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FINAL REPORT ON No. 1

CONTRACT/[redacted] NO DA 92-557-FEC-37961

INCLUSIVE DATES 15 March 1965 TO 14 March 1966

SUBJECT OF INVESTIGATION

STUDIES ON
MAMMALIAN AND HUMAN
PYRUVATE AND α-KETOGLUTARATE
DEHYDROGENATION COMPLEXES

RESPONSIBLE INVESTIGATOR

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Abstract

1. A coenzyme A- and NAD-linked pyruvate and α-ketoglutarate dehydrogenase complexes have been isolated from pig heart muscle as multienzyme units with molecular weights of approximately 9 and 2.7 million, respectively. Pyruvate dehydrogenase complex contains approximately 67 moles of protein-bound lipoic acid and 17 moles of bound FAD. α-Ketoglutarate dehydrogenase complex contains approximately 10 moles of protein-bound lipoic acid, 9 moles of FAD and 6 moles of thiamine-PP.

2. Both complexes were activated by Ca^{2+} as the same extent as Mg^{2+} which had been considered as one of the typical metal activators of oxidative decarboxylation reaction of α-keto acid. These activating effects were in good agreement with the results of the metal contents obtained by the atomic absorption analysis. Pyruvate dehydrogenase complex was strongly inhibited by EDTA at low concentration, but on the contrary α-ketoglutarate dehydrogenase complex was little inhibited by EDTA and rather obviously inhibited by 8-hydroxyquinoline.

3. Attempts have been made to dissolve both complexes into three essential components containing each coenzyme, but it did not still go well to restore pyruvate decarboxylase activity. Two other components; lipoic reductase-transacetylase and lipoamide dehydrogenase were isolated in pure status.

4. Preliminary experiment to determine the structural organization of the mammalian pyruvate and α-ketoglutarate dehydrogenase complexes have been made and hopeful results were obtained.

Studies on Mammalian and Human Pyruvate and

α-Ketoglutarate Dehydrogenation Complexes

Contents of the Final Report No. 1

1. A statement and analysis of the problem.

Enzyme systems which catalyze CoA- and NAD-linked oxidative decarboxylation of pyruvate and α-ketoglutarate (Reaction 1) have been isolated from extracts of *Escherichia coli* as organized units with molecular weights of about 4.8 million and 2.4 million, respectively (1-3). The *E. coli* pyruvate

$$RCOCO_2H + CoA\text{-}SH + NAD^+ \longrightarrow RCO\text{-}S\text{-}CoA + CO_2 + NADH + H^+ \quad (1)$$

dehydrogenation complex has been resolved into three essential components: (a) pyruvic carboxylase (dependent on added thiamine-PP), (b) lipoic reductase-transacetylase (contains protein-bound lipoic acid), and (c) a flavoprotein (dihydrolipoic dehydrogenase, contains bound FAD) (4). The molecular weights of these components are approximately 183,000, 1.6 million and 112,000, respectively. These components reassociate spontaneously to produce a large unit resembling the original complex in composition and enzymatic activities. The picture of the structural organization of the pyruvate dehydrogenation complex which emerged from biochemical studies is that of an organized mosaic of enzymes in which each of the component enzymes is uniquely located to permit efficient implementation of a consecutive reaction sequence. This picture has been confirmed and extended by correlative electron microscope studies (5). Electron micrographs of the complex negatively stained with phosphotungstate indicate that it has a polyhedral structure with a diameter of about 300 Å and a height of about 200 Å. The lipoic reductase-transacetylase aggregate occupies the central portion of the polyhedron. The subunits of this aggregate appear to be arranged into 4 stacks, comprising a tetrad. Surrounding this tetrad are the 16 molecules of pyruvic carboxylase and 8 molecules of dihydrolipoic dehydrogenase apparently arranged into two rings laid one above the other. It is tentatively concluded that each ring contains 8 molecules of carboxylase and 4 molecules of dehydrogenase in an alternating sequence.

An α-ketoglutarate dehydrogenase complex has been isolated by several investigators from pig heart as an organized unit of high molecular weight. Recently CoA- and NAD-linked pyruvate and α-ketoglutarate dehydrogenase complexes has been succesfully isolated as a soluble organized unit of high molecular weight from pig heart (6). Recent work has been concentrated on the following studies: (a) convenient purification procedure, properties and the electron microscopic macromolecular structures of the complexes; (b) the mechanism of oxidative decarboxylation of α-keto acids; (c) the resolution of the complexes into their essential components; (d) the reconstitution of the complexes from its components parts to produce a large enzyme unit resembling the original complex in composition, enzymatic activities and their macromolecular structure.

2. Outline of experimental procedure.

a. Materials--CoA, NAD, NADH, FAD, FMN, Pyruvic acid, thiamine-PP, ATP (potassium salt) and crystalline bovine serum albumine were purchased from the Sigma Chemical Company. Potassium pyruvate was prepared by the method of Korkes *et al.* (7). L-Cystein, protamine sulfate (salmine) and lactic dehydrogenase, 2 x recrystallized from rabbit skeletal muscle were purchased from Nutritional Biochemicals Corporation. The activity of lactic dehydrogenase were determined by the method of Ochoa et al. (8). Oxidized and reduced lipoic acid and its derivatives were generous gifts of Drs. Tatsuoka, H. Nawa and H. Hirano. A cell-free extract prepared from dried cells of Clostridium kluyveri (Worthington Biochemical Corporation) by the method of Stadtman (9) was routinely as a source of phosphotransacetylase. Its activity was determined by the method of Stadtman (10). Crystalline D-amino acid oxidase was prepared by the method of Yagi and Ozawa (11), and its apoenzyme was prepared by the Negelein and Brömel (12).

b. Procedures--The activity of D-amino acid oxidase was measured by using a Beckman Clark type oxygen electrode with a model 160 physiological gas analyzer. Protein was determined by both biuret method (13) and the phenol method of Lowry et al. (14), with crystalline bovine serum albumin was served as the standard. Calcium phosphate gel suspended on Whatman standard grade cellulose powder was prepared as described by Price and Greenfield (15). The lipoic acid content (calculated as (+)-lipoic acid) of several preparation of the complex was determined manometrically with lipoic acid-deficient Streptococcus faecalis 10Cl cells (16). The sample was autoclaved in 0.1 N sodium hydroxide in sealed test tube at 15 lb for 3 hours under nitrogen (17). FAD content of the enzyme was determined according to the method of Beinert and Page (18) by measuring the absorbance of neutralized trichloroacetic acid extract at 450 mμ before and after reduction with dithionite. Acid extractable FAD was identified by paper chromatography (19) and the full activation of apo-D-amino acid oxidase system. The absorption spectrum and optical density change in kinetic study were carried out with Beckman DB spectrophotometer with Sargent SRL recorder or Shimadzu QR-50 spectrophotometer with Shimadzu ARP-21 potentiometric recorder. Thiamine-PP was determined by the modified procedure of Kajiro (20) and Green et al. (21). Cuvetts with 1-cm light path were used. Electrophoretic run was made in a Hitachi model HTD-1 Tiselius apparatus at 4° or in Zone electrophoretic apparatus with Cyanogum 41 gel according to the method of Raymond et al. (22). Sedimentation velocity, diffusion and Archibald procedure for measurement of molecular weight were carried out with Beckman model E analytical ultracentrifuge. Some diffusion runs were carried out in Spinco model H electrophoresis-diffusion apparatus with the Schlieren optics. Contents of protein-bound metal ions were determined on a Hitachi Perkin-Elmer model 139 spectrophotometer with an atomic absorption attachment. Electron microscopic analysis was carried out with JEM6C electron microscope.

c. Enzyme assay--(1) Pyruvate dismutation activity based on Reaction 2 was essentially the same as that described by Reed, Leach and Koike (23). The reaction mixture contained 100 μmoles of potassium phosphate buffer (pH 7.0),

$$2 \text{ Pyruvate} + \text{Phosphate} \xrightarrow{\text{CoA, NAD}} \text{Acetyl phosphate} + CO_2 + \text{Lactate} \qquad (2)$$

25 μmoles of potassium pyruvate, 0.05 μmoles of CoA, 0.24 μmoles of NAD, 6.4 μmoles of L-Cysteine, 3 units of phosphotransacetylase, 2,000 unit of lactic dehydrogenase, 0.2 μmole of thiamine-PP, 0.8 μmole of magnesium sulfate and 2 mg of bovine serum albumin, in a final volume of 1 ml. The mixture was incubated for 30 minutes at 37° and then assayed for acetyl phosphate (24). Specific activity is expressed as micromoles of acetyl phosphate formed per hour per mg of protein.

(2) Dihydrolipoic transacetylase activity based on Reaction 3 was determined with a reaction mixture containing 100 μmoles of Tris buffer (pH 7.0), 10 μmoles of acetyl phosphate, 0.1 μmole of CoA, 10 μmoles of dihydrolipoamide, 2 units of phosphotransacetylase and 2 mg of bovine serum albumin, in a final volume of 0.9 ml (23). The mixture was incubated for 30 minutes at 37°, 0.1 ml of 1 N HCl was added, and the mixture was cooled, and then was

$$CH_3COOPO_3^{2-} + Lip(SH)_2amide \xrightarrow[PTA]{CoA} CH_3\text{-}CO\text{-}S\text{-}Lip\text{-}SH\ amide + HPO_4^{2-} \quad (3)$$

assayed for heat-stable thioester (24). Specific activity is expressed as micromoles of heat-stable thioester formed per hour per mg of protein.

(3) Lipoamide dehydrogenase activity based on Reaction 4 was determined at room temperature with spectrophotometer as described by Massey (25) with the following modification. The reaction mixture contained 100 μmoles of potassium phosphate buffer (pH 6.5), 0.2 μmole of NAD, 0.8 μmole of lipoamide and 2.5 μmoles of EDTA, in a final volume of 2 ml. Specific activity is expressed as micromoles of NADH decomposed per hour per mg of protein.

$$Lip\ S_2\ amide + NADH + H^+ \longrightarrow Lip(SH)_2amide + NAD^+ \quad (4)$$

(4) The pyruvate and α-ketoglutarate decarboxylase assay, based on Reaction 5, is a modification of that described by Hager (26). The reaction mixture contained 150 μmoles of potassium phosphate buffer (pH 6.5), 25 μmoles of potassium ferricyanide, 0.2 μmole of thiamine-PP, 1.0 μmole of calcium chloride, 50 μmoles of potassium pyruvate and 2 mg of bovine serum albumin, in a final volume of 1.4 ml. The mixture was incubated at 37° for 30 minutes, and then 1 ml of 10% trichloroacetic acid solution was added. Ferrocyanide was determined in the deproteinized mixture as Prussian Blue (26). Specific activity is expressed as one-half of the total micromoles of ferrocyanide formed per hour per mg protein.

$$R\cdot CO\cdot CO_2H + 2\ Fe(CN)_6^{3-} + H_2O \xrightarrow[Ca^{2+}]{TPP} CH_3COOH + CO_2 + 2\ Fe(CN)_6^{4-} + 2H^+ \quad (5)$$

(5) α-Ketoglutarate dehydrogenase assay for the overall oxidation of α-ketoglutarate, based on Reaction 6, is a modification of that described by Massey (25). The reaction mixture contained 150 μmoles of potassium phosphate buffer (pH 7.2), 0.08 μmole of CoA, 0.3 μmole of NAD, 5 μmoles of L-Cysteine (free base), 0.5 μmole of calcium chloride and 5 μmoles of potassium α-ketoglutarate. The reaction was begun by the addition of enzyme at 25°. Specific activity is expressed as μmole of NADH formed per hour per mg protein.

$$\alpha\text{-Ketoglutarate} + CoA\text{-}SH + NAD^+ \longrightarrow \text{Succinyl CoA} + CO_2 + NADH + H^+ \quad (6)$$

(6) Lipoic transsuccinylase assay, based on Reaction 7, is a modification of that described by Knight and Gunsalus (27). The reaction mixture con-

$$\text{Succinate} + ATP + Lip(SH)_2amide \longrightarrow \text{Succinyl-S-LipSH} + ADP + Pi \quad (7)$$

tained 100 μmoles of Tris buffer, pH 7.2, 10 μmoles of $MgCl_2$, 10 μmoles of ATP (potassium salt), 5 units of succinic thiokinase (28), 180 μmoles of potassium succinate, 10 μmoles of $lip(SH)_2amide$ in 0.5 ml of 95% ethanol, 13 μmoles of L-Cystein (free base), 0.1 μmole of CoA and 2 mg of bovine serum albumine. The mixture was incubated for 30 minutes at 30° and assayed for thioester (24). Specific activity is expressed as micromoles of thioester formed per hour per mg of protein.

3. Result

a. Isolation and properties of pyruvate and α-ketoglutarate dehydrogenase complexes from pig heart muscle

(1) Preparation of pig heart particles and E. coli complexes (as internal standard)--Pig hearts were collected and chilled as soon as possible after slaughter, minced, and stored at -20°. Pig heart particles were prepared according to the procedure of Sanadi et al. (29), and frozen and thawed three times. The protein coagulated by this treatment was removed by centrifugation for 30 minutes at 16,000 x g, an amber-colored extract was obtained. The E. coli complexes were isolated essentially as described previously (1) from sonic extracts of E. coli (Crookes strain) cells.

(2) Preparation of pyruvate and α-ketoglutarate dehydrogenase complexes--An amber-colored extract (3.65 mg of protein per ml) is adjusted to pH 6 with 1 N acetic acid, and the pyruvate and α-ketoglutarate dehydrogenase complexes, respectively, are precipitated by addition of 0.0 to 0.015 volume and 0.015 to 0.03 volume of 2% protamine sulfate solution (pH 5.0). Each precipitate is eluted with 0.1 M potassium phosphate, pH 7.0 and the eluates are dialyzed overnight against 0.05 M potassium phosphate, pH 7.0, and then centrifuged. The elutes, designated protamine precipitate eluate, are centrifuged for 2 hours at 198,000 x g in the No. 50 rotor of a Beckman model 2-L ultracentrifuge. The yellow pellet obtained was dissolved in 0.05 M potassium phosphate, pH 7.0, and purified by chromatography on a calcium phosphate gel-cellulose column. Yellow fractions are eluted with 4% ammonium sulfate in 0.1 M potassium phosphate, pH 7.5. The pyruvate dehydrogenase complex is precipitated with solid ammonium sulfate between 0.29 and 0.36 saturation and the α-ketoglutarate dehydrogenase complex precipitated between 0.24 and 0.29. A summary of the data obtained from a typical purification and recovery of enzyme activity is given in Table 1 and 2.

(3) Enzymatic activities of complex--The pyruvate dismutation, dihydrolipoic transacetylase, pyruvate decarboxylase and lipoamide dehydrogenase were determined throughout the purification (Table 1). All the four activities were associated with the highly purified pyruvate dehydrogenase complex. The ratio of dihydrolipoic transacetylase activity to pyruvate dismutation activity was constant over the 280-fold range of purification achieved. This relatively constant ratio of both activities that dihydrolipoic transacetylase is an integral part of the pyruvate dehydrogenase complex.

α-Ketoglutarate dehydrogenase, α-ketoglutarate decarboxylase and lipoamide dehydrogenase were determined throughout the purification (Table 2). All three activities were associated with the highly purified α-ketoglutarate dehydrogenase complex over the 68-fold range of purification achieved.

(4) Physicochemical properties

(a) Electrophoretic analysis--The highly purified preparations of the pyruvate and α-ketoglutarate dehydrogenase complexes, corresponding to the ammonium sulfate precipitate, were dialyzed for 16 hours against 1 liter of 0.05 M potassium phosphate buffer (pH 7.0). The electrophoresis run was made in a Tiselius apparatus at 4°. The protein concentration was 1.33 g and 1.32 g per 100 ml, respectively. The schlieren patterns obtained with the complexes (Fig. 1 and 2) show single ascending and descending boundaries. The pyruvate and α-ketoglutarate dehydrogenase complexes show the mobilities of -4.82×10^{-5} and $-9.95 \times 10^{-5} cm^2 volt^{-1} sec^{-1}$, respectively.

(b) Ultracentrifugal analysis and molecular weight--1. Sedimentation velocity--Sedimentation coefficients were calculated and corrected as described by Schachman (30). The patterns obtained with the highly purified complexes are shown in Fig. 3 and 4. These patterns are typical of several different highly purified preparations examined. The values extrapolated to infinite dilution ($S°_{20,w}$) are 67.5 S and 35.7 S, respectively.

2. Molecular weight determined by the method of approach to sedimentation equilibrium--These runs were performed at 2,333 rpm in an An-J analytical rotor, using about 0.5 ml of the sample in a synthetic boundary cell as recommended by Ehrenberg (31). The initial protein concentration in the cell was determined from a synthetic boundary cell at 8,225 rpm under identical optical conditions. The molecular weight was calculated at the meniscus with the equation proposed by Klainer and Kegeles (32). From those data the average molecular weights are calculated to be 9.0 million for pyruvate dehydrogenase complex and 2.7 million for α-ketoglutarate dehydrogenase complex. As an internal standard, the molecular weight of the E. coli pyruvate dehydrogenase complex was determined under the same condition mentioned above. The molecular weight was calculated to be 4.9 million. This value is in good agreement with the molecular weight of 4.8 million, which was calculated from $S°_{20,w}$ and $D_{20,w}$ (1).

3. Diffusion studies and molecular weight--Diffusion studies were carried out at 4.65° in the Spinco model H electrophoresis-diffusion apparatus. Photographs of the schlieren pattern of the diffusing boundary were taken at intervals over a period of 21 hours. Diffusion coefficient was calculated and corrected as described by Svensson (33). The highly purified preparations of the pyruvate and α-ketoglutarate dehydrogenase complexes was dialyzed against 0.05 M potassium phosphate buffer (pH 7.0) overnight at 0°. The dialyzed preparations were diluted to the protein concentration of 0.58 and 0.25 g per 100 ml with the dialyzate, respectively. The corrected diffusion coefficient ($D_{20,w}$) for these preparations were 0.62×10^{-7} and $1.18 \times 10^{-7} cm^2 sec^{-1}$, respectively. With use of these values for the molecular weights of both complexes were calculated to be 9.7 million and 2.8 million, respectively. Hydrodynamic parameter of both complexes were summarized in Table 3.

(5) Contents of bound-coenzymes--The contents of bound-coenzymes are summarized in Table 4.

(a) Protein-bound lipoic acid--It is apparent from the data in Table 1 and 2 that protein-bound lipoic acid concentrates with the complexes during the purification. The average amounts of protein-bound lipoic acid found in several preparations of purified pyruvate and α-ketoglutarate dehydrogenase complexes were 7.4×10^{-9} and 3.7×10^{-9} moles of bound lipoic

An α-ketoglutarate dehydrogenase complex has been isolated by several investigators from pig heart. Recently convenient procedures have been devised in this laboratory for simultaneous isolation of the pig heart pyruvate and α-ketoglutarate dehydrogenase complexes in a highly purified state. This complex in the present study resembles not only complex isolated by the methods of Sanadi et al. (29) or Massey (25) but also the E. coli α-ketoglutarate dehydrogenase complex in composition and enzymatic activities. The ratio among bound lipoic acid, FAD and thiamine-pyrophosphate in the pig heart α-ketoglutarate complex is approximately 1:1:0.6, whereas the ratio is approximately 1:1:0.8 in the E. coli complex.

The molecular weights of the complexes determined by Archibald method are in good agreement with those calculated from sedimentation and diffusion constants. As an interval control, the molecular weight of the E. coli pyruvate dehydrogenase complex determined by Archibald method under the same condition is in good agreement with value, which was calculated from sedimentation and diffusion constants as shown in Table 3. This is the first example of the molecular weight determination of the charged giant molecules (order of 2.5-9 million) by Archibald method. It is yet unknown whether this procedure is suitable for such giant molecule or not. Besides these studies the determination of size, shape and molecular weight by light scattering procedure are under way, so that it will be appeared in some journals in near future (39, 40, 41).

b. Metal ion activators of α-keto acid dehydrogenase complex

Enzyme systems which catalyze the oxidative decarboxylation of pyruvate and α-ketoglutarate have been reported to include Mg^{2+} as one of the components of enzyme complexes, and some of them are activated by additional Mg^{2+} (39, 40, 41). Pigeon breast pyruvate dehydrogenase complex was activated by Mn^{2+} in place of Mg^{2+}, but strongly inhibited by Cu^{2+} and Zn^{2+}, and attempts to prepare Mg^{2+}-free enzyme by dialysis against various chelating agents (pyrophosphate, 8-hydroquinoline) failed. On the oxidative decarboxylation of pyruvate with crude pig heart enzyme, it was reported that the addition of Mg^{2+}, Mn^{2+} or thiamine-PP was not necessary (37). In previous paper (6), the highly purified pyruvate dehydrogenase complex was free from thiamine-PP, but on the contrary α-ketoglutarate dehydrogenase complex showed only a little response to added thiamine-PP. The effects of several divalent cations, especially activating effect of Ca^{2+}, and the inhibitory effect of EDTA and other ligands on the oxidative decarboxylation of α-keto acid with both complexes have been studied. The contents of some protein-bound metals of the complexes were determined by the atomic absorption spectrophotometry.

(1) Effects of divalent metal ions on the oxidative decarboxylation activities--As the results reported already by some authors, low concentration of Mn^{2+} was able to replace Mg^{2+} in pig heart pyruvate carboxylase assay, however, in both pig heart α-ketoglutarate and E. coli pyruvate carboxylase assays Mn^{2+} exhibited inhibitory effect. Cu^{2+}, Zn^{2+} and Hg^{2+} were strongly inhibitory in both pig heart carboxylase assays. Fe^{2+} and Pb^{2+} had no effect. These results were summarized in Table 5. In both pig heart carboxylase assays Ca^{2+} was strongly stimulatory to the same extent as Mg^{2+}. Considering that calcium is situated in the same group IIA of the periodic chart of the elements with magnesium, the activating effect of Ca^{2+} on the oxidative de-

carboxylation of α-keto acids is an interesting evidence. E. coli pyruvate dehydrogenase complex was activated by Mg^{2+} as well as pig heart pyruvate dehydrogenase complex and α-ketoglutarate dehydrogenase complex, but Ca^{2+} had no effect on it and Mn^{2+} exhibited complete inhibition of its activity at the concentration of 7×10^{-4} M.

Highly purified enzymes even in the absence of any metal activator showed 55-60% (pyruvate dehydrogenase complex) and 70-75% (α-ketoglutarate dehydrogenase complex) of their activities which were obtained in each carboxylase assay containing 0.3 μmole of Ca^{2+}. The trial to prepare metal-free enzyme protein by dialyzing against 0.01 M EDTA in 0.05 M phosphate buffer, pH 7.0, at 0° for 48 hours had been made, but it failed.

(2) Effects of several chelating agents on the oxidative decarbosylation activities--On the other hand, the inhibitory effects of some chelating agents are given in Table 6. Pig heart pyruvate dehydrogenase complex was strongly inhibited by EDTA, however, α-ketoglutarate dehydrogenase complex activity was little inhibited by EDTA as contrast with pyruvate dehydrogenase complex, and it was rather obviously inhibited by 8-hydroxyquinoline and O-phenanthroline. Other chelating agents including glycine, histidine, pyrophosphate, KCN and KF were almost ineffective on their activities at the concentration of 7×10^{-4} M.

In Table 7 it was demonstrated that the inhibitory effect of EDTA was reversed by the addition of either Mg^{2+} or Ca^{2+}.

(3) Metal contents in α-keto acid dehydrogenase complexes--These results mentioned above suggested that metal ions were concerned positively to the oxidative decarboxylation reaction of α-keto acid and they seemed to combine strongly to enzyme protein. Then it was tried to determine the contents of protein-bound metal ions directly by the atomic absorption spectrophotometry. The enzyme samples were previously dialyzed against 0.01 M EDTA in phosphate buffer to dialyze out free metal ions existing in the enzyme solution. As shown in Table 8 magnesium and calcium contents in both complexes were concentrated during the purification though these absolute quantities were low. These results supported the evidence of the active participation of Ca^{2+} and Mg^{2+} in oxidative decarboxylation reaction of α-keto acid. In E. coli pyruvate dehydrogenase complex calcium was not detected and this result fits in no activating effect on its activity with Ca^{2+} alone. In addition, even the simultaneous presence of Mg^{2+} and Ca^{2+} on the pyruvate decarboxylase assay had no multiplication effects as shown in Table 5, suggesting that both pig heart complexes were activated by either Mg^{2+} or Ca^{2+} according to the same mechanism.

(4) Conclusion--Both pig heart complexes were activated by Ca^{2+} as the same extent as Mg^{2+} which had been considered as one of the typical metal activators of oxidative decarboxylation reaction of α-keto acid. E. coli pyruvate dehydrogenase complex was, however, activated only by Mg^{2+} and not by Ca^{2+}. These activating effects were in good agreement with the results of the metal contents obtained by the atomic absorption analysis. Pig heart and E. coli pyruvate dehydrogenase complex were strongly inhibited by EDTA at low concentration, but on the contrary pig heart α-ketoglutarate dehydrogenase complex was little inhibited by EDTA and rather obviously inhibited by 8-

hydroxyquinoline. Further investigation of the mechanism of the oxidative decarboxylation of α-keto acids, for instance, interactions among thiamine-PP, metal activators and enzyme protein is now under way.

c. Resolution of mammalian α-keto acid dehydrogenase complex

(1) Pyruvate dehydrogenase complexes--So far at present little is known concerning to the resolution of pyruvate dehydrogenase complex in the presence of 4 M urea or 0.02 M ethanolamine (pH 9-9.5). In the presence of 4 M urea pyruvate dehydrogenase complex is quite stable. For example, pyruvate dehydrogenase complex was incubated at 0° with 1-5 M urea for 2 hours, but the complex retained full activity during this period. Examination of a solution of the complex in 4 M urea in a Beckman model E analytical ultracentrifuge revealed the presence of two components; (1) faster moving boundary assuming carboxylase-lipoic reductase-transacetylase complex, (2) slower moving boundary assuming a flavoprotein associated with yellow color. These results indicated a dissociation of pyruvate dehydrogenase complex into at least two components. After a number of trials, a satisfactory procedure will be developed, involving fractionation on a calcium phosphate gel-cellulose column (15) in the presence of urea like resolution of E. coli pyruvate dehydrogenase complex (4).

When pyruvate dehydrogenase complex was allowed to stand at 0° in contact with an ethanolamine-phosphate buffer, pH 9.5, which was 0.02 M with respect to ethanolamine and approximately 0.01-0.03 M with respect to potassium phosphate, only 15-25% of the NAD^+-linked pyruvate dehydrogenase activity was destroyed in one hour. During 2 hours incubation with this buffer, pyruvate dehydrogenase complex lost only 30% of its activity. In the presence of excess of thiamine-PP or pyruvate, pyruvate dehydrogenase complex did not lose any activities during 2 hours incubation with this buffer. Examination of a freshly prepared mixture in the analytical ultracentrifuge showed two components; (1) faster moving yellow component assuming lipoic reductase-transacetylase-flavoprotein complex, and (2) slower moving colorless component assuming pyruvic decarboxylase (5.7 S). This pattern in analytical centrifuge indicated a possibility of the dissociation of pyruvate dehydrogenase complex into at least two components. As a preliminary experiment, separation of the two components was achieved by fractionation on calcium phosphate gel-cellulose in the presence of the ethanolamine-phosphate buffer. The column (3 x 2 cm) of calcium phosphate gel-cellulose was washed with the ethanolamine-phosphate buffer until the pH of the effluent was approximately 9.0-9.3. A solution of 26.8 mg of pyruvate dehydrogenase complex and 1.4 μm of thiamine-PP in 4 ml of ethanolamine-phosphate buffer (final 0.02 M ethanolamine and 0.025 M potassium phosphate) was applied to the column. The column was then washed with approximately 38 ml of the same buffer. A colorless protein fraction was eluted, leaving a broad yellow fluorescent band on the column. The latter band was eluted with a solution of 4% ammonium sulfate in 0.1 M potassium phosphate, pH 7.5. A colorless fraction was precipitated immediately, after coming off from the column, with ammonium sulfate between 0 and 50% saturation and dialyzed against 0.05 M phosphate buffer containing thiamine-PP (40 mg per 250 ml of this buffer). Yellow fraction was also precipitated with ammonium sulfate between 0 and 50% saturation and dialyzed against 0.05 M potassium phosphate buffer, pH 7. The recovery of the protein in the two fractions was 14 mg and 4 mg, respectively. First colorless fraction exhibited very weak pyruvate decarboxylase activity. Its activity seems to be destroyed during this process. The latter fraction exhibited dihydrolipoic transacetylase activity and also dihydrolipoic dehydrogenase activities. Especially the specific activity of yellow fraction was approximately three times that of the original complex in dihydrolipoic transacetylase assay. This yellow fraction was essentially homogeneous upon ultracentrifugation in a Beckman model E analytical centrifuge with the sedimentation coefficients ($S^{\circ}_{20,w}$) of 37 S. Its molecular weight was determined by Archibald method and it is calculated to be 4 million from these data. This yellow fraction was also separated into two enzymes, exhibiting a dihydrolipoic transacetylase and lipoamide dehydrogenase activities, respectively, under the same condition described by Koike et al. (4). The latter enzyme, lipoamide dehydrogenase showed homogeneous pattern in the analytical ultracentrifugation with $S^{\circ}_{20,w} = 5.7$ S. The molecular weight of this enzyme was determined by Archibald method and its value is calculated to be 123,000. This enzyme also contains two moles of FAD per mole of enzyme. Convenient separation procedure of the dihydrolipoic transacetylase is in progress. At present the main problem is how to retain the carboxylase activity during the course of ethanolamine fractionation of complex. The other hand the complex, which is free from flavoprotein and exhibites pyruvate decarboxylase and dihydrolipoic transacetylase activities, was subjected to the similar type of resolution. There is, however, no good resolution of this complex into two components.

Recently pig heart pyruvate dehydrogenase complex, 0.8 ml (13.44 mg) was mixed with 0.8 ml of 1 M potassium iodide in 0.05 M potassium phosphate buffer, pH 7.0 and this mixture was examined in analytical centrifuge. The following two components were observed in this run: (a) faster moving yellow component assuming lipoic reductase-transacetylase-flavoprotein complex, and (b) slower moving colorless component assuming pyruvate decarboxylase. One-half part of this mixture and the other part of mixture recovered from analytical run were dialyzed against three changes of 0.05 M potassium phosphate buffer, pH 7.0 for overnight. Dialyzed mixture was subjected to analytical centrifugation. From these runs it was observed that two separated components in the presence of 0.05 M potassium iodide at pH 7.0 were reconstituted to produce a large unit giving the same sedimentation velosity as before mentioned treatment. Both dialyzed pyruvate dehydrogenase complexes after treatment showed over 60% activity of original for pyruvate decarboxylase assay. This reagent might be hopeful one to reserve pyruvate decarboxylase activity when the complex is dissolved.

(2) α-Ketoglutarate dehydrogenase complex--There is so far, no available information and successful procedure for the resolution of α-ketoglutarate dehydrogenase complex. By Massey (25), α-ketoglutarate dehydrogenase complex was separated into a flavoprotein and colorless fractions with high molecular weight in the presence of 2.5 M urea on calcium phosphate gel-cellulose. However he could not retain α-ketoglutarate decarboxylase activity and could not reconstitute both components, either. With E. coli α-ketoglutarate dehydrogenase complex I have been atempting to dissolve it into the similar fractions like mammalian α-ketoglutarate dehydrogenase complex, but decarboxylase activity was destroyed during the course of this fractionation. Detergents, the changes of both ioning strength and pH etc., did not give any promising results for the separation of the complex. Resolution of E. coli α-ketoglutarate dehydrogenase complex was accomplished on a

column of calcium phosphate gel-suspended in cellulose by Mukherjee _et al._ (42). Similar procedure is adopted with a little modification for the resolution of mammalian α-ketoglutarate dehydrogenase complex, however, there is no light on its resolution experiment so far.

d. Electron microscopic and biochemical studies of the pyruvate and α-ketoglutarate dehydrogenase complexes, and its subunit

To confirm the structural organization of the complexes and its subunits electron microscope studies were introduced. _E. coli_ pyruvate dehydrogenase complex has provided a unique opportunity to correlate functional properties as revealed by biochemical analysis with ultrastructure as revealed by electron microscopy. The picture of the structural organization of the pyruvate dehydrogenase complex which emerged from the biochemical studies of Koike and Reed is that of an organized mosaic of enzymes in which the component enzyme is uniquely located to permit efficient implementation of a consecutive reaction sequence. This picture has been confirmed and extended by correlative electron microscope studies carried out in collaboration with Fernández-Morán (5).

In this laboratory original negative staining procedure of enzyme molecule is in progress to prove our multienzyme concept from macromolecular structural stand point of view. Moreover, through systematic application of improved preparation techniques, important structural details of individual multienzyme complexes could be directly observed, hereby disclosing novel feature of the molecular architecture.

(1) The principal preparation procedure of specimen can be classfied as followed: 1. Negative staining techniques with horse cytochrome C and yeast cytochrome C-mercury complex, and conventional negative staining procedure with buffered phosphotungstate (pH 7.5), 2. Positive staining technique with 1-3% aqueous uranyl acetate, 3. Shadowing with Pt-C or Pt-Pd.

(a) Negative staining methods: Ultrathin carbon film specimen grid is used for this procedure. Specimens are prepared by two methods; 1. microdroplet spraying techniques--1% cytochrome C solution previously ultracentrifuged for one hour in Spinco Mode L-2 or 1% buffered potassium phosphotungstate (pH 7.5) and varying concentrations of complexes solution or subunits solution are mixed in the spraying tube at 0° just before spraying. The tip of the capillaries of sprayer bended right angle is mounted on the holder in the horizontal position about one foot above the specimen grid level. The mixture is sprayed on the grid placed at 2-3 feet distance from the sprayer. After spraying the grid is dried under freezing in vacuo, 2. drop techique--at first the mixture of the complex and 1% buffered potassium phosphotungstate are placed on the carbon film grid with a capillary pipet. After removing excess of the mixture with filter paper the grid is allowed to be dried.

(b) Positive staining method: On the carbon film grid adequate concentration of complex is placed by spraying or dropping and is partially dried. 1-3% of aqueous uranyl acetate solution is dropped on it with a capillary pipet. After removing excess of staining reagent, the grid is allowed to be dried.

(c) Shadowing method [illegible] placing the complex on ultrathin formvar film grid, the grid is shadowed with Pt-C or Pt-Pd at varied angles by conventional procedure. Two sizes of polystyrene molecule are used for a control for this method.

(2) Results--Electron micrographs of complexes obtained with shadowing with Pt-Pd indicate that both complexes have a prolate ellipsoid structure with an average diameter 600 to 650 Å for pyruvate dehydrogenase complex and 350 to 400 Å for α-ketoglutarate dehydrogenase complex rather than polyhedral structure like E. coli pyruvate dehydrogenase complex (Fig. 7 and 8). Electron micrographs of pyruvate dehydrogenase complex negatively stained with potassium phosphotungstate and positively stained with uranyl acetate indicate a flower like shape with 10 to 20 petals, suspecting the possibility of resolution into subunits (Fig. 9 and 10). Electron micrographs of α-ketoglutarate dehydrogenase complex stained with potassium phosphotungstate and uranylacetate indicate tetrad structure (Fig. 11 and 12). There is no explanation of these electron micrographs of α-ketoglutarate dehydrogenase complex. The molecular weights of the complexes calculated with these diameter according to Scheraga's equation (S=M (1-V)/ Nf; V=volume, f=transitional coefficient, S=sedimentation coefficient) are very agreeable with those calculated with hydroparametric data.

There is no convincing evidence to support the polyhedral structure of E. coli pyruvate dehydrogenase complex shown in our earlier work. I suspect that the polyhedral structure of the complex, which were observed at low temperature with specimen negatively stained with buffered phosphotungstate solution by Fernández-Morán's cross-spraying procedure, seems to be artifact of strong deproteinizing reagent against highly purified giant protein molecule. Fernández-Morán stated that phosphotungstate did not chemically modify the structure of mitochondria in his report. Chemical composition of mitochondria is quite different from pure enzyme protein and we know that the over-all activity of complex irreversibly lost their activity immediately, after mixing with phosphotungstate or uranyl acetate. 1% buffered potassium phosphotungstate, which is one of the good deproteinized reagents, gives apparently unidentified changes of the shape of pyruvate and α-ketoglutarate dehydrogenase complexes. In this laboratory a cross-spraying apparatus devised by Fernández-Morán was set and the negative staining with potassium methyl-phosphotungstate is now in progress. As an internal control E. coli pyruvate and α-ketoglutarate dehydrogenase complexes and polystyrene, latex molecule which calibrated a diameter and obtained from Dow Chemical Co., U.S.A., are used for this purpose. In stead of shadowing with Pt-Pd, Pt-carbon will be introduced to preserve native shape under cooling. Electron microscope studies are in progress, and should shed further light on its structural organization.

4. Conclusion

a. A coenzyme A- and NAD-linked pyruvate and α-ketoglutarate dehydrogenase complexes have been isolated from pig heart as multienzyme units with molecular weights of approximately 9 million and 2.7 million, respectively. The pyruvate dehydrogenase complex containes approximately 67 moles of protein-bound lipoic acid and 17 moles of bound FAD. The ratio of bound lipoic acid to FAD in this complex is 5:1. The highly purified complex is free of thiamine-PP and the activity in the dismutation assay was restored by added thiamine-PP, Km=4.2 x

5. List of References

1. Koike, M., Reed, L. J., and Carroll, W. R., J. Biol. Chem., 235, 1924 (1960).
2. Koike, M., and Reed, L. J., J. Biol. Chem., 235, 1931 (1960).
3. Koike, M., Shah, P. C., and Reed, L. J., J. Biol. Chem., 235, 1939 (1960).
4. Koike, M., Reed, L. J., and Carroll, W. R., J. Biol. Chem., 238, 30 (1963).
5. Fernández-Morán, H., Reed, L. J., Koike, M., and Willms, C. R., Science, 145, 930 (1964).
6. Hayakawa, T., Muta, H., Hirashima, M., Ide, S., Okabe, K., and Koike, M., Biochem. Biophys. Res. Commun., 17, 51 (1964).
7. Korkes, S., del Campillo, A., Gunsalus, J. C., and Ochoa, S., J. Biol. Chem., 193, 721 (1951).
8. Ochoa, S., Mehler, A. H., and Kornberg, A., J. Biol. Chem., 174, 979 (1948).
9. Stadtman, E. R., and Barker, H. A., J. Biol. Chem., 180, 1085 (1949).
10. Stadtman, E. R., in Colowick, S. P., and Kaplan, N. O. (Editors), Methods in enzymology, New York, 1, 596 (1955).
11. Yagi, K., and Ozawa, T., Biochem. Biophys. Acta, 56, 413 (1962).
12. Negelein, E., and Brömel, H., Biochem. Z., 300, 225 (1939).
13. Layne, E., in Colowick, S. P., and Kaplan, N. O. (Editors), Methods in enzymology, New York, 3, 447 (1957).
14. Lowry, O. H., Rosenbrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem., 193, 265 (1951).
15. Price, V. E., and Greenfield, R. E., J. Biol. Chem., 209, 363 (1954).
16. Gunsalus, I. C., Dowlin, M. I., and Struglia, L., J. Biol. Chem., 194, 849 (1952).
17. Wagner, A. F., Walton, E., Boxer, G. E., Prus, M. P., Holly, F. W., and Folkers, K., J. Am. Chem. Soc., 78, 5079 (1956).
18. Beinert, H., and Page, E., J. Biol. Chem., 225, 479 (1957).
19. Huennekens, F. M., and Felton, S. P., in Colowick, S. P., and Kaplan, N. O. (Editors), Methods in enzymology, New York, 3, 950 (1957).
20. Kajiro, Y., J. Biochem., 44, 827 (1957).
21. Green, D. E., Herbert, D., and Subrahmanyan, V., J. Biol. Chem., 138, 327 (1941).
22. Raymond, S., and Wang, Y., Anal. Biochem., 1, 391 (1960).
23. Reed, L. J., Leach, F. R., and Koike, M., J. Biol. Chem., 232, 123 (1958).
24. Lipmann, F., and Tuttle, L. C., J. Biol. Chem., 159, 21 (1945).

25. Massey, V., Biochem. Biophys. Acta, 38, 447 (1960).

26. Hager, L. P., Thesis, University of Illinois (1953).

27. Knight, E. Jr., and Gunsalus, I. C., in Colowick, S. P., and Kaplan, N. O. (Editors), Methods in enzymology, New York, 5, 651 (1962).

28. Hager, L. P., J. Am. Chem. Soc., 79, 4865 (1957).

29. Sanadi, D. R., Littlefield, J. W., and Bock, R. M., J. Biol. Chem., 197, 851 (1952).

30. Schachman, H. K., in Colowick, S. P., and Kaplan, N. O. (Editors), Methods in enzymology, New York, 4, 32 (1957).

31. Ehrenberg, A., Acta Chem. Scand., 11, 1257 (1957).

32. Klainer, S. M., and Kegeles, G., J. Phys. Chem., 59, 952 (1955).

33. Svensson, H., and Thompson, T. E., in Alexander, P., and Block, R. J. (Editors), Analytical methods of protein chemistry, 3, 57 (1961).

34. Levitas, N. Robinson, J., Rosen, F., Huff, J. W., and Perlzweig, W. A., J. Biol. Chem., 167, 169 (1947).

35. Sato, T., J. Japan. Biochem. Soc., in Japanese, 35, 331 (1963).

36. Jagannathan, V., and Schweet, R. S., J. Biol. Chem., 196, 551 (1952).

37. Schweet, R. S., Katchman, B., Bock, R. M., and Jagannathan, V., J. Biol. Chem., 196, 563 (1952).

38. Korkes, S., del Campillo, A., and Ochoa, S., J. Biol. Chem., 195, 541 (1952).

39. Schweet, R. S., and Cheslock, K., J. Biol. Chem., 199, 749 (1952).

40. Goldman, D. S., Biochem. Biophys. Acta, 27, 506 (1958).

41. Linderstrom, E. S., J. Bacteriol., 65, 565 (1953).

42. Mukherjee, B. B., Matthews, J., Horney, D. L., and Reed, L. J., J. Biol. Chem., 240, PC2268 (1965).

Table I

Purification of Pig Heart Pyruvate Dehydrogenase Complex

Fraction	Total protein	Dismutation	Total units	De-car-boxyl-ase	Dihydro-lipoic trans-acetylase	Lipo-amide dehydro-genase	Bound lipoic acid
	g	μmoles/hr/mg protein	μmoles/hr	μmoles/hr/mg protein			μg/mg protein
Homogenate	4,020	0.35	1,420	0.04	0.22	-	-
Particle	219	1.2	263	0.15	1.3	-	-
Amber-colored extract	23	5.5	127	0.57	6.6	79.6	0.19
Protamine ppt. eluate	1.97	23.2	45	1.9	30.8	48.0	0.41
Pellet	0.94	47.2	44	4.3	85.0	104	1.38
$AmSO_4$ ppt. (0.29-0.36)	0.15	99.6	15	5.7	96.1	370	1.53
Colorless fraction	0.31	6.7	-	6.2	97.0	6.6	1.57

Table 2

Purification of Pig Heart α-Ketoglutarate Dehydrogenase Complex

Fraction	Total protein	De-carboxylase	Total units	De-hydrogenase	Lipoic trans-succinylase	Lipoamide dehydrogenase	Bound lipoic acid
	g	μmoles/hr/mg protein	μmoles/hr	μmoles/hr/mg protein			μg/mg protein
Homogenate	1,200	0.38	470	–	–	–	–
Particle	67	1.9	130	–	–	–	–
Amber-colored extract	7.1	4.7	33	35	2.5	80	0.19
Protamine ppt. eluate	1.5	10	16	89	6.3	160	0.24
Pellet	0.49	15	7.5	120	11	330	0.91
$AmSO_4$ ppt. (0.24-0.29)	0.12	26	3	200	16	480	0.77

Table 3

Hydrodynamic Parameter of
α-Keto Acid Dehydrogenase Complexes

	Pyruvate Dehydrogenase Complex		α-Ketoglutarate Dehydrogenase Complex	
	Pig heart	*E. coli*	Pig heart	*E. coli*
1. a. $S^{\circ}_{20,w}$	67.5	64.1	35.7	40
b. $D_{20,w}$	0.62	1.2	1.18	1.51
c. Molecular weight (x 10^6)	9.7	4.8	2.8	2.4
2. Molecular weight by Archibald method (x 10^6)	9.0	4.9	2.7	-

Table 4

Coenzyme Contents

of α-Keto Acid Dehydrogenase Complexes

	Pyruvate Dehydrogenase Complex		α-Ketoglutarate Dehydrogenase Complex	
	Pig heart	*E. coli*	Pig heart	*E. coli*
	moles/mole of enzyme		moles/mole of enzyme	
Lipoic acid	67	54	10	10
FAD	14	17	9	10
Thiamine-PP	0 ($K_m = 4.2 \times 10^{-6}$M)	-	6	10

Table 5. Effects of Divalent Metal Ions on the Oxidative Decarboxylation Activities

	pig heart PDC			*E. coli* PDC	pig heart KGDC	
cations (.3 μmole)	decarboxylase	CO_2 evolution	dismutation	decarboxylase	decarboxylase	CO_2 evolution
None	100	100	100	100	100	100
Mg^{++}	144	142	101	119	104	101
Ca^{++}	179	139	98	108	138	128
Mg^{++}(.15 μmole) Ca^{++}(.15 μmole)	177	-	-	-	-	-
Mn^{++}	152	-	98	79	88	-
Mn^{++} (1 mole)	68	-	-	0	-	-
Co^{++}	87	-	99	92	52	-
Cu^{++}	39	-	16	71	45	-
Zn^{++}	43	-	95	81	62	-
Hg^{++}	54	-	56	-	32	-

PDC: Pyruvate Dehydrogenase Complex
KGDC: α-Ketoglutarate Dehydrogenase Complex

Table 6. Effects of Several Chelating Agents on the Oxidative Decarboxylation Activities

ligands	µmoles	pig heart PDC carbox-ylase	pig heart PDC dismu-tation	*E. coli* PDC carbox-ylase	pig heart KGDC carbox-ylase
None		100	100	100	100
EDTA	.1	0	74	36	-
	.5	-	46	-	-
	1	-	27	0	96
	5	-	9	-	88
	10	-	-	-	83
8-hydroxy-quinoline	1	69	-	75	0
O-phenan-throline	1	100	-	96	93
	5	91	85	85	55

PDC: Pyruvate Dehydrogenase Complex
KGDC: α-Ketoglutarate Dehydrogenase Complex

Table 7. Reactivation of Pyruvate Carboxylase Activity Inhibited by EDTA*, by the Addition of Mg^{++} and Ca^{++}

Cations	µmoles of cations				
	0	0.5	1.0	1.5	2.0
			%**		
Mg^{++}	0	65	86	95	100
Ca^{++}	0	38	88	98	100

* Reaction mixtures were preincubated in the presence of EDTA at 0° for 10 min. before addition of each metal ion.

** Represents the ratio of $\frac{\text{activity with EDTA (1 µmole)}}{\text{activity without EDTA}} \times 100$

Table 8. Metal Contents in α-Keto Acid Dehydrogenase Complexes[a]

Metals / Fraction	Mg	Ca	Mn	Fe	Cu	Zn
Pig heart Amber-colored ext,	2.2	1.6	b	2.3	0.07	0.73
PDC Protamine ppt.	0.54	0.56	b	1.4	b	0.24
Pellet	0.71	0.74	b	4.6	b	0.23
AmSO ppt. (.29-.36)	0.98	1.6	b	3.1	b	b
KGDC Protamine ppt.	0.69	1.1	b	1.5	0.07	0.18
Before column	0.52	0.97	b	1.3	0.06	0.24
AmSO ppt. (.24-.29)	1.7	1.7	b	0.21	b	b
E. coli PDC	0.48	b	-	-	-	-

a; Metal contents are in mole (x 10^{-9})/mg·protein.
b; Not detected.
PDC; Pyruvate Dehydrogenase Complex.
KGDC; α-Ketoglutarate Dehydrogenase Complex.

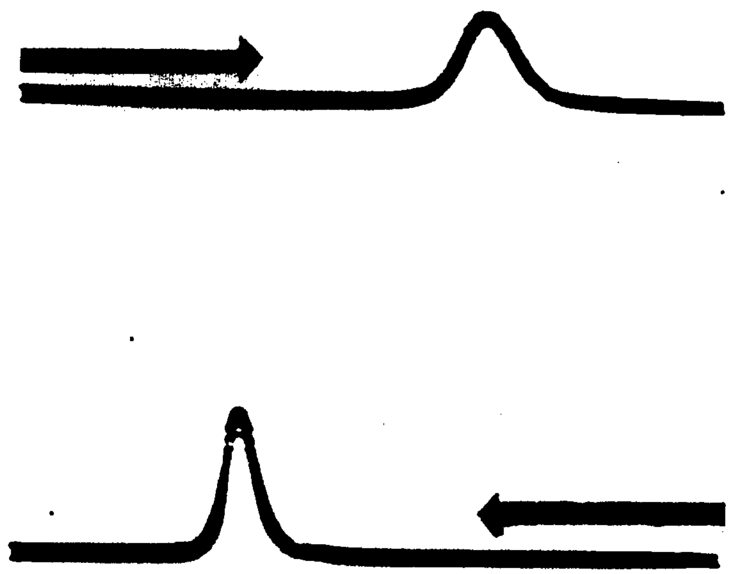


Fig. 1. Electrophoretic schlieren pattern, descending (top) and ascending (bottom), obtained with a preparation of the pyruvate dehydrogenase complex, 13.3 mg per ml of 0.05 M potassium phosphate buffer, pH 7.0, after 182 minutes (descending) and 180 minutes (ascending) at 4.6 volt cm^{-1}.

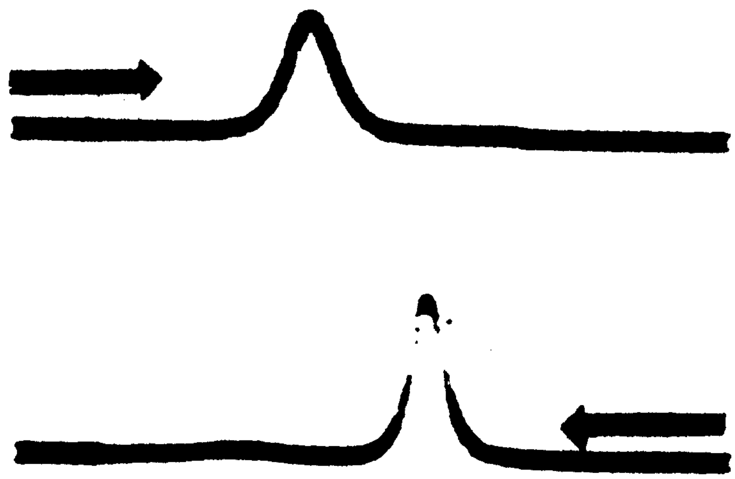


Fig. 2. Electrophoretic schlieren pattern, descending (top) and ascending (bottom), obtained with a preparation of the α-ketoglutarate dehydrogenase complex, 1.32 mg per ml of 0.05 M potassium phosphate buffer, pH 7.0, after 60 minutes (descending) and 61 minutes (ascending) at 4.6 volt cm^{-1}.

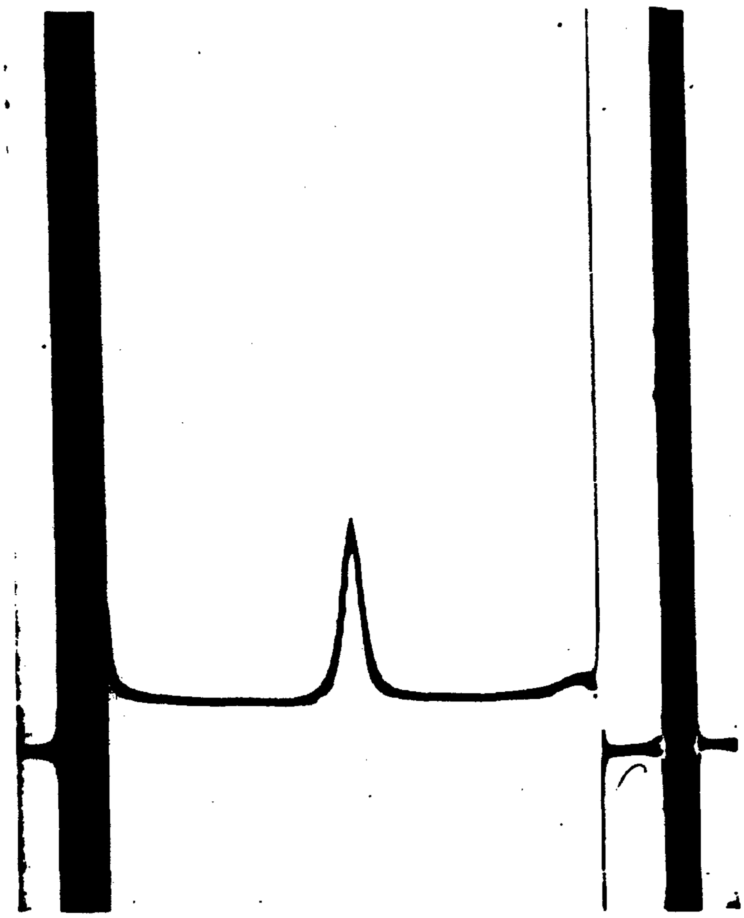


Fig. 3. Ultracentrifuge schlieren pattern obtained with the pyruvate dehydrogenase complex, 4.19 mg per ml of 0.05 M potassium phosphate buffer, pH 7.0, after 24 minutes at 35,600 rpm, bar angle, 60°.

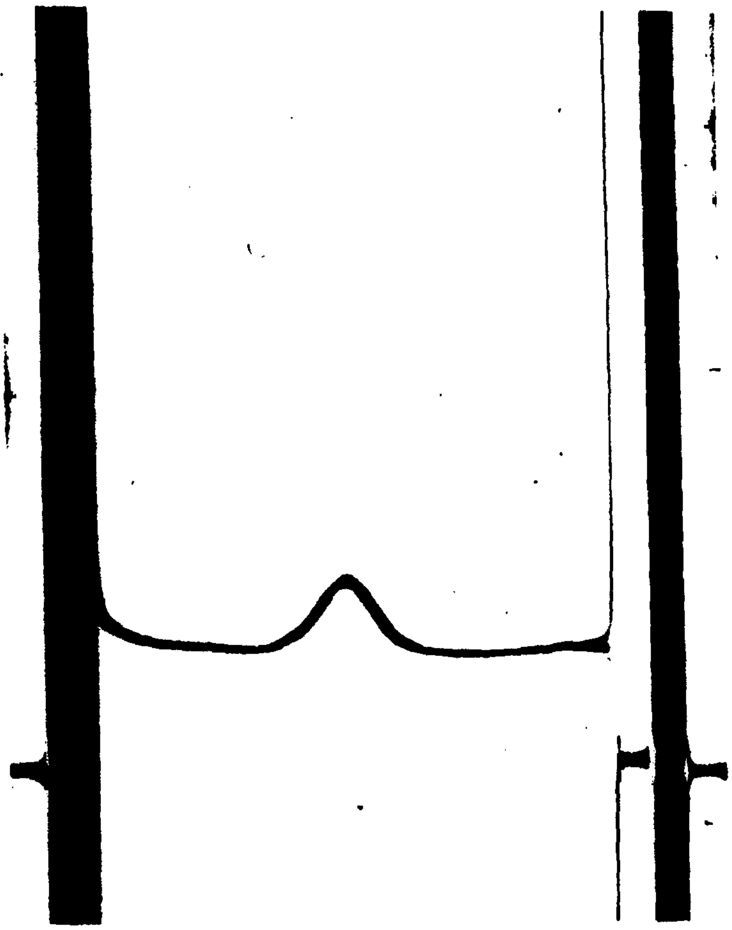
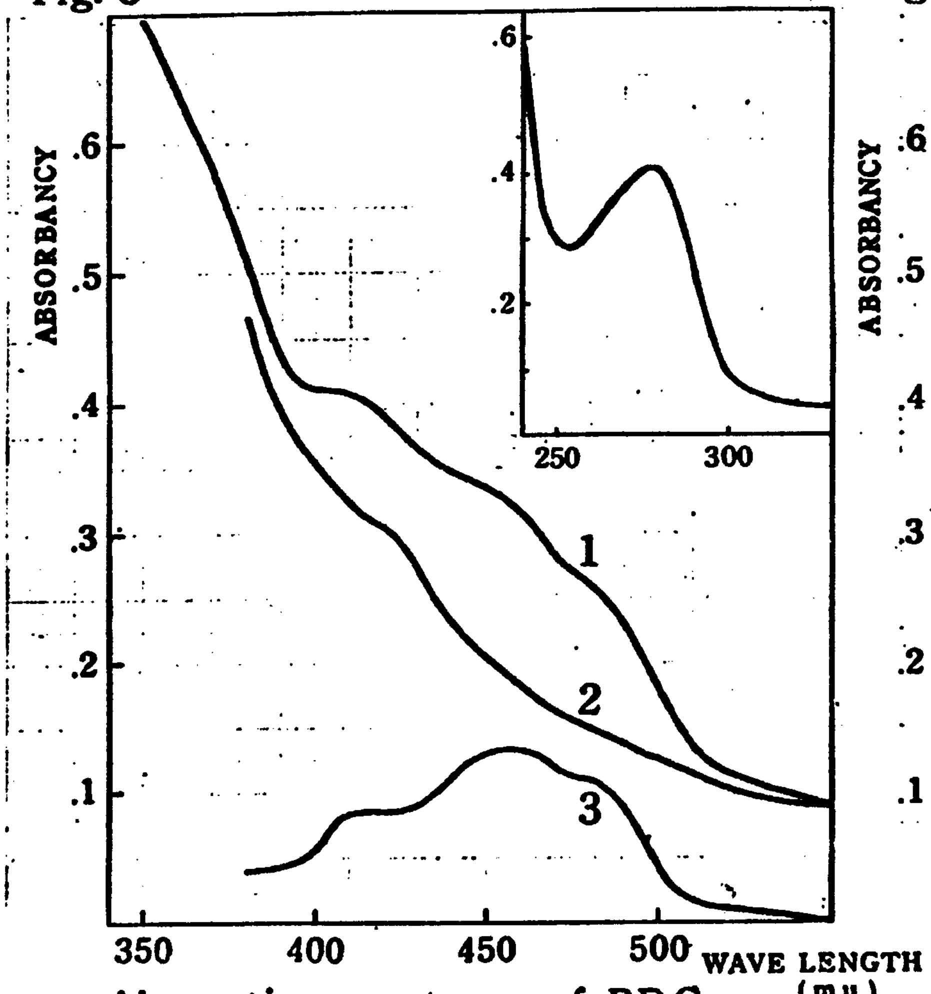


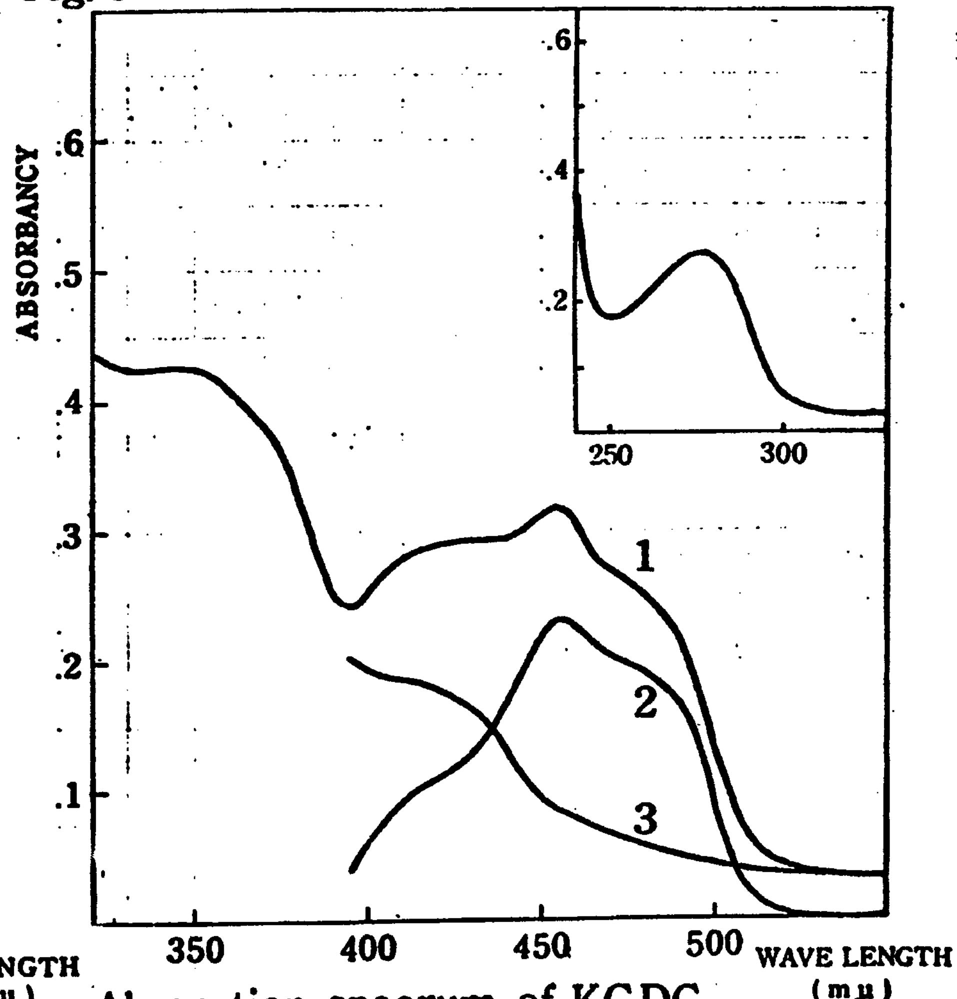
Fig. 4. Ultracentrifuge schlieren pattern obtained with the α-ketoglutarate dehydrogenase complex, 4.8 mg per ml of 0.05 M potassium phosphate buffer, pH 7.0, after 27 minutes at 47,660 rpm, bar angle 55°.

Fig. 5



Absorption spectrum of PDC.
16.4 mg protein/2 ml 1, oxidized form; 2, after addition of dithionite; 3, differens spectrum

Fig. 6



Absorption specrum of KGDC.
11.1 mg protein/2 ml 1, oxidized form; 2, after addition of dithionite; 3, differens spectrum

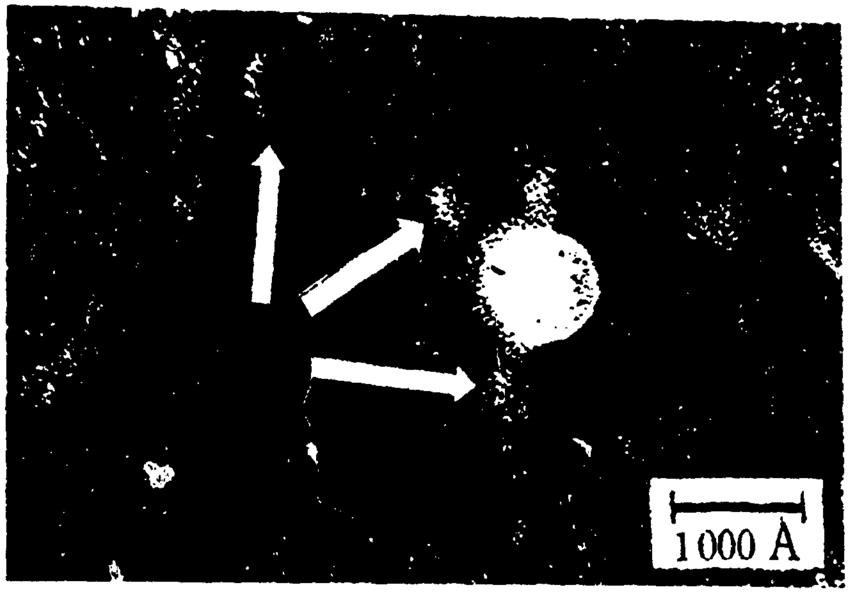


Fig. 7. Electron micrograph of the pyruvate dehydrogenase complex shadowcasted with Pt-Pd. (x 180,000)

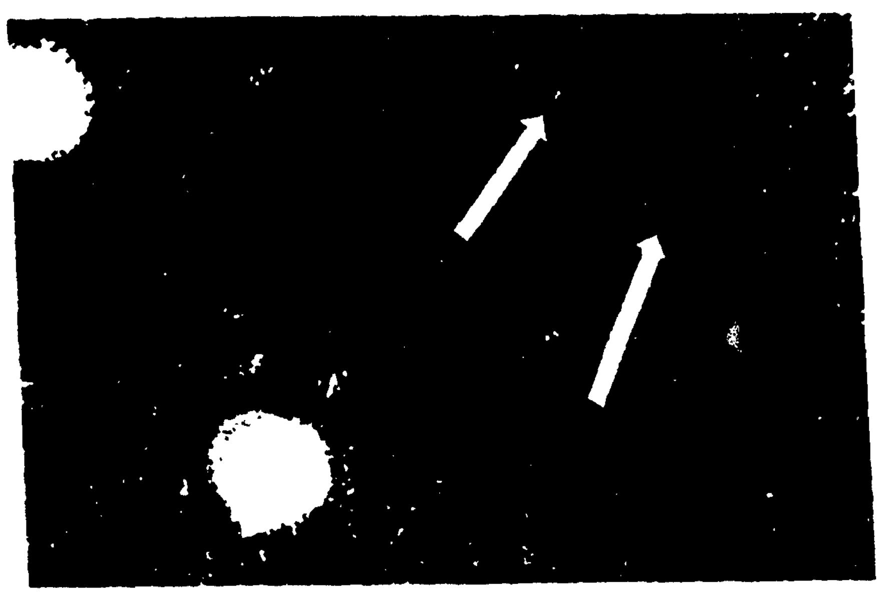


Fig. 8. Electron micrograph of the α-ketoglutarate dehydrogenase complex shadowcasted with Pt-Pd. (x 180,000)

Fig. 9. Electron micrograph of the pyruvate dehydrogenase complex positively stained with uranyl acetate. (x 300,000)

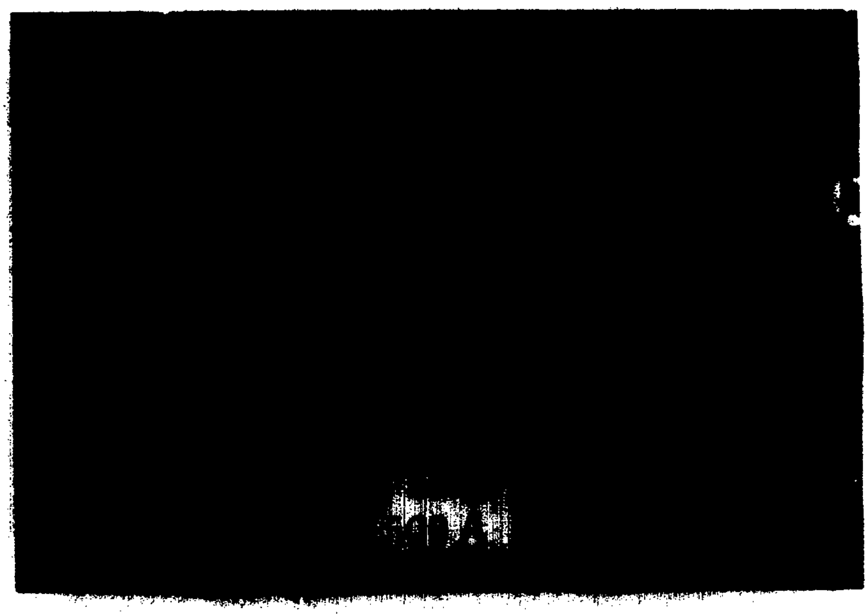


Fig. 10. Electron micrograph of the pyruvate dehydrogenase complex negatively stained with potassium phosphotungstate (pH 7.5). (x 300,000)

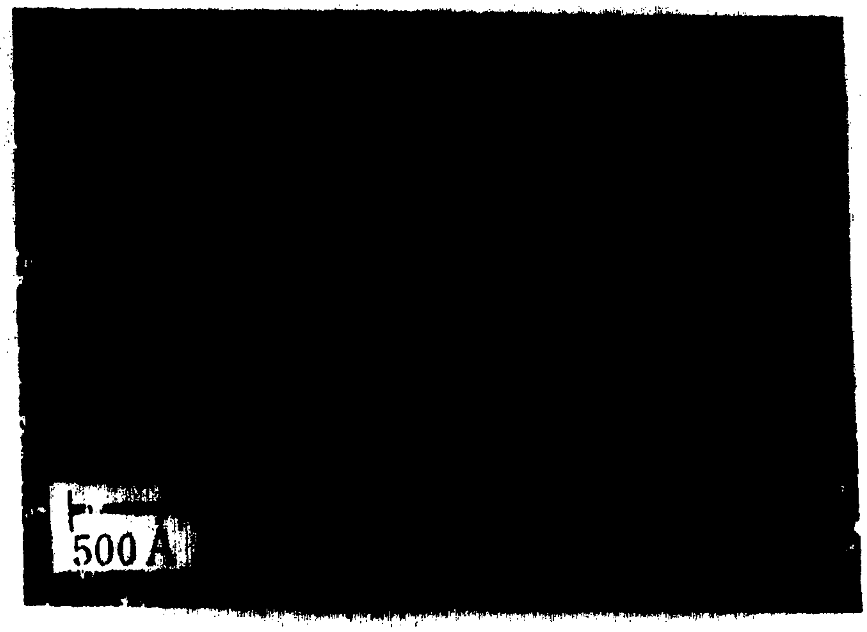


Fig. 11. Electron micrograph of the α-ketoglutarate dehydrogenase complex stained with potassium phosphotungstate (pH 7.5). (x 300,000)

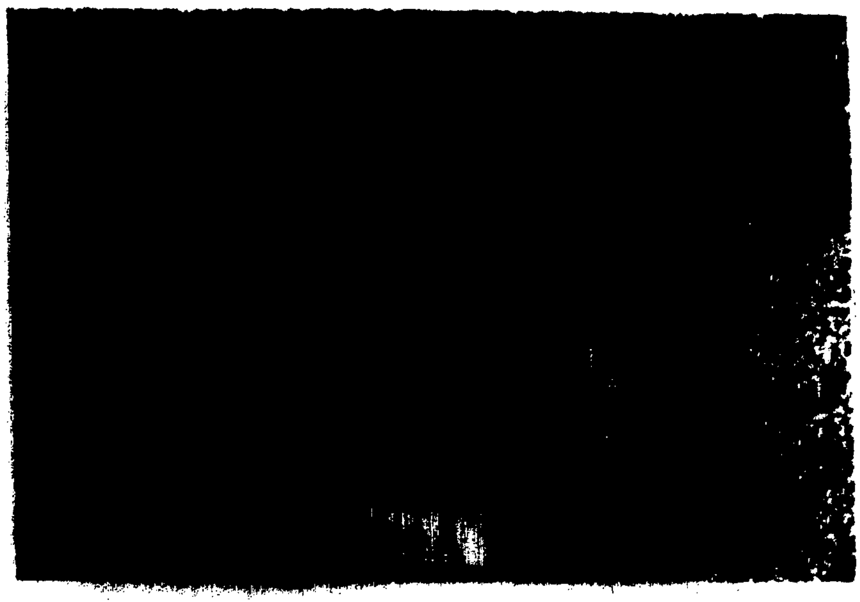


Fig. 12. Electron micrograph of the α-ketoglutarate dehydrogenase complex stained with uranyl acetate. (x 300,000)

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1. ORIGINATING ACTIVITY (Corporate author) Atomic Disease Institute School of Medicine, Nagasaki Univ. Nagasaki, Japan	2a. REPORT SECURITY CLASSIFICATION Unclassified 2b. GROUP

3. REPORT TITLE

STUDIES ON MAMMALIAN AND HUMAN PYRUVATE AND α-KETOGLUTARATE DEHYDROGENATION COMPLEXES (U)

4. DESCRIPTIVE NOTES (Type of report and inclusive dates)

Final report, 15 March 1965 - 14 March 1966

5. AUTHOR(S) (Last name, first name, initial)

Koike, Masahiko

6. REPORT DATE	7a. TOTAL NO. OF PAGES	7b. NO. OF REFS
2 May 1966	31	42

8a. CONTRACT OR GRANT NO. DA-92-557-FEC-37961 b. PROJECT NO. 2N014501B71D c. Task 00 028FE d.	9a. ORIGINATOR'S REPORT NUMBER(S) J-232 9b. OTHER REPORT NO(S) (Any other numbers that may be assigned this report)

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13. ABSTRACT

1. A coenzyme A- and NAD-linked pyruvate and α-ketoglutarate dehydrogenase complexes have been isolated from pig heart muscle as multienzyme units with molecular weights of approximately 9 and 2.7 million, respectively. Pyruvate dehydrogenase complex contains approximately 67 moles of protein-bound lipoic acid and 17 moles of bound FAD. α-Ketoglutarate dehydrogenase complex contains approximately 10 moles of protein-bound lipoic acid, 9 moles of FAD and 6 moles of thiamine-PP.

2. Both complexes were activated by Ca^{2+} as the same extent as Mg^{2+} which had been considered as one of the typical metal activators of oxidative decarboxylation reaction of α-keto acid. These activating effects were in good agreement with the results of the metal contents obtained by the atomic absorption analysis. Pyruvate dehydrogenase complex was strongly inhibited by EDTA at low concentration, but on the contrary α-ketoglutarate dehydrogenase complex was little inhibited by EDTA and rather obviously inhibited by 8-hydroxyquinoline.

3. Attempts have been made to dissolve both complexes into three essential components containing each coenzyme, but it did not still go well to restore pyruvate decarboxylase activity. Two other components; lipoic reductase-transacetylase and lipoamide dehydrogenase were isolated in pure status.

4. Preliminary experiment to determine the structural organization of the mammalian pyruvate and α-ketoglutarate dehydrogenase complexes have been made and hopeful results were obtained. (Author)

DD FORM 1 JAN 64 1473

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14. KEY WORDS	LINK A		LINK B		LINK C	
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13. **ABSTRACT:** Enter an abstract giving a brief and factual summary of the document indicative of the report, even though it may also appear elsewhere in the body of the technical report. If additional space is required, a continuation sheet shall be attached.

It is highly desirable that the abstract of classified reports be unclassified. Each paragraph of the abstract shall end with an indication of the military security classification of the information in the paragraph, represented as *(TS), (S), (C)*, or *(U)*.

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