Relationship between Phosphorylation and Activity of Pyruvate Dehydrogenase in Rat Liver Mitochondria and the Absence of Such a Relationship for Pyruvate Carboxylase*

(Received for publication, November 14, 1977)

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Linn et al. (Linn, T. C., Pettit, F. H., and Reed, L. J. (1969) Proc. Natl. Acad. Sci. U. S. A. 62, 234–241) have shown that purified preparations of pyruvate dehydrogenase complex can be inactivated by phosphorylation and reactivated by dephosphorylation. The present study shows that these processes also take place in rat liver mitochondria. In addition, we have compared such changes with possible parallel phosphorylation of pyruvate carboxylase. Mitochondria were incubated with $^{32}$P, the pyruvate dehydrogenase and pyruvate carboxylase were isolated by immunoprecipitation and sodium dodecyl sulfate-acrylamide gel electrophoresis, and the $^{32}$P content was determined. Incubation of mitochondria with fluoride, ioniophore A23187, and ethylene glycol bis(β-aminoethyl ether) $N,N'$-tetraacetic acid to inhibit pyruvate dehydrogenase phosphatase led to greater than 97% inactivation of the complex and incorporation of up to 2.0 mol of phosphate per mol of pyruvate dehydrogenase. When mitochondria were incubated with dichloroacetate to inhibit pyruvate dehydrogenase kinase, maximal activity of the complex was observed and less than 0.01 mol of phosphate was incorporated per mol of enzyme. Under the same conditions, neither treatment resulted in significant changes in the maximal catalytic activity of pyruvate carboxylase nor was there any phosphorylation of the enzyme. We conclude that phosphorylation plays a major role in the control of pyruvate dehydrogenase complex in liver mitochondria but that this mechanism is probably not a factor in pyruvate carboxylase regulation.

* This work was supported in part by Grants AM 12245 and GM 00685 of the National Institutes of Health. A preliminary report of this work has appeared previously: Leiter, A., Weinberg, M., Utter, M. F., and Linn, T. C. (1975) Fed. Proc. 35, 1412. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

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ase complex and pyruvate carboxylase as pointed out by Reed (16). Several reports have noted that gluconic acid formation from pyruvate is consistent with activation of pyruvate carboxylase (17). Studies of changes in levels of intermediary metabolites following gluconic acid perturbation indicate a "crossover" between pyruvate and oxaloacetate. It also has been reported that gluconic acid treatment of hepatocytes leads to increased rates of CO₂ fixation by mitochondria although the maximal level of pyruvate carboxylase activity is unaffected (19). The foregoing studies are consistent with possibility that pyruvate carboxylase can be phosphorylated although other interpretations are possible. For example, the reported phosphorylation and inactivation undergone by pyruvate kinase (21) could also explain the effect of gluconic acid.

**EXPERIMENTAL PROCEDURES**

**Materials**

Pyruvate carboxylase was purified from chicken liver and stored as described by Leib et al. (23) and was stored lyophilized at -70°. Each of these two enzymes was judged to be at least 90 to 95% pure according to electrophoretic analysis on SDS-acrylamide gels. All measurements of protein were made using a standard protein procedure. Firefly lantern extracts were obtained from Sigma, Isoporphosphate SD-187 was a generous gift of Dr. Robert Hamill of Eli Lilly and Co., Indianapolis, Ind. It was dissolved in 25% dimethylformamide, 75% ethanol. All reagents were of the highest purity available. All enzymes were obtained from Boehringer Mannheim Co. unless noted otherwise.

**Methods**

**Preparation of Mitochondria** - Rat liver mitochondria were prepared by homogenizing livers from male Sprague-Dawley rats (200 to 300 g body weight) in 9 volumes of buffer at 4° (mM): 0.2 M sucrose, 0.07 M Mops, 0.005 M EGTA, 0.0005 M defatted albumin, 0.5 mM MgCl₂, pH 7.4. The homogenate was centrifuged twice at 10,000 × g for 10 min and the combined supernatant solutions centrifuged at 9000 × g for 10 min to give a mitochondrial pellet. The pellet was washed twice with the homogenizing buffer (EGTA omitted) and resuspended to a final protein concentration of about 100 mg/ml.

In the experiments to be presented mitochondria were incubated under specified conditions, rapidly centrifuged, and the resultant pellets were quick frozen in a dry ice-ethanol bath. Details are given in legends to Figs. 2 and 3. Mitochondrial extracts for enzyme assays and isolations were prepared from the frozen pellets as described in the text or appropriate figure legends.

**Assay of Pyruvate Dehydrogenase and Pyruvate Carboxylase** - Pyruvate dehydrogenase activity was assayed as follows. Assay mix (0.2 ml) containing potassium phosphate, 50 mM, pH 8.0; MgCl₂, 2 mM; EDTA, 2 mM; thiamin pyrophosphate, 0.1 mM; NAD, 2.5 mM; dithiothreitol, 1 mM; CoASH, 0.5 mM; lactic dehydrogenase, 10 μg/ml; and phosphotransacetylase, 1 μg/ml was added to vented reaction vessels made by shortening test tubes (10 × 75 mm) and cutting out vents. These were suspended from rubber stoppers which were then used to cover snap cap scintillation vials containing 0.2 ml hydroxide of hyamine. The stopper was closed over the vial which was then placed in a New Brunswick "Aquatherm" water bath at 90° with continuous gentle gyration shaking. All further additions to the reaction vessels were made through the septum of the stopper with Hamilton syringes fitted with PB-600 repeating dispensers. Mitochondrial extracts were prepared by thawing frozen mitochondria in a 20° water bath followed by suspension in ice cold 20 mM sodium phosphate, pH 7.0, to obtain a protein concentration of 10 to 20 mg/ml. Within 3 min after completion of this process, 10 μl of the suspension were added to each of three to five vials. Two minutes later the reaction was started by addition of 11-12 "Ci"pyruvate (Amersham/Searle) to a concentration of about 0.5 mM (specific activity, 150 to 450cpm/nmol). The assay was terminated by the action of 50 μl of 2 N HCl containing 0.1% 2,4-dinitrophenylhydrazine. The assay was linear with respect to the amount of protein added with time up to 30 min and was proportional to the fraction of up to 70% of the added pyruvate. After an additional 45 min of shaking to allow complete diffusion of CO₂ to the hyamine, the serum stopper was removed and 10 ml of tolune-based scintillant (5,5-diphenyloxazoloxo (PPO) 0.3 g/liter, 1,4-bis[2-(4-methyl-5-phenyl benzene)loxazoloxo] 0.3 g/liter) was added. The 14C-containing material was counted in a Packard Tri-Carb liquid scintillation spectrometer. Blank assays used extracts in which pyruvate dehydrogenase had been inactivated by incubation of mitochondria with fluoride. Coenzyme A was also omitted from these control assays. Pyruvate carboxylase activity was assayed as described by Scrutton and White (24) or Scrutton et al. (25).

**Preparation of Intramitochondrial Perchloric Acid Extracts for Analysis of Mitochondrial ATP** Intramitochondrial perchloric acid extracts were prepared by placing 0.1 ml of mitochondrial suspension over the following layers: 0.1 ml of incubation medium containing fluoride (part Wacker, Siliconol AR150), 0.1 ml of perchloric acid; all in a 500-ml Eppendorf microcentrifuge tube. The tube was centrifuged for 20 s at top speed in an Eppendorf centrifuge, the upper layers were removed, and the acid supernatant solution was neutralized with potassium carbonate. The potassium perchlorate was removed by centrifugation.

**Immunoochemical Techniques** - Antibodies were prepared by immunizing goats with five or six subcutaneous injections of 5 mg each of highly purified preparations of either beef kidney pyruvate dehydrogenase or chicken liver pyruvate carboxylase emulsified in Freund's adjuvant. The IgG fractions of the respective antisera were purified by precipitation with 40% saturated ammonium sulfate followed by chromatography on DEAE-cellulose (27). Each antibody was capable of neutralizing completely the catalytic activity of its antigen or the respective rat liver mitochondrial counterparts. However, 4 to 6 times as much anti-pyruvate carboxylase was required to inhibit the rat liver enzyme compared with the chicken liver enzyme.

**Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis** - SDS gel electrophoresis was performed essentially as described by Weber et al. (28). Pyruvate carboxylase immunoprecipitates were run on 100-mm gels with 5% acrylamide while pyruvate dehydrogenase immunoprecipitates were run on gels containing 7.5% or 8.0% acrylamide. In any given experiment gels were stained in Coomassie brilliant blue R-250 for a uniform period of time (4 to 12 h). Gels were destained by soaking in several changes of 33% methanol, 10% acetic acid solution. Destaining was discontinued as soon as the background on the gels was clear. The gels were then placed in 1% acetic acid overnight. During the course of these experiments, variability in the intensity of Coomassie blue staining of similar gels was occasionally observed. For this reason, triplicate gels were always run to give a more accurate estimate of the amount of protein present. This variability could be reduced by presoaking the gels in several changes of 10% trichloroacetic acid prior to protein staining.

To determine the distribution of 32P in the isolated polypeptides, duplicate gels were run initially, one was stained for protein and the other was sliced into 2-mm sections which were processed as follows for 32P analysis. Each slice was incubated in 0.5 ml of a 91 NCS reagent (Amersham/Searle):H₂O mixture for 2 h at 50°. After cooling, 4 ml of tolune-based scintillant (24 g of 2,5-diphenyloxazoloxo, 0.5 g of 1,4-bis[2-(5-phenyloxazoloxo) benzene] per gallon) was added and the 32P was determined in a Packard scintillation counter. Following weighings were done as described previously (29). In later experiments, the stained polypeptide bands corresponding to either the α chain of pyruvate dehydrogenase or to the polypeptide of pyruvate carboxylase were cut out and digested for 4 h at 50° in 0.2 ml of 30% H₂O, under complete digestion of the gels, 3.5 ml of Acrosol (New England Nuclear Corp.) was added and the 32P was counted in the integral mode, gain setting 25%, on a Packard Tri-Carb scintillation counter. Similarly treated portions of the same gel not containing any protein served as blanks.
RESULTS

**Demonstration of Phosphate Incorporation into Pyruvate Dehydrogenase** — Three requirements for demonstrating whether pyruvate carboxylase and pyruvate dehydrogenase undergo parallel simultaneous phosphorylations in intact mitochondria are: 1) the mitochondria must be treated so as to place pyruvate dehydrogenase in the phosphorylated-inactive forms; 2) the phosphorylated proteins must be isolated in the small amounts that occur in mitochondria; 3) the identity of the isolated phosphorylated proteins must be established.

In the experiments about to be described, mitochondria were incubated with $^{32}$P. After the equilibration with the intramitochondrial adenine nucleotides had been established, either dichloroacetate or fluoride was added. Incubation with dichloroacetate, as is shown in Fig. 1, an inhibitor of pyruvate dehydrogenase kinase (29), leads to activation of the complex. On the other hand, incubation with fluoride (6), an inhibitor of pyruvate dehydrogenase phosphatase, leads to inactivation. Following extraction from mitochondria by freezing and thawing, the pyruvate dehydrogeenase complex was isolated by immunoprecipitation. The precipitates were dissolved in SDS and subjected to acrylamide gel electrophoresis. Maximal precipitation of the complex was determined by the amount of pyruvate dehydrogeenase (α and β chains) present in SDS-acrylamide gels similar to the ones presented in Fig. 3. A typical resultant precipitin curve is shown in Fig. 2. It was calculated that between 70% and 100% of the expected pyruvate dehydrogenase was recovered in these precipitates under optimal conditions. This calculation is based on an activity of 0.025 unit of activated pyruvate dehydrogenase complex per mg of mitochondrial protein at 30° as observed by us. The calculation also assumes that the purified liver enzyme has a specific activity of 15 units/mg and that like the kidney complex, the liver enzyme is approximately 45% pyruvate dehydrogenase (30).

Analysis by SDS-gel electrophoresis of a precipitate from fluoride-treated mitochondria is shown in Fig. 3. The upper trace is a spectrophotometric scan of a gel stained for protein. The lower trace shows the distribution of $^{32}$P in 0.5-mm slices of a duplicate gel. Only one peak of $^{32}$P is observed which appears to be associated with a single polypeptide even though five protein bands are observed in all. Comparing the mobility of this band with mobilities of similarly treated polypeptides of known size, the molecular weight was estimated to be 40,500. Since Reed and associates (3) have shown that only the 41,000 molecular weight α chain of purified pyruvate dehydrogenase is phosphorylated, we conclude that the observed radioactivity is associated with that protein. The other polypeptides seen in Fig. 3 can also be tentatively identified on the basis of their estimated molecular weights (from right to left: light chain, γ-globulin, 25,000; β chain, pyruvate dehydrogenase, 35,500; α chain, pyruvate dehydrogenase, 40,500; heavy chain, γ-globulin, 50,000; and dihydrolipoyl transacetylase, 62,000). This mobility of the transacetylase component agrees with our observations on purified beef kidney complex. The dihydrolipoyl dehydrogenase component (M, 53,000) is not seen as a separate peak because of the large amount of γ-globulin heavy chain present. The sum of the areas of the peaks on spectrophotometric gel scans of the α and β chains. The results are normalized and expressed as constant amount of extract incubated with increasing amounts of antibody.

To determine whether the $^{32}$P associated with the 41,000 molecular weight polypeptide is covalently attached, two identical immunoprecipitations were carried out with extracts from fluoride-treated mitochondria. After washing, one precipitate was dissociated with SDS as described in the legends to Figs. 2 and 3 while the other was resuspended in 5% trichloroacetic acid. Following centrifugation, the precipitate was dissolved in SDS. After gel electrophoresis, the radioactivity in the α chain of pyruvate dehydrogenase was determined for both experiments. The same amount of radioactivity was associated with the α chain of pyruvate dehydrogenase with or without acid precipitation, strongly suggesting that the $^{32}$P is covalently bound to the protein.

Treatment of mitochondria with dichloroacetate, an inhibitor of pyruvate dehydrogenase kinase (29) leads to activation of the complex (see Table II). As shown in Fig. 4, enzyme that is isolated by immunochromic means from these mitochondria contains very little $^{32}$P as compared with equivalent amounts of material obtained from the fluoride-treated mitochondria.

**Test for Phosphorylation of Pyruvate Carboxylase** — The optimal amount of antibody necessary to precipitate pyruvate carboxylase from mitochondrial extracts was determined by...
incubated for 1 h at 30° and 12 h at 4° with about 3.3 mg of the IgG described in the legend to Fig. 2. One milliliter of extract was
were quick-frozen at -78° in dry ice-ethanol bath and were stored at
chilled to 0° in an ice water bath for 3 min. Aliquots of 1.4 ml were
centrifuged for 10 to 15 s in an Eppendorf model 3200 centrifuge.
Then the tubes were immediately placed in an ice water bath, the
supernatant solution was removed, and the mitochondrial pellets
were incubated at 30° for 1 h and at 4° for 24 h. The precipitates were
washed three times as described in Fig. 2 and were dissolved in 100
~1 of 2% SDS-mercaptoethanol for 10 min at 100°. Each of these
dissociated precipitates were divided into three portions and run on
three gels containing 5% acrylamide. The amount of pyruvate
carboxylase was estimated from the area of its peak on spectropho-
tometric gel scans relative to peaks produced by known amounts of
purified chicken liver pyruvate carboxylase. Other experimental
details are as described in the text and earlier figure legends. The
upper panel of this figure is a spectrophotometric gel scan showing
the distribution of protein in a gel stained with Coomassie brilliant
blue. The lower panel shows the distribution of 32P in 2 mM slices of
a duplicate gel superimposed.

FIG. 3. Distribution of 32P and protein in 8% SDS-acrylamide gels
of pyruvate dehydrogenase immunoprecipitates. Mitochondria were
added (4 to 6 mg of protein per ml) to KCl medium (80 mM KCl, 50
mm Mops, 2 mM MgCl2, 0.5 mM EDTA, defatted bovine serum albumin, 1 mg/ml, [32P]potassium phosphate, 0.2 mM; specific activity
about 2 x 106 cpm/mmol, pH 7.4). Following a 1/2 to 2-min preincubation to permit equilibration of 32P with mitochondrial
adenine nucleotides, sodium fluoride (20 mM) was added to inhibit
pyruvate dehydrogenase phosphatase. After 6 min, the flasks were
chilled to 0° in an ice water bath until use, usually within 24 h. Extracts were prepared as described in the legend to Fig. 2. One milliliter of extract was
incubated for 1 h at 30° and 12 h at 4° with about 3.3 mg of the IgG fraction of the goat antiserum. These tubes
were incubated at 30° for 1 h and at 4° for 24 h. The precipitates were
washed three times as described in Fig. 2 and were dissolved in 100
µl of 2% SDS-mercaptoethanol for 10 min at 100°. Each of these
dissociated precipitates were divided into three portions and run on
three gels containing 5% acrylamide. The amount of pyruvate
carboxylase was estimated from the area of its peak on spectropho-
tometric gel scans relative to peaks produced by known amounts of
purified chicken liver pyruvate carboxylase. Other experimental
details are as described in the text and earlier figure legends. The
results are normalized and expressed as constant amount of extract
incubated with increasing amounts of antibody.

FIG. 5. Precipitation of rat liver pyruvate carboxylase from mi-
ochondrial extracts with goat antibody to chicken liver pyruvate
carboxylase. Mitochondrial extracts were prepared as described in
Figs. 2 and 3. The protein concentration was about 20 mg/ml before
centrifugation. Between 45 and 100 µl of the clear extract was added
to about 2 mg of IgG fraction of the goat antiserum. These tubes
were incubated at 30° for 1 h and at 4° for 24 h. The precipitates were
washed three times as described in Fig. 2 and were dissolved in 100
µl of 2% SDS-mercaptoethanol for 10 min at 100°. Each of these
dissociated precipitates were divided into three portions and run on
three gels containing 5% acrylamide. The amount of pyruvate
carboxylase was estimated from the area of its peak on spectropho-
tometric gel scans relative to peaks produced by known amounts of
purified chicken liver pyruvate carboxylase. Other experimental
details are as described in the text and earlier figure legends. The
results are normalized and expressed as constant amount of extract
incubated with increasing amounts of antibody.

SDS-gel electrophoretic analyses of the immunoprecipitates. The precipitin curve for rat liver mitochondrial pyruvate
carboxylase with goat antibody to the chicken liver enzyme is shown in Fig. 5. Up to 100% of the expected pyruvate carboxy-
lase is present in the precipitates in the linear portion of the
curve. This calculation assumes 0.16 unit of pyruvate carboxy-

FIG. 6. Distribution of 32P and protein in a 5% acrylamide-SDS
gel of a pyruvate carboxylase immunoprecipitate from fluoride-
treated mitochondria. Mitochondrial treatment and preparation of
extracts is as described in the legend to Fig. 3. About 2.1 mg of the
IgG fraction of goat antiserum to chicken pyruvate carboxylase was
added to 0.1 ml of the clear mitochondrial extract. The immunopre-
cipitates were processed as described in the legends of Figs. 2, 3, and
5. The upper trace is a of spectrophotometric scan of a gel stained for
protein. The lower trace shows superimposed the distribution of 32P
in 2 mM slices of a duplicate gel. Experimental details are given
under "Methods."
A linear relationship exists between the area produced by
the incorporation of pyruvate dehydrogenase in the pyruvate carboxylase
in the same experiment containing known amounts of highly
radioactive pyruvate dehydrogenase. If control goat IgG and rabbit anti-
goat IgG were added to the mitochondrial extract, this small
peak was also observed in the immunoprecipitates. Therefore,
we feel that this peak appeared due to nonspecific co-precipitation
of pyruvate dehydrogenase in the pyruvate carboxylase
immunoprecipitate.

Determination of the Stoichiometry of Phosphate Incorporation into Pyruvate Dehydrogenase—In order to determine whether the phosphate incorporation into pyruvate dehydrogenase is stoichiometrically significant, one must know the specific radioactivity of the terminal phosphate of intramitochondrial ATP. In addition, it is necessary for the specific radioactivity of the mitochondrial ATP to reach a constant value prior to the beginning of the experiment. The time for equilibration was determined by withdrawing samples at various times after adding mitochondria to the incubation mixture. The mitochondria in these samples were centrifuged rapidly through a layer of silicone oil into perchloric acid and the specific activity of the ATP in these extracts was determined. Firefly lantern extracts were used to measure the amount of ATP present in these extracts as outlined below. The amount of
ATP present in these extracts was determined as follows: Tris-Cl, 0.3 M, pH 8.0; glucose, 6 mM; MgCl₂, 6 mM; yeast hexokinase (Boehringer Mannheim), 60 µg/ml. After 1/2 h at
30°, the tubes were chilled, the pH was adjusted to 9.0 to 9.2 with
NaOH, and the solutions were applied to a 1-ml Dowex 1-X8 column.
Some of the glucose 6-phosphate did not adhere to the column and
most of the remainder eluted with 2 ml of 0.2 N HCl. The 32P
content was determined. Recovery was checked by spectrophotometric assay using NADP and glucose-6-phosphate dehydrogenase (34).

Stoichiometric calculations also require knowledge of the amount of protein present in the SDS gels. The amount of pyruvate dehydrogenase and pyruvate carboxylase present in the SDS gels of immunoprecipitates was estimated spectrophotometrically at 565 nm by scanning the Coomassie blue-stained gels in a Gilford 2410 linear transport. The areas of the peaks for either pyruvate carboxylase or both the α and β chains of pyruvate dehydrogenase were determined by triangulating both the α and β chains areas on the areas produced in gels run
in the same experiment containing known amounts of highly
radioactive chicken liver pyruvate carboxylase or bovine kidney
pyruvate dehydrogenase. Comparable results were obtained using a Dupont model 300 curve resolver to determine areas. A linear relationship exists between the area produced by scanning the pyruvate dehydrogenase chains and the amount added as is shown in Fig. 8. A similar linear relationship exists for pyruvate carboxylase (not shown) although the slope is different.

In addition to allowing the intramitochondrial pool of ATP to reach isotopic equilibrium with the added 32P, it is also necessary to establish the same condition for the phosphate on pyruvate dehydrogenase before meaningful calculations con-

Fig. 7. Time course of change in 32P specific radioactivity in the terminal phosphate of ATP in rat liver mitochondria after addition of 32P. At zero time, 32P (52,000 cpm/nmol) was added to 25-ml Erlenmeyer
flasks containing mitochondria (20 mg of protein) in 4 ml of incubation
medium. At the times indicated, samples were withdrawn and
mitochondria were sedimented through a layer of silicone oil into
perchloric acid as soon as possible as described under "Methods." The specific radioactivity of the terminal phosphate of the ATP in these extracts was determined as outlined below. The amount of ATP present in these extracts was determined by using the firefly
luminescence assay essentially as described by others (Refs. 32 and
33). The radioactivity of the terminal phosphate was determined by
adding 80 to 100 pmol of ATP from the neutralized perchloric acid
electrolytes to 200 nmol of unlabeled ATP in a volume of 2 ml. This
was applied to a 0.2-ml Dowex 1-X8 column. Following washes with 1.25 ml each of 0.01 N HCl and 0.05 N HCl, the nucleoside triphosphates
were eluted with 1.25 ml of 0.2 N HCl. Following neutralization with
K₂CO₃, the glucose 6-phosphate was formed by adding 1/4 volume of
the following: Tris-Cl, 0.3 M, pH 8.0; glucose, 6 mM; MgCl₂, 6 mM;
and yeast hexokinase (Boehringer Mannheim), 60 µg/ml. After 1/2 h at
30°, the tubes were chilled, the pH was adjusted to 9.0 to 9.2 with
NaOH, and the solutions were applied to a 1-ml Dowex 1-X8 column.
Some of the glucose 6-phosphate did not adhere to the column and
most of the remainder eluted with 2 ml of 0.2 N HCl. The 32P
content was determined. Recovery was checked by spectrophotometric assay using NADP and glucose-6-phosphate dehydrogenase (34).

Fig. 8. Relationship between the amount of purified pyruvate dehydrogenase placed on SDS-acrylamide gels and the area of the peaks on spectrophotometric gel scans of the two polypeptide chains. The indicated amounts of purified pyruvate dehydrogenase were subjected to SDS-gel electrophoresis. All gels were stained as described under "Methods." The gels were scanned at 565 nm in a Gilford model 2410 linear transport. The total area of the peaks was estimated by triangulating both the α and β chain peaks and subtracting any overlap from their sum.
During the preincubation the nonradioactive phosphate of the pyruvate dehydrogenase should exchange with that of the [\( ^{32}P \)]ATP through the combined actions of the phosphatase and kinase. At the end of the designated preincubation period, NaF was added to inhibit the phosphatase and thus prevent further dephosphorylation. In the subsequent incubation period, any remaining unphosphorylated serine residues on the dehydrogenase would be phosphorylated with \( ^{32}P \) as a result of sufficiently long, all of the phosphate originally present on the dehydrogenase should be replaced by \( ^{32}P \) and a maximal value for \( ^{32}P \) incorporation should be reached. As shown in Table I, over 1 mol of \( ^{32}P \) per mol of dehydrogenase (as the tetramer) has been incorporated by the end of the shortest time period, 2 min. The incorporation of \( ^{32}P \) increases with longer preincubation periods and appears to be leveling off in the range of 2 mol of phosphate per mol of dehydrogenase. These results do not permit us to decide whether the increase in \( ^{32}P \) incorporation is due to an increase in the total number of phosphate residues per tetramer or simply reflects the time necessary to bring the phosphate residues present on the dehydrogenase to isotopic equilibrium with the \( ^{32}P \)ATP. In other experiments it has been shown that essentially all of the oxidative activity of the pyruvate dehydrogenase complex has been lost under any of the conditions described in Table I.

In Experiment IV of Table I, another approach to the problem of establishing isotopic equilibrium has been tried. Mitochondria were pre-incubated with \( ^{32}P \) for 2 min. Dichloroacetate was then added to inhibit the kinase. This should allow the unopposed phosphatase to remove any nonradioactive phosphate from the dehydrogenase. A small sample was taken for assay of the complex after the dichloroacetate treatment. The oxidative activity was about 20 milliunits/mg of mitochondrial protein, indicating that the enzyme was almost completely activated. The rest of the mitochondria were then centrifuged and resuspended in new \( ^{32}P \)-containing medium to which NaF had been added. With the phosphatase inhibited, unopposed kinase activity should result in complete phosphorylation. As seen in Table I, the amount of \( ^{32}P \) incorporated into pyruvate dehydrogenase under these conditions is comparable to that observed in Groups II and III, suggesting that the total incorporation of \( ^{32}P \) will approach 2 mol of dehydrogenase and that 5 to 12 min of preincubation is sufficient to achieve the same end.

The foregoing methods permit the calculation of the number of phosphates incorporated per mol of enzyme. Table II compares enzymatic activity and phosphorylation of pyruvate dehydrogenase complex and pyruvate carboxylase from rat liver mitochondria treated with either fluoride or dichloroacetate. The latter treatment resulted in the expected activation of pyruvate dehydrogenase complex and with almost no phosphate incorporation into the enzyme. This effect was presumably due to inhibition of pyruvate dehydrogenase kinase (29). Phosphatase inhibition, resulting from incubating mitochondria with sodium fluoride and ionophore A23187 and EGTA (to remove calcium) (61), led to almost complete inactivation of the complex with incorporation of up to 2 mol of phosphate per mol of the tetrameric pyruvate dehydrogenase component of the complex (molecular weight of the tetramer = 154,000 (3)).

### Table I

<table>
<thead>
<tr>
<th>Treatment of mitochondria</th>
<th>Pyruvate dehydrogenase in immunoprecipitate</th>
<th>( ^{32}P ) incorporation</th>
<th>Mol ( ^{32}P ) incorporated per mol enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \mu g ) protein</td>
<td>net counts/min</td>
<td></td>
</tr>
<tr>
<td>Pre-incubation with ( ^{32}P ) before addition of NaF for:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 2 min</td>
<td>A</td>
<td>4.6 ± 0.06</td>
<td>1250 ± 380</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>4.93 ± 0.22</td>
<td>7970 ± 159</td>
</tr>
<tr>
<td>II 5 min</td>
<td>A</td>
<td>4.43 ± 0.29</td>
<td>17580 ± 280</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>5.30 ± 0.37</td>
<td>11900 ± 1085</td>
</tr>
<tr>
<td>III 12 min</td>
<td>A</td>
<td>4.70 ± 0.35</td>
<td>19200 ± 494</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>5.51 ± 0.07</td>
<td>19330 ± 354</td>
</tr>
<tr>
<td>IV 2 min + dichloroacetate</td>
<td>A</td>
<td>2.63 ± 0.10</td>
<td>12520 ± 473</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>4.83 ± 0.24</td>
<td>10980 ± 579</td>
</tr>
</tbody>
</table>

* Following preincubation, mitochondria incubated for 5 min with dichloroacetate. The mitochondria were collected by rapid centrifugation and were resuspended in new medium containing fluoride and \( ^{32}P \).

### Table II

<table>
<thead>
<tr>
<th>Activity</th>
<th>( ^{32}P )/mol PDH</th>
<th>Activity</th>
<th>( ^{32}P )/mol PC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyruvate dehydrogenase complex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dichloroacetate</td>
<td>25.8 ± 2.7 (N = 3)</td>
<td>0.006 ± 0.004 (N = 3)</td>
<td>156</td>
</tr>
<tr>
<td>Fluoride + A23187</td>
<td>0.32 ± 0.1 (N = 8)</td>
<td>1.89 ± 0.06 (N = 4)</td>
<td>158</td>
</tr>
<tr>
<td>+ EGTA</td>
<td></td>
<td></td>
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</table>

* Milliunits per mg of mitochondrial protein, 30°.
In incubation of mitochondria with fluoride or dichloroacetate had no effect on maximal pyruvate carboxylase activity measured from mitochondria treated in this fashion. Similarly, the activation of pyruvate carboxylase by acetyl-CoA was not changed (data not shown). However, fluoride treatment appeared to enhance the negative cooperativity of the pyruvate kinetics (35) while dichloroacetate appeared to diminish the negative cooperativity. These changes were not stable and were not apparent in mitochondria that has been frozen and stored nor were they observed in partially purified enzyme preparations. The significance of these latter observations must await further study.

**Discussion**

This study presents direct evidence that the kind of phosphorylation-dephosphorylation observed with purified pyruvate dehydrogenase complex by Reed, Linn, and associates (1,2) occurs in rat liver mitochondria. It is likely that the degree of phosphorylation of the enzyme at any particular time depends on the relative activities of pyruvate dehydrogenase kinase and phosphatase. Incubation of mitochondria with inhibitors of either pyruvate dehydrogenase kinase or phosphatase leads to changes in the degree of phosphorylation consistent with altered activities of the kinase and/or the phosphatase.

The idea that pyruvate carboxylase undergoes phosphorylation and dephosphorylation simultaneously with pyruvate dehydrogenase is teleologically an attractive idea. Phosphorylation with possibly opposite changes in activity could provide a well controlled means of regulating energy metabolism since pyruvate is at a metabolic branch point and both enzymes occur in the mitochondrial matrix. However, the data that we have presented do not support the idea that pyruvate carboxylase is modified by either pyruvate dehydrogenase kinase or phosphatase. The present study does not rule out the possibility that the stimulation by glucagon of hepatic gluconeogenesis from pyruvate results from changes in pyruvate carboxylase activity secondary to phosphorylation or dephosphorylation. A more likely explanation of this phenomenon may be the changes in pyruvate kinase activity, which were possibly due to phosphorylation, observed in perfused rat liver by Blair et al. (36).

Studies from many laboratories report wide variations in pyruvate dehydrogenase complex activity from rat liver mitochondria depending on the treatment. These activity changes have been assumed to reflect differences in the degree of phosphorylation. This assumption is probably correct in most cases. However, several laboratories have reported "maximal" activities which are about one-half those observed by us (8,10). A part of this difference with one study (10) may be due to our selection of assay blanks in the radioactive assay which are lower than blanks used in that report in which enzyme was omitted. The most probable explanations, otherwise, are either incomplete dephosphorylation of pyruvate dehydrogenase or failure to assay the mitochondrial extract immediately after preparation since the complex is unstable in this state (31).

These data support and extend the observations of Schuster et al. (14) and Hughes and Denton (15) who presented evidence that pyruvate dehydrogenase in heart mitochondria (14) and in adipose tissue mitochondria (15) can be phosphorylated and that more active preparations show less phosphorylation. Our work shows that pyruvate dehydrogenase in rat liver mitochondria can also be phosphorylated in situ. In addition, by determining both the specific radioactivity of intramitochondrial [γ-32P]ATP and the amount of enzyme protein in our immunoprecipitates, we have shown that the observed phosphorylation is stoichiometrically significant. Up to 2 mol of 32P were incorporated per mol of pyruvate dehydrogenase in the inactivated complex. In contrast, it has been reported in studies with the purified enzyme that up to 4 mol of phosphate can be incorporated per mol (38).

Reed and co-workers (39) have shown that pyruvate dehydrogenase possesses 3 different serine residues which may be phosphorylated. After proteolytic digestion, the incorporation of 32P into the different sites can be estimated by examination of the radioactivity of the tryptic peptides. Lightly phosphorylated complex preparations (less than 50% inactivation) show 32P incorporation into primarily one site while more heavily phosphorylated preparations have increasing amounts of isotope in all 3 serine residues. Preliminary investigations of the distribution of 32P in the dehydrogenase in experiments similar to those of Table I suggest that the 32P is present in all three sites in the dehydrogenase even at the earliest time tested.

We have previously reported that purified pig and rat liver pyruvate carboxylases are composed of four polypeptide chains of molecular weight 130,000 (31). In contrast, Gottschalk et al. (40) reported that rat liver pyruvate carboxylase is composed of polypeptides with a molecular weight of 70,000. McClure et al. (35) have reported dissociation of the 130,000-dalton subunit into a mixture of smaller polypeptides following reduction and carboxymethylation. In our study, pyruvate carboxylase precipitated with antibody from rat liver mitochondrial extracts and subjected to SDS-gel electrophoresis reveals the presence of only one major polypeptide with a molecular weight of 130,000. Besides the two antibody chains, only trace amounts of smaller polypeptides are observed. It is not clear whether or not these are related to pyruvate carboxylase. Finally, Warren and Tipton (41) have reported that the 130,000-dalton subunit of pig liver pyruvate carboxylase can be dissociated into polypeptides of 47,000 daltons. If the rat liver enzyme has a similar structure, we would have difficulty visualizing such polypeptides on our gels of immunoprecipitates because of the presence of the antibody heavy chain (50,000 daltons). However, this is unlikely since all of the pyruvate carboxylase protein expected to be present on the basis of the observed activity (assuming specific activity = 22 units/mg at 30° for the purified enzyme) can be accounted for in the 120,000-dalton polypeptide. The basis for these discrepancies between the results of this laboratory and other studies is not apparent. No mention was made of precautions taken to avoid proteolytic degradation in any of the latter studies. In studies of other enzymes, immunoprecipitation has proved to be a valuable tool in the isolation of native, unproteolyzed enzymes and these results lend support to our earlier observations (31) that the 130,000-dalton polypeptide is the naturally occurring protomer of rat liver peptide carboxylase.

**Acknowledgment**—We wish to thank Dr. Lester Reed for very helpful discussions concerning this manuscript and the experiments described therein.

**References**


2 A. Leiter, M. F. Utter, L. J. Reed, and S. Yeaman (1977) unpublished observations
Pyruvate Dehydrogenase:Pyruvate Carboxylase in Mitochondria

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