α-KETO ACID DEHYDROGENASE COMPLEXES, V. MACROMOLECULAR ORGANIZATION OF PYRUVATE AND α-KETOGLUTARATE DEHYDROGENASE COMPLEXES ISOLATED FROM BEEF KIDNEY MITOCHONDRIA*

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Communicated by Roger J. Williams, June 17, 1966

Enzyme systems that catalyze a coenzyme A- and diphosphopyridine nucleotide-linked oxidative decarboxylation of pyruvate and α-ketoglutarate (reaction 1)

$$RCOCO_2H + CoA\text{-}SH + DPN^+ \rightarrow RCO\text{-}S\text{-}CoA + CO_2 + DPNH + H^+ \quad (1)$$

have been isolated as multienzyme complexes with molecular weights of several million from pigeon breast muscle,[1, 2] pig heart muscle,[3, 4] and *Escherichia coli*.[5] Two classes of complexes have been obtained, one specific for pyruvate, the other for α-ketoglutarate. The *E. coli* pyruvate dehydrogenase complex (PDC) has been separated into three enzymes—pyruvate decarboxylase, dihydrolipoyl transacetylase, and a flavoprotein, dihydrolipoyl dehydrogenase. The complex has been reconstituted from the isolated enzymes.[6] The *E. coli* α-ketoglutarate dehydrogenase complex (KGDC) also has been separated into three enzymes, analogous to those obtained from PDC, and it too has been reassembled from the isolated enzymes.[7] The three enzymes are α-ketoglutarate decarboxylase, dihydrolipoyl transsuccinylase, and a flavoprotein, dihydrolipoyl dehydrogenase. The individual enzymes are linked in the two complexes by noncovalent bonds.

Biochemical and electron microscopic studies on the *E. coli* PDC and its component enzymes indicate that the dihydrolipoyl transacetylase is a self-assembling system composed of identical subunits that are situated at the eight vertices of a cube.[8, 9] The structure of the transacetylase apparently determines the structure of the PDC. The molecules of pyruvate decarboxylase and of flavoprotein appear to be aligned, respectively, on the 12 edges and in the six faces of the cube. It appears that the macromolecular organization of the *E. coli* KGDC is similar to that of the *E. coli* PDC.[10]

In view of the insight gained into the macromolecular organization of the bacterial PDC and KGDC, a profitable comparison with the corresponding mammalian complexes can now be made. This paper describes the purification of pyruvate and α-ketoglutarate dehydrogenase complexes from beef kidney mitochondria and the results of electron microscopic studies on the macromolecular organization of these two mammalian complexes. The mammalian complexes exhibit enzymatic activities similar to those observed with the bacterial complexes. The basic similarity in mechanism of α-keto acid oxidation observed with the bacterial and mammalian complexes led us to anticipate similarities in macromolecular organization of these complexes. This indeed appears to be the case. The macromolecular organization of the mammalian KGDC closely resembles that of the two bacterial complexes. The macromolecular organization of the mammalian PDC differs from that of the other three complexes in that the subunits of the transacetylase component of the

former complex appear to be situated at the twenty vertices of a pentagonal dodecahedron. The molecules of pyruvate decarboxylase and of flavoprotein are probably aligned on the edges and in the faces of the dodecahedron in the intact mammalian PDC.

Methods.—Enzyme assays: All enzyme assays were carried out essentially as described in previous publications.[7, 11] Units are expressed as micromoles of substrate utilized or product formed per hour and specific activities as units per mg of protein. The protein content of suspensions was determined by the biuret method,[12] that of soluble preparations by the method of Lowry *et al.*[13] or by absorption at 280 mμ.

Preparation of beef kidney mitochondria: Beef kidneys were collected within 10–15 min after slaughter and were chilled immediately. All subsequent operations were carried out at 0–5°. About 800 gm of washed, minced cortical tissue was suspended in sufficient 0.5 *M* sucrose to give a final volume of 2 liters, and the suspension was passed through a continuous-flow homogenizer.[14] The mitochondrial fraction was isolated essentially as described by Crane, Glenn, and Green.[15]

Purification of PDC: Method 1: The mitochondrial paste was homogenized in sufficient 0.02 *M* potassium phosphate buffer, pH 7.0 (phosphate buffer) to give a protein concentration of about 40 mg/ml. The homogenate was subjected to sonic oscillation in a Raytheon 10-kc oscillator for 30 sec. The suspension was centrifuged for 30 min at 30,000 rpm in the no. 30 rotor of a Spinco model L ultracentrifuge. To 200 ml of the amber-colored supernatant fluid was added dropwise with stirring 7.5 ml of a 1% solution of protamine sulfate. The mixture was centrifuged for 105 min at 50,000 rpm in the no. 50 rotor, and the supernatant fluid was discarded. The pellet was suspended in phosphate buffer with the aid of a glass-Teflon homogenizer, the mixture was centrifuged for 10 min at 40,000 rpm, and the residue was discarded. The supernatant fluid was recentrifuged for 80 min at 40,000 rpm in the no. 40 rotor. The amber-colored pellet was dissolved in phosphate buffer. About 0.35 ml of 1% protamine sulfate per 100 mg of protein was added dropwise with stirring. The pH was adjusted to 5.6 with 1 *M* acetic acid, the mixture was centrifuged for 10 min at 10,000 rpm in an International model HR-1 centrifuge, and the supernatant fluid was discarded. The precipitate was suspended in phosphate buffer, and the mixture was centrifuged for 10 min at 10,000 rpm. The pH of the opalescent, yellow solution was carefully adjusted to 5.6 by dropwise addition, with stirring, of 1% acetic acid. The mixture was centrifuged for 10 min at 10,000 rpm, and the precipitate was dissolved in phosphate buffer. The pH was adjusted to 5.8 as described above. The precipitate was separated by centrifugation and was discarded. The pH of the supernatant fluid was lowered to 5.3. The yellow precipitate was collected by centrifugation and was dissolved in phosphate buffer. Solutions of the purified PDC have been stored at −15° for several weeks without significant loss of activity.

Purification of KGDC and PDC: Method 2: Little or no over-all α-ketoglutarate dehydrogenase activity (reaction 1) was detected in extracts of sonicated mitochondria prepared as described above. However, significant amounts of the individual enzymatic activities were detected following sucrose density gradient centrifugation of the mitochondrial extracts. This observation suggested that the extracts contained a substance or substances which caused dissociation of KGDC. When mitochondria were washed once with 0.02 *M* phosphate buffer, pH 7.0, and then sonicated, the resulting extract exhibited significant α-ketoglutarate dehydrogenase activity (reaction 1). However, this activity decreased relatively rapidly during storage of the extract at 4°. Repeated "washing" of the mitochondria with phosphate buffer as described below removed the interfering substance.[16] The mitochondrial paste was homogenized in sufficient 0.02 *M* phosphate buffer, pH 7.0, to give a protein concentration of about 20 mg per ml. The homogenate was centrifuged for 15 min at 30,000 rpm, and the supernatant fluid was discarded. The "washing" procedure was repeated 3 times with 0.02 *M* phosphate buffer, pH 6.3. The final residue was homogenized in the same buffer containing 0.0005 *M* ethylenediaminetetraacetate (phosphate-EDTA buffer), and the protein concentration was adjusted to 40–50 mg/ml. The homogenate was stored at −15° for 2 days and then thawed. The suspension was homogenized and then centrifuged for 20 min at 30,000 rpm. The clear, yellow supernatant fluid[17] was recentrifuged for 3 hr at 40,000 rpm. The yellow pellet was suspended in phosphate-EDTA buffer, pH 7.0, with the aid of a glass-Teflon homogenizer, the mixture was centrifuged for 10 min at

TABLE 1

PURIFICATION OF PDC

Step	Volume, ml	Protein, mg	Specific activity*	Recovery, %
Mitochondrial suspension	700	28,000	—	—
Extract	600	14,500	0.35†	(100)
Ultracentrifuge pellet	30	333	16†	106
Protamine eluate	17	94	46	83
Precipitate, pH 5.6	10	51	59	57
Precipitate, pH 5.8–5.3	1	7	204	28

* Micromoles of DPNH formed per hour per mg of protein. The assay was carried out essentially as described for the *E. coli* KGDC.[7]

† Amytal (20 μM/ml) was included in the assay medium to inhibit DPNH oxidase.

40,000 rpm, and the pellet was discarded. The supernatant fluid was recentrifuged for 2.5 hr at 40,000 rpm. The yellow pellet was dissolved in phosphate-EDTA buffer, pH 7.0. This solution contained both KGDC and PDC. Separation of the two complexes was achieved by centrifugation in sucrose density gradients as described below.

Electron microscopy: An aliquot of the protein solution was added to cold, 0.25% sodium phosphotungstate, pH 7.0, to give a final protein concentration of 50–100 μg/ml. The mixture was sprayed on carbon or Butvar 98 (Shawinigan Resins Corp., Springfield, Mass.) film which was supported on a carbon-stabilized net of Butvar 72A mounted on a copper grid. After about 5 min the specimen was examined in a Siemens-Elmiskop I operating at 60 kv. The micrographs were taken at a magnification of 80,000×.

Results.—Purification of PDC: Method 1: A summary of the purification is presented in Table 1. Further purification was achieved by sucrose density gradient centrifugation under conditions described in Figure 2. The sedimentation velocity pattern of the purified PDC is shown in Figure 1*A*; $S^{\circ}_{20,w} = 62S$.

Purification of KGDC and PDC: Method 2: A summary of the purification is presented in Table 2. A sucrose density gradient profile of the purified preparation is shown in Figure 2. Fractions exhibiting maximal pyruvate dehydrogenase activity and minimal α-ketoglutarate dehydrogenase activity were pooled and centrifuged for 2 hr at 50,000 rpm. The yellow pellet was dissolved in phosphate-EDTA buffer, pH 7.0, and recentrifuged in a 20–5 per cent sucrose density gradient for 6 hr at 25,000 rpm. The active fractions were worked up as described above to obtain a highly purified preparation of PDC.[18] The sedimentation velocity pattern of this preparation is shown in Figure 1*B*; $S_{20,w} = 80S$.

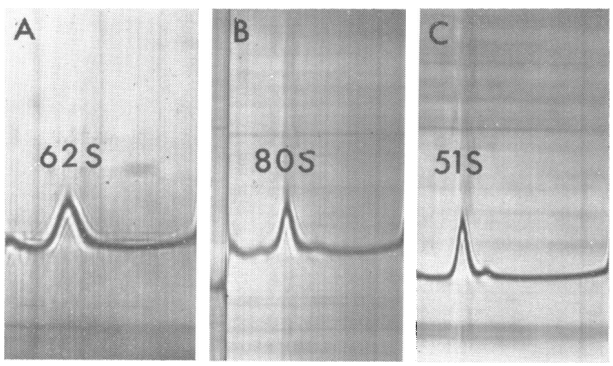

FIG. 1.—Sedimentation velocity patterns obtained with purified preparations of PDC and KGDC in 0.02 *M* potassium phosphate buffer, pH 7.0, at 42,040 rpm and 20°. Sedimentation coefficients are corrected to water at 20° and to zero protein concentration. (*A*) PDC obtained by method 1; (*B*) PDC, and (*C*) KGDC obtained by method 2.

Isolation of KGDC was achieved by centrifugation of the partially purified preparation (step 4, Table 2) in a 20–5 per cent sucrose gradient for 10 hr at 25,000 rpm. Under these conditions PDC sediments as a pellet. The fractions exhibiting maximal α-ketoglutarate dehydrogenase activity were

TABLE 2

PURIFICATION OF KGDC AND PDC

Step	Volume, ml	Protein, mg	Specific Activities* KGDC	PDC	Recovery, % KGDC	PDC
1. Mitochondrial suspension	185	8,515	—	—	—	—
2. Extract	144	2,320	11	3	(100)	(100)
3. First ultracentrifugation	48	355	66	15	92	74
4. Second ultracentrifugation	3	106	172	40	72	49

* Micromoles of DPNH formed per hour per mg of protein.

pooled and centrifuged for 3 hr at 50,000 rpm. The yellow pellet was dissolved in phosphate-EDTA buffer, pH 7.0. The sedimentation velocity pattern of this preparation is shown in Figure 1*C*; $S^{\circ}_{20,w} = 51S$.

Enzymatic activities of PDC and KGDC: The highly purified PDC exhibited pyruvate decarboxylase, dihydrolipoyl transacetylase, and dihydrolipoyl dehydrogenase activities (Table 3). Highly purified preparations of KGDC exhibited analogous activities, namely α-ketoglutarate decarboxylase, dihydrolipoyl transsuccinylase, and dihydrolipoyl dehydrogenase.

Isolation of dihydrolipoyl transacetylase from PDC: A solution of PDC in 0.02 *M* ethanolamine-phosphate buffer, pH 10.0, was centrifuged in a 20–5 per cent sucrose gradient containing the same buffer (Fig. 3*A*). Essentially all of the pyruvate decarboxylase and part of the flavoprotein were separated from the transacetylase. The decarboxylase was inactivated under these conditions.[6] Additional flavoprotein was released from the transacetylase by treatment with ammonium sulfate and

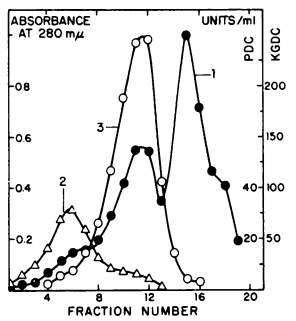

FIG. 2.—Sucrose density gradient profile of a purified preparation of PDC and KGDC (step 4, Table 2); *curve 1*, absorbance at 280 mμ; *curve 2*, pyruvate dehydrogenase activity; *curve 3*, α-ketoglutarate dehydrogenase activity. The sucrose density gradient (20–5%) contained 0.02 *M* potassium phosphate buffer, pH 7.0. Protein (22 mg) was centrifuged for 5 hr at 5° and 25,000 rpm in the SW25 rotor of a Spinco model L centrifuge.

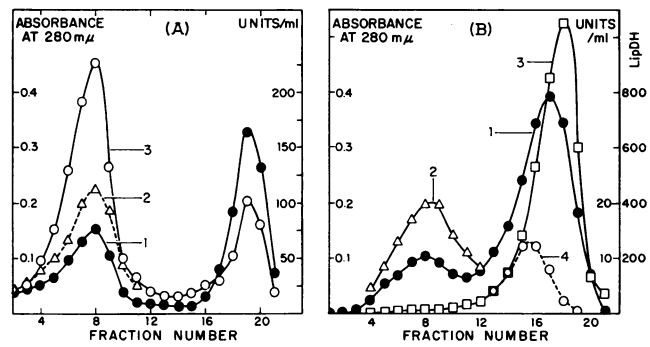

FIG. 3.—(*A*) Distribution of protein (*curve 1*), dihydrolipoyl transacetylase activity (*curve 2*), and dihydrolipoyl dehydrogenase activity (*curve 3*) following centrifugation of purified PDC in a 20–5% sucrose gradient containing 0.02 *M* phosphate-ethanolamine buffer, pH 10.0. Protein (4.3 mg) was centrifuged for 10 hr at 5° and 25,000 rpm. None of the fractions exhibited activity in the over-all pyruvate dehydrogenase reaction (eq. 1). (*B*) Distribution of protein (*curve 1*), dihydrolipoyl transsuccinylase activity (*curve 2*), dihydrolipoyl dehydrogenase activity (LipDH, *curve 3*) and α-ketoglutarate decarboxylase activity (*curve 4*) following centrifugation of purified KGDC in a 20–0% sucrose gradient containing 0.3 *M* potassium iodide and 0.02 *M* phosphate buffer, pH 7.0. Protein (4.8 mg) was centrifuged for 15 hr at 5° and 25,000 rpm. To avoid interference by potassium iodide in the transsuccinylase assay, fractions 4–12 were centrifuged individually for 4.5 hr at 50,000 rpm. The tubes were rinsed quickly with cold 0.02 *M* phosphate buffer, pH 7.0, and the pellets were dissolved in 0.1 *M* phosphate buffer, pH 8.0, and then assayed for transsuccinylase activity.

TABLE 3

ENZYMATIC ACTIVITIES OF PDC AND KGDC

Complex	DPN reduction	Ferricyanide reduction*	Transacylase†	Lipoamide reduction
	(μmoles/hr/mg protein)			
PDC	290	1.6	209	458
KGDC	532	408	24	1,251

* A modification of the spectrophotometric assay described by Massey[19] was used to determine the initial rate of ferricyanide reduction.

† Dihydrolipoamide transacetylase and dihydrolipoamide transsuccinylase activities were measured with PDC and KGDC, respectively.

urea. Fractions 4–10 (Fig. 3*A*) were neutralized, combined, and centrifuged for 4 hr at 50,000 rpm. The pale yellow pellet was dissolved in 1 ml of 0.02 *M* phosphate buffer, pH 7.0. To this solution were added 240 mg of urea and 300 mg of solid ammonium sulfate. The precipitate was dissolved in 0.02 *M* phosphate buffer, pH 7.0. It exhibited a specific activity of 328 in the transacetylase assay and only 10 per cent of the dihydrolipoyl dehydrogenase activity of the native PDC (cf. Table 3).

Isolation of dihydrolipoyl transsuccinylase from KGDC: Samples of KGDC were centrifuged in a 20–0 per cent sucrose gradient containing 0.3 *M* potassium iodide and 0.02 phosphate buffer, pH 7.0. The fractions obtained from one centrifuge tube were used for enzyme assays (Fig. 3*B*). It is evident that most of the decarboxylase and flavoprotein were separated from the transsuccinylase under these conditions. The appropriate fractions (4–10) from a second centrifuge tube were combined for isolation of the transsuccinylase. The combined fractions were centrifuged for 4.5 hr at 50,000 rpm. The pellet was dissolved in 0.1 *M* phosphate buffer, pH 8.0. It exhibited a specific activity of 53 in the transsuccinylase assay and only 5 per cent of the α-ketoglutarate decarboxylase activity and 10 per cent of the dihydrolipoyl dehydrogenase activity of the native KGDC.

Electron microscopy of PDC: Preparations of the mammalian PDC and KGDC and the transacetylase and transsuccinylase components isolated therefrom were examined in the electron microscope. A typical field of transacetylase particles negatively stained with phosphotungstate is shown in Figure 4*A*. Selected individual images, showing various orientations of the transacetylase particle, are shown at a higher magnification in Figure 4*B*. The diameter of these particles is approximately 210 Å. The appearance of the transacetylase particle suggests that it is composed of subunits situated at the 20 vertices of a pentagonal dodecahedron. In the model shown in Figure 4 the subunits are represented as spheres.[20] In these photographs the model is viewed down (*C*) a twofold axis, (*D*) a threefold axis, and (*E*) a fivefold axis.

The mammalian PDC tends to dissociate under the negative staining conditions used. Electron micrographs of PDC showed images very similar to those obtained with the isolated transacetylase and, in addition, much smaller particles, presumably molecules of pyruvate decarboxylase and dihydrolipoyl dehydrogenase, in the background (Fig. 4*F*). Other images were seen in which the small particles appeared to be attached to and to surround the transacetylase particle (Fig. 4*G*, *H*, *I*). These latter images, with a diameter about 310 Å, probably correspond to the relatively intact PDC.

Electron microscopy of KGDC: A typical field of transsuccinylase particles, iso-

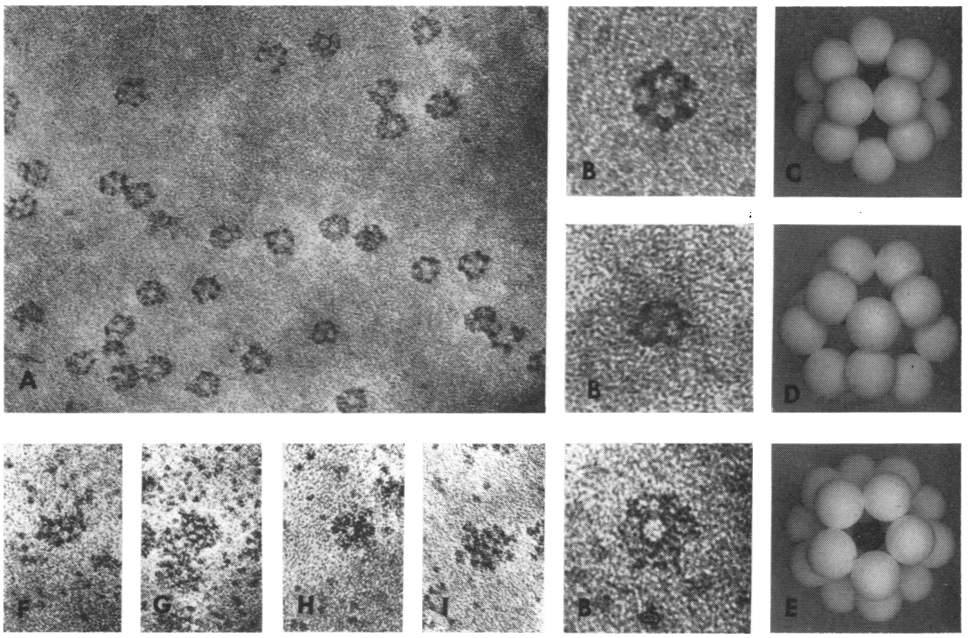

FIG. 4.—(*A*) Electron micrograph of mammalian dihydrolipoyl transacetylase negatively stained with sodium phosphotungstate, pH 7.0; reversed contrast print. ×200,000. (*B*) Selected individual images of the transacetylase particle. ×400,000. (*C–E*) Tentative model of mammalian transacetylase consisting of 20 spheres at the vertices of a pentagonal dodecahedron, viewed down (*C*) a twofold axis, (*D*) a threefold axis, and (*E*) a fivefold axis. These views of the model correspond to the orientations of the transacetylase particle shown in (*B*). (*F–I*) Electron micrographs of mammalian PDC negatively stained with phosphotungstate; reversed contrast prints. ×200,000. (See text for interpretation.)

lated from KGDC, is shown in Figure 5*A*. Two types of image cans be seen, and are enlarged in Figures 5*B* and *C*. There are tetramers (*B*) with a side of 125–150 Å, and images having the appearance of two parallel rows of subunits (*C*) with a length of 150–180 Å. These two types of images are strikingly similar to those seen with the *E. coli* dihydrolipoyl transacetylase and transsuccinylase.[8–10] The interpretation of these data, based on analogy with the bacterial transacylases, is that the mammalian transsuccinylase is composed of subunits situated at the eight vertices of a cube. In the model shown in Figures 5*D* and *E* the subunits are represented as spheres.[20] In these photographs the model is viewed down a fourfold axis (*D*) and a twofold axis (*E*).

The mammalian KGDC also tends to dissociate during negative staining with phosphotungstate. Selected images which are believed to represent the intact, or relatively intact, KGDC are shown in Figure 5*F*. The particles have the appearance of polyhedrons with a diameter about 250 Å. The core of the polyhedron has the same average dimension as, and closely resembles, the isolated transsuccinylase particle (Fig. 5*A*). The peripheral subunits presumably correspond to the molecules of α-ketoglutarate decarboxylase and dihydrolipoyl dehydrogenase.

Discussion.—The macromolecular organization of the mammalian transsuccinylase,[21] as indicated by electron microscopy, is strikingly similar to that of the *E. coli* transacetylase and transsuccinylase.[8–10] The gross appearance of the

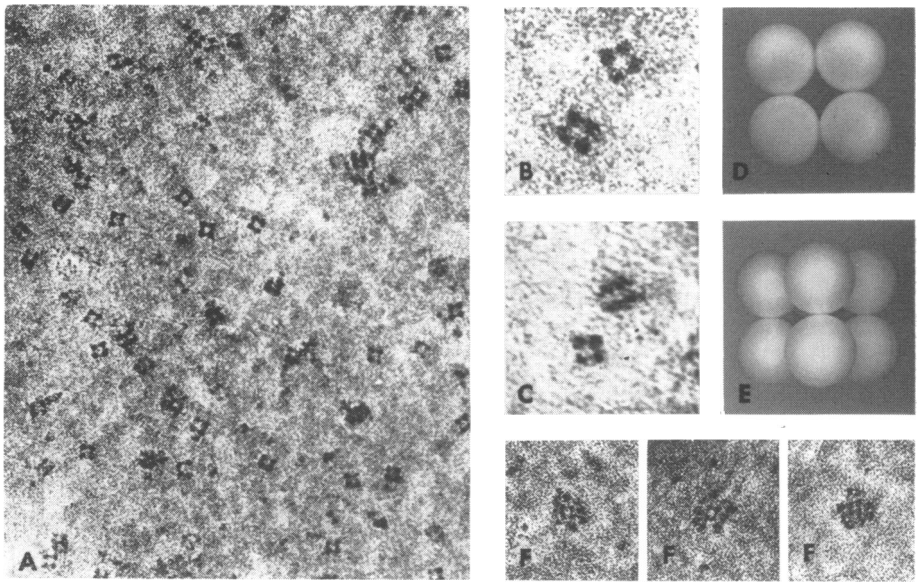

FIG. 5.—(*A*) Electron micrograph of mammalian dihydrolipoyl transsuccinylase negatively stained with sodium phosphotungstate, pH 7.0; reversed contrast print. ×200,000. (*B, C*) Selected individual images showing tetramers (*B*) and two parallel rows of subunits (*C*). ×400,000. (*D, E*) Tentative model of mammalian transsuccinylase consisting of eight spheres at the vertices of a cube, viewed down (*D*) a fourfold axis and (*E*) a twofold axis. These views of the model correspond to the orientations of the transsuccinylase particle shown in (*B*) and (*C*). (*F*) Electron micrographs of mammalian KGDC negatively stained with phosphotungstate; reversed contrast print. ×200,000.

mammalian KGDC is also similar to that of the *E. coli* PDC and KGDC. It appears that the subunits of the mammalian transsuccinylase, by analogy with the *E. coli* transacetylase and transsuccinylase, are situated at the eight vertices of a cube. The molecules of α-ketoglutarate decarboxylase and of flavoprotein appear to be disposed in an orderly arrangement around the transsuccinylase aggregate, presumably along the 12 edges and in the six faces of the cube.

The macromolecular organization of the mammalian transacetylase[21] differs from that of the *E. coli* transacetylase and the *E. coli* and mammalian transsuccinylases in that the subunits of the former enzyme appear to be situated at the vertices of a pentagonal dodecahedron, rather than at the vertices of a cube. The molecules of pyruvate decarboxylase and of flavoprotein are probably grouped in an orderly arrangement around this dodecahedron, presumably along the 30 edges and in the 12 faces, in the intact PDC. The difference in the three-dimensional structure of the mammalian transacetylase and the other three transacylases should not obscure the basic similarities in the molecular architecture of the mammalian and bacterial α-keto acid dehydrogenase complexes. It appears that the principles of macromolecular organization of these four multienzyme complexes are similar and may well be elaborations of the principles of self-assembly observed with the coat proteins of spherical viruses.[22] It was suggested previously[23] that the mammalian transacetylase may be composed of 24 subunits arranged in a polyhedral structure consisting of 12 pentagonal faces and two hexagonal faces. If the subunits are found to be identical, as anticipated, a pentagonal dodecahedron would be a more reasonable structure.[24]

The difference between the two preparations of the mammalian PDC, with sedimentation coefficients of 62*S* and 80*S*, is not clear. It is probable that the 80*S* complex is more "intact" than the 62*S* complex, i.e., the latter complex may not contain the full complement of pyruvate decarboxylase and of flavoprotein. It should be noted in this context that the procedure used to obtain the 80*S* complex is milder than that used to obtain the 62*S* complex.

The authors are indebted to Mrs. Setsuko Ishikawa, Jack Smith, and Fred Hoffman for expert technical assistance, and to Dr. Daniel Ziegler for assistance in developing some of the steps of the purification procedure.

* Supported in part by a grant (GM-06590) from the U.S. Public Health Service.

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[16] Incubation of a partially purified preparation of KGDC with the mitochondrial washings at 4° resulted in a relatively rapid loss of over-all activity (reaction 1) and a concomitant dissociation of the complex, as indicated by activity profiles following centrifugation in a sucrose gradient. The interfering substance is heat-labile.

[17] Extracts obtained by freezing and thawing "washed" mitochondria contained approximately twice as much α-ketoglutarate dehydrogenase activity and approximately the same amount of pyruvate dehydrogenase activity as extracts obtained by sonic oscillation. Furthermore, the former extracts contained little if any hemoproteins, in contrast with the latter extracts.

[18] Highly purified preparations of PDC with similar properties also have been obtained following centrifugation of the original mitochondrial extract (step 2, Table 2) in a 20–5% sucrose gradient for 6 hr at 25,000 rpm.

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[20] It is recognized that in the actual transacetylase and transsuccinylase molecules the subunits are in all probability not uniform spheres, and may well consist of more than one peptide chain.

[21] The appearance in the electron microscope of dihydrolipoyl transacetylase and dihydrolipoyl transsuccinylase from pig heart is very similar to that of the corresponding enzymes from beef kidney (M. Koike, T. Hayakawa, and M. Hirashima, private communication, 1965).

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[24] It is of interest to note that X-ray and chemical evidence indicate that apoferritin, the protein component of the iron storage protein ferritin, probably consists of 20 identical subunits situated at the vertices of a pentagonal dodecahedron.[25]

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