 sec, the reaction was terminated by the addition of 1 ml of benzene and the tube was vigorously shaken to extract the acetylated dihydrolipoamide. The two phases were separated by centrifugation for 15 sec on a Beckman microfuge and 500 μl of the upper benzene layer was transferred to a scintillation vial to which 10 ml of scintillation fluid consisting of 4 g of PPO and 0.1 g POPOP/l of toluene was added. The samples were counted in a Nuclear-Chicago 720 scintillation counter. A blank consisting of an assay minus the enzyme was performed and all samples were corrected for the small amount of unreacted [1-^{14}C]acetyl-CoA extracted into the benzene phase. There was no reaction in the absence of dihydrolipoamide.

Succinate dehydrogenase was assayed by a modified method of Hiatt (8). The assay mixture contained 50 mM phosphate buffer (pH 7.4), 3.7 mM succinate, 0.3 mM DCIP, 3.3 mM KCN, and 0.3 mg/ml PMS in a total volume of 3 ml. The assay mixture, minus PMS, was incubated for 15 min at 25 C to allow for substrate activation of the enzyme and the reaction was then initiated by the addition of PMS. The reaction was followed by measuring the reduction of PMS at 600 nm.

Triose-P isomerase was used as a marker for proplastids (26). The assay mixture contained 200 mM tris-HCl (pH 7.6), 0.093 mM NADH, 5 μg α-glycerol-P dehydrogenase, and 0.3 mM D,L-glyceraldehyde-3-P in a total volume of 3 ml. The reaction rate was measured by following the oxidation of NADH at 340 nm.

Acetyl-CoA carboxylase has also been used as a marker for proplastids. The assay for this enzyme was identical to that described by Scott-Burden and Canvin (20).

Catalase, a microbody marker (14), was assayed by following O_2 production using a Yellow Springs instrument model 5331 O_2 electrode. The reaction mixture contained 170 mM K-phosphate (pH 7) and 1.2 mM H_2O_2 in a total volume of 2.5 ml. Oxygen was removed from the assay mixture by bubbling N_2 through it.

In all cases, the reaction rates were constant for time periods in excess of the assay time, and all assays were linear with protein concentration over the range used in the assay.

Chemicals. All biochemicals were purchased from the Sigma Chemical Co. The RNase-free sucrose was obtained from Schwarz/Mann and radiochemicals from New England Nuclear. All other chemicals were of analytical grade. Dihdrolipoamide was synthesized from lipoamide by the borohydride reduction method of Reed *et al.* (16).

Protein Determination. Protein was determined by the method of Lowry (11) after precipitation of the protein by 10% trichloroacetic acid. A standard curve was prepared using BSA (fraction V, Sigma).

RESULTS

Stability, Extraction, and Purification of the Mitochondrial Complex. Comparison of pyruvate dehydrogenase complex activity in mitochondria disrupted with deoxycholate or sonication, with pyruvate dehydrogenase complex activity from acetone powder, showed that the acetone treatment of the mitochondrial fraction caused a loss of about 20% of the total complex activity. Pyruvate dehydrogenase complex activity could be removed quantitatively from the acetone powder. The highest initial activity was obtained when the powder was resuspended with TES buffer.

The complex was not very stable on extraction from the acetone powder and lost 35% of the activity after 2 days and 73% after 16 days when stored as a frozen solution in 25 mM TES buffer (pH 7.5), which was found to give the greatest stability of the enzyme.

Purification of the Pyruvate Dehydrogenase Complex. The purification of the complex is shown in Table I. The over-all purification obtained was 22.6-fold from the supernatant of the resuspended acetone powder. This, however, does not take into account the fact that 90% of the protein from the acetone powder does not resuspend, so that a further 10-fold purification is obtained at this step. Also, the preparation of mitochondria from the crude homogenate represents a significant purification. The specific activity of the complex is approximately 10% of that found for the complex from other sources, probably reflecting the greater instability of the complex from plants. During ultracentrifugation, 55% of the activity before centrifugation was recovered in the pellet and 10 to 20% in the supernatant. This could not be recovered by longer periods of centrifugation, by adding 50 mM NaCl or 0.01 volume of 0.2% protamine sulfate or 1 mM NAD^+ to the buffer.

The glycerol gradients were velocity gradients and were not centrifuged to equilibrium. After centrifugation of the gradients, a yellow band was found in the gradient which corresponded to the pyruvate dehydrogenase complex activity (Fig. 1). Lipoate transacetylase activity also was coincident with this peak and was not found elsewhere on the gradient. Dihydrolipoamide dehydrogenase activity was coincident with the yellow band but a large amount was found at the upper end of the gradient. A large amount of protein remained on top of the gradient and a peak of protein was coincident with the complex activity. In some gradients, a second protein peak was found at a higher glycerol concentration. This peak had no complex activity and no dihydrolipoate transacetylase or dihydrolipoamide dehydrogenase activities. Other techniques were used in attempts to purify the complex further but the instability of the complex made these procedures unsuccessful.

Recoveries of Complex Activity and Component Enzyme Activities. The activities of the complex and component enzymes relative to those in the crude extract are given in Table II. Only 19% of the dihydrolipoamide dehydrogenase sediments with the complex and a large percentage appears in the supernatant. This

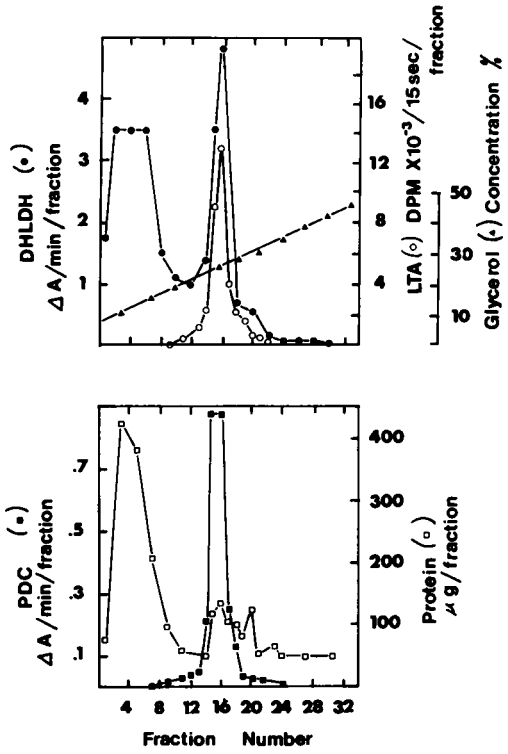

FIG. 1. Activities of the pea mitochondrial pyruvate dehydrogenase complex (PDC), lipoamide transacetylase (LTA), and dihydrolipoamide dehydrogenase (DHLDH) on a glycerol gradient.

TABLE I

Purification of the Pyruvate Dehydrogenase Complex from Pea Mitochondria

Purification Fraction	Total Activity	Total Protein	Specific Activity	Purification	Recovery
	E.C. Units	mg	E.C. Units	Fold	%
Acetone powder extract	6.92	157.3	0.044	1.0	100.0
Protamine sulfate supernatant	6.79	141.5	0.048	1.1	98.1
Pelleted enzyme	3.97	10.6	0.376	8.6	57.4
Pooled glycerol gradient fractions	2.88	2.93	0.984	22.6	41.7

E.C. units are μmole NADH formed/min/mg protein.

TABLE II

Recovery of Component Enzymes during Purification of the Pyruvate Dehydrogenase Complex from Pea Mitochondria.

Purification Fraction	PDC[a]		LTA[b]		DHLDH[c]	
	Units	%	Units	%	Units	%
Acetone powder extract	6.9	100 (0.044)[f]	13	100 (0.084)[f]	271	100 (1.730)[f]
Pelleted enzyme[d]	4.6	66 (0.454)	10	76 (1.000)	52	19 (5.220)
SW-27 supernatant[d]	0.8	12	2	17	163	60
Pooled gradient fractions[e]	4.9	71	5	38	95	35

[a] Pyruvate dehydrogenase complex.
[b] Lipoate transacetylase.
[c] Dihydrolipoate dehydrogenase.
[d] Expressed as a percentage of the activity present in the acetone powder extract.
[e] Expressed as a percentage of the activity applied to the gradient.
[f] Specific activity of the complex and component enzymes (E.C. units).

TABLE III

The Effect of Magnesium Ions on the Percentage of Total Protein and Various Enzyme Activities Found in the Supernatant and 10,000 x g Pellet Fraction of Castor Bean Endosperm.

Activity	SUPERNATANT		500–10,000 x g Pellet	
	+Mg^{2+}	Control	+Mg^{2+}	Control
	% Total	% Total	% Total	% Total
Total protein	42 (10.76)[a]	47 (9.3)	58 (15.0)	53 (10.6)
Succinic dehydrogenase	10 (.0538)[b]	27 (.168)	90 (.479)	73 (.444)
Triose phosphate isomerase	83 (18.3)[b]	93 (37.1)	17 (3.71)	7 (5.9)
Acetyl-CoA carboxylase	2 (.606)[c]	4 (.738)	98 (24.16)	96 (15.98)
Pyruvate dehydrogenase complex	38 (.444)[b]	46 (.289)	62 (.719)	54 (.338)

[a] Total protein in mg.
[b] Total activity in μmoles/min.
[c] Total activity in nmoles/min.

activity may be due to dissociation of this enzyme from the complex during sedimentation but it has also been reported that much of this enzyme exists in the free form (12). The lipoamide transacetylase has about twice the specific activity of the entire complex before and after the centrifugation whereas the dihydrolipoamide dehydrogenase has a specific activity 39-fold higher in the crude and 11.5-fold higher in the pelleted enzyme, indicating that this is a great excess (Table II).

An attempt was made to assay the first enzyme of the complex using ferricyanide as the electron acceptor but the rates were too small to measure. An attempt was made to assay the pyruvate dehydrogenase component using [1-^{14}C]pyruvate and measuring the release of $^{14}CO_2$. This reaction would not proceed, however, in the absence of CoA and NAD^+ indicating the hydroxyethyl-TPP was not released from the enzyme. The rate was, therefore, dependent on the activity of the whole complex and was not just a measure of the activity of the first enzyme.

Determination of an $s_{20,w}$ Value for the Complex. Sucrose density gradients were used to determine the $s_{20,w}$ values using the method of McEwen (13). However, only 1.3% of the added activity was recovered, indicating that the complex was unstable in the presence of sucrose. The activity which remained was found in a yellow band which could be seen midway down the tube. From this it was calculated that the complex had an $s_{20,w}$ of 47.5 but this value is highly tentative considering the instability of the complex in sucrose.

Identification of the Pyruvate Dehydrogenase Complex in Proplastids from Castor Beans. It was found that inclusion of 1 mM $MgCl_2$ in the grinding medium increased the percentage of total protein, triose-P isomerase, succinate dehydrogenase, acetyl-CoA carboxylase, and the pyruvate dehydrogenase complex in the pellet which was formed by centrifugation at 10,000*g* for 20 min (Table III). This may indicate that the membranes are more intact in the presence of Mg^{2+} ions. Table III also shows, in agreement with Scott-Burden and Canvin (20) that acetyl-CoA carboxylase is a better marker for proplastids than triose phosphate isomerase since only 2% of this activity was found in the soluble phase. In all subsequent work, Mg^{2+} ions were included in the grinding medium to maximize the yield of proplastid components.

The optimum centrifugal force for sedimentation of proplastids was determined by measuring the ratio of acetyl-CoA carboxylase which was found only in the proplastids to succinate dehydrogenase, since this ratio indicates the contamination of proplastids with mitochondria. As is shown in Table IV, the ratio of succinate dehydrogenase to acetyl-CoA carboxylase was lowest in the 500 to 2,000*g* pellet and increased as the centrifugal force was raised. This indicates that proplastids are denser than mitochondria as shown by Scott-Burden and Canvin (20) and can be sedimented at a lower centrifugal force. As judged by the acetyl-CoA carboxylase, only 60% of the total proplastids sedimented at 2,000*g*, so that a 500 to 5,000*g* pellet was normally used since this contained 92% of the proplastids. The contamination of mitochondria increased from 31 to 68% at this higher force.

The ratio of succinate dehydrogenase to pyruvate dehydrogenase complex increased in the fractions isolated at increasing centrifugal forces (Table IV). Both of these enzymes are tightly

TABLE IV

A Comparison of the Ratios of Specific Activities of Succinate Dehydrogenase, Acetyl-CoA Carboxylase and Pyruvate Dehydrogenase Complex after Three Differential Centrifugations of a Homogenate from Castor Bean Endosperm.

Fraction	SDH[a] / Carboxylase[b]	PDC[c] / Carboxylase[b]	SDH[a] / PDC[c]
	x 10^6		x 10^2
500 - 2,000 x g	2.4	93	2.6
2,000 - 5,000 x g	5.2	125	4.2
5,000 -10,000 x g	19.3	135	14.3

[a]Succinate dehydrogenase activity in Δ absorbance/min/mg protein.

[b]Acetyl-CoA carboxylase activity in DPM/mg protein.

[c]Pyruvate dehydrogenase complex activity in Δ absorbance/min/mg protein.

bound in the mitochondria so that if they were confined to the mitochondrion, the ratio should remain constant. The fact that the ratio increases indicates that the pyruvate dehydrogenase complex is also present in the proplastid. The ratio of the pyruvate dehydrogenase complex to the acetyl-CoA carboxylase increased but not to the same extent as the succinate dehydrogenase to acetyl-CoA carboxylase ratio (Table IV), again indicating that the pyruvate dehydrogenase complex is not confined to the mitochondrion.

Isolation of Proplastids on Sucrose Density Gradients. Separation of the components in a 500 to 10,000*g* pellet on a continuous sucrose density gradient which did not contain Mg^{2+} ions is shown in Figure 2. The gradient was centrifuged for 3 hr to ensure that the particles reached equilibrium. Three major protein peaks were found. The protein peak on top of the gradient contained triose-P isomerase which may be from broken proplastids but there might also have been some soluble enzyme from the cytosol. There was succinate dehydrogenase in this peak which probably represented broken mitochondria. A peak with a density of 1.18 g/ml contained most of the succinate dehydrogenase activity that moved into the gradient and was the location of the mitochondria. The protein peak which sedimented with a density of 1.21 g/ml contained the triose-P isomerase activity which moved into the gradient. A density of 1.21 g/ml has been reported for proplastids (14, 20, 25). The separation of proplastids and mitochondria was not complete since the triose-P isomerase and succinate dehydrogenase peaks overlap. Pyruvate dehydrogenase complex activity was found in both the mitochondrial and proplastid peak. It is unlikely that this amount of complex activity could be a result of mitochondrial contamination of the proplastids.

It has been reported that 1 mM $MgCl_2$ increases the bouyant density of proplastids and this has been used to separate proplastids from mitochondria effectively (14, 20). Inclusion of 1 mM $MgCl_2$ in the continuous sucrose density gradient caused the proplastids to move much further into the gradient after 3 hr of centrifugation (Fig. 3). A very distinct separation of the proplastids and mitochondria occurred, as determined by the triose-P isomerase and succinate dehydrogenase peaks. There was a complete separation of the pyruvate dehydrogenase complex activity in the proplastid peak and since this contained no succinate dehydrogenase activity, it is clear that this complex must be present within the proplastid. A third peak of pyruvate dehydrogenase complex activity was found which had moved a little way into the gradient. This was probably soluble complex activity from broken organelles. The pyruvate dehydrogenase complex is large enough to move a short distance into the gradient after 3 hr of centrifugation. There was soluble triose-P isomerase and succinate dehydrogenase on top of the gradient indicating that broken organelles were present. The characterization of the

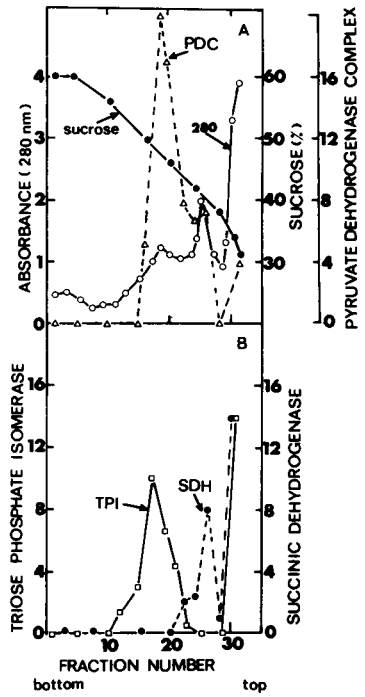

FIG. 2. Distribution of protein (*A* at 280 nm), pyruvate dehydrogenase complex (PDC), triose-P isomerase (TPI), and succinic dehydrogenase (SDH) activities on a continuous sucrose density gradient in the absence of $MgCl_2$. Pyruvate dehydrogenase complex activity is μmol NAD^+ reduced/hr/fraction × 10, triose-P isomerase activity is μmol NADH oxidized/hr/fraction, and succinic dehydrogenase is μmol DCIP reduced/hr/fraction. A 500 to 10,000*g* fraction was placed on the gradient.

pyruvate dehydrogenase complex from proplastids was performed on organelles separated by a discontinuous sucrose velocity gradient technique, the details of which have already been published (17), since larger amounts of material can be obtained with this technique.

Release of the Pyruvate Dehydrogenase Complex from Proplastids. It was possible to detect only a small amount of pyruvate dehydrogenase complex activity in freshly prepared proplastids. The activity could be released by treatment of the proplastids with Triton X-100 (Fig. 4). At 25 C in 0.1% Triton X-100, full activity was not expressed for 60 min when no further increase in activity was found. At 25 C, the activity was stable for 2.5 hr when it began to decline. At 0 C, the activity was stable for at least 4 hr and the complex could be kept for long periods at minus 10 C. This behavior of the complex indi-

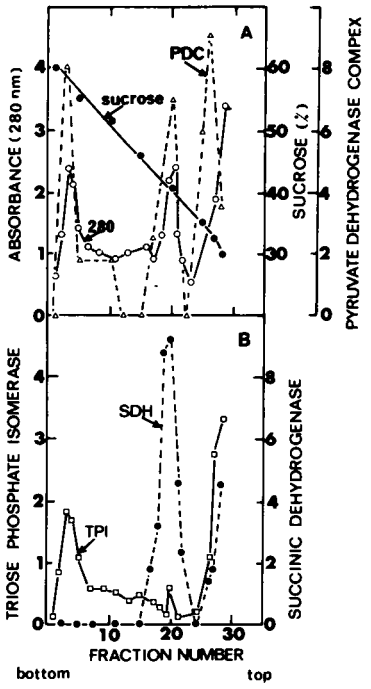

FIG. 3. Distribution of protein (*A* at 280 nm), pyruvate dehydrogenase complex (PDC), triose-P isomerase (TPI), and succinic dehydrogenase (SDH) on a continuous sucrose density gradient in the present of 1 mM $MgCl_2$. Pyruvate dehydrogenase complex activity is μmol NAD^+ reduced/hr/fraction × 10, triose-P isomerase activity is NADH oxidized/hr/fraction × 10^{-1}, and succinic dehydrogenase activity is μmol DCIP reduced/hr/fraction. A 500 to 10,000*g* fraction was placed on the gradient.

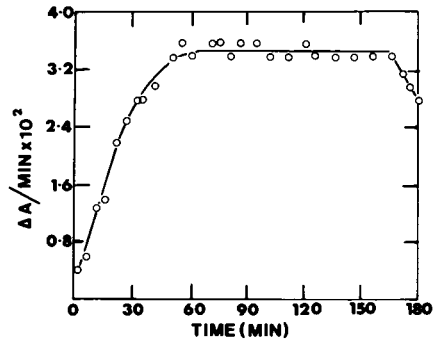

FIG. 4. Release of pyruvate dehydrogenase complex from castor bean endosperm proplastids at 25 C in the presence of 50 mM tris (pH 7.5) and 0.1% Triton X-100.

cates that it is within the proplastid and is not just absorbed on the surface.

Effect of pH on Mitochondrial and Proplastid Complex Activity. The effect of pH on the activity of the complex from mitochondria and proplastids is shown in Figure 5. The pH optimum for the mitochondrial complex is from 6.5 to 7.5 with the activity falling off sharply at higher and lower values. The complex from proplastids has a very sharp pH optimum and the maximum activity is between pH 7.5 and 8. There is a difference in the pH optimum of the complex from the two sources, indicating that they may be different. The pH optimum of the mammalian complex is between pH 8 and 9 (9). However, that of the brewers' yeast complex (23) and the *Neurospora* complex (7) is similar to the plant mitochondrial complex. Since the complex is composed of three component enzymes, pH profiles are difficult to interpret.

Requirement for Components of the Assay Mixture and Substrate Specificity. Both complexes had an absolute requirement for NAD^+, pyruvate, and CoA. Neither complex, as isolated, required Mg^{2+} for activity but inclusion of Mg^{2+} in the assay increased the rate by 25 to 50%. This suggests that even after the high speed centrifugation, Mg^{2+} was still bound to the mitochondrial complex. Addition of EDTA to the assay for this complex immediately inhibits the reaction completely and subsequent addition of Mg^{2+} restores the activity showing an absolute requirement for Mg^{2+} in the reaction.

There is an absolute requirement for TPP in the case of the partially purified mitochondrial complex, and TPP also increases the activity of the proplastid complex, indicating that TPP can dissociate. At higher concentrations, TPP inhibits the proplastid complex (Fig. 6). Cysteine is also required for optimal rates and this can be replaced by dithioerythritol. A reducing agent may be required to maintain the CoA in the reduced state. The first enzyme of the *E. coli* complex also has essential −SH groups involved in TPP binding (19). Both are absolutely specific for NAD^+ as the electron acceptor, there being no activity with $NADP^+$. This is the same as the complex from other sources which are also specific for NAD^+ (23). The mitochondrial complex is also absolutely specific for pyruvate as the keto acid, and shows no activity with a range of keto acids including α-ketoglutarate, oxaloacetate, and glyoxylate.

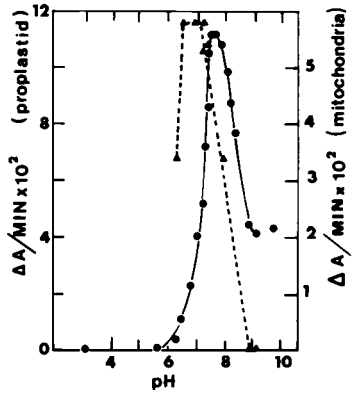

FIG. 5. Effect of pH on the activity of the pea mitochondrial (▲- - -▲) and castor bean endosperm proplastid (●——●) pyruvate dehydrogenase complexes. The mitochondrial complex was purified to the high speed pellet stage. Velocities were measured using the standard assay mixture adjusted to the indicated pH.

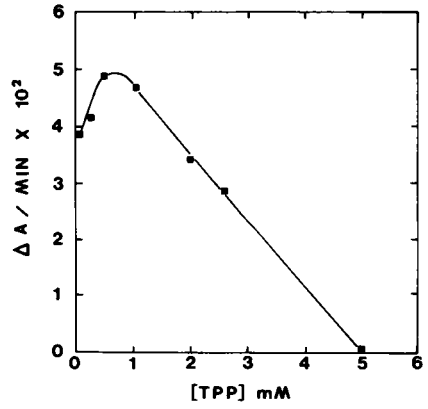

FIG. 6. Effect of thiamine pyrophosphate (TPP) on the activity of the pyruvate dehydrogenase complex from castor bean endosperm proplastids.

DISCUSSION

The pyruvate dehydrogenase complex from pea mitochondria has been partially purified and characterized. The total activity and specific activity are much higher than those reported earlier for the potato enzyme (3) where extremely low activities were measured. The complex is very unstable and dissociates during purification. This is especially true of the third enzyme of the complex, dihydrolipoamide dehydrogenase. The measured size of the complex is smaller than that described for the mammalian, bacterial, and *Neurospora* complexes (1, 2, 6, 7, 10), but this is probably due to partial dissociation of the complex on the gradients.

The specific activities of the lipoamide transacetylase and dihydrolipoate dehydrogenase suggest that the rate-limiting step in the reaction sequence is the pyruvate dehydrogenase component. No attempt has been made to dissociate the complex into its component enzymes and to reform the functional complex. The instability of the complex compared with the complex from other sources would make this more difficult to achieve.

The demonstration that pyruvate dehydrogenase complex activity can be detected in large amounts in proplastids, purified by continuous and discontinuous sucrose density gradients, indicates that this complex is an integral part of the proplastid. The absence of succinic dehydrogenase activity shows that the complex activity is not resulting from contamination by mitochondria. The amount of complex activity in the proplastid fraction as compared with the mitochondrial fraction varied and in some cases (*e.g.* Fig. 2), there appeared to be more in the proplastids than in the mitochondria. The level of complex activity in the proplastid peak may be very dependent on the stage of development of the proplastid. The concentration of the component enzymes during development is being studied further. The proplastids also appear to be very fragile so that small variations in their preparation may determine the ratio of intact proplastids to mitochondria resulting in apparently different amounts of pyruvate dehydrogenase complex present in the proplastids.

The fact that pyruvate dehydrogenase complex activity cannot be detected in proplastids until after treatment with Triton X-100 indicates that the activity is firmly bound in the organelle and is not just absorbed onto the surface. The site of synthesis of the complex is not known.

Preliminary characterization of the two complexes indicates that they may be different. The pH curve of the proplastid complex is remarkably sharp compared with the pea mitochondrial complex and the complex from other sources. The reason for this type of curve has not been determined.

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