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

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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, ionophore A23187, and ethylene glycol bis(\(\beta\)-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 \((M_r = 154,000)\). 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.

Reed, Linn and coworkers (1-3) have clearly established that purified preparations of pyruvate dehydrogenase complex are inactivated by phosphorylation of its first component, pyruvate dehydrogenase, catalyzed by pyruvate dehydrogenase kinase. The phosphoenzyme is reactivated by another enzyme, also associated with the complex, pyruvate dehydrogenase phosphatase. The degree of phosphorylation of the complex has been shown to depend on the ATP/ADP ratio (4), the NADH/NAD ratio (5), the acetyl-CoA/CoA ratio (6), and the concentrations of pyruvate (8), calcium, and magnesium (7). It has been further shown that pyruvate, ADP, NAD, and CoASH inhibit the kinase whereas acetyl-CoA and NADH are activators of this enzyme (5). Phosphatase activity is inhibited by NADII (5) and is stimulated by high concentrations of magnesium and calcium (7).

Numerous laboratories have demonstrated wide variations in the activity of pyruvate dehydrogenase measured in extracts of mitochondria and tissues. The various factors such as ATP/ADP ratio and others mentioned above that have been suggested to affect the activity of the isolated complex have been implicated as regulatory factors in mitochondria as well (8-13). These changes have been assumed to reflect changes in the relative degree of phosphorylation of the complex. However, there has been no direct demonstration of phosphorylation in mitochondria or cells with the exception of two recent studies (14, 15). Schuster et al. (14) showed that pyruvate dehydrogenase complex can be phosphorylated in beef heart mitochondria and that less phosphate was incorporated under conditions where the enzyme was more active. Hugghe and Denton (15) found that mitochondria from rat fat pads incorporated \(^{32}P\) into pyruvate dehydrogenase.

In the present study, we have attempted to demonstrate phosphorylation of pyruvate dehydrogenase in rat liver mitochondria and to establish the approximate stoichiometry of this process. In addition, we have investigated whether pyruvate carboxylase simultaneously undergoes similar modifications since it has been suggested (16) that a reciprocal relationship may exist between the activities of the two enzymes.

There is no direct evidence which suggests that pyruvate carboxylase is regulated by a phosphorylation-dephosphorylation mechanism although the idea is teleologically attractive in light of the comparative properties of pyruvate dehydrogen-
ase complex and pyruvate carboxylase as pointed out by Reed (16). Several reports have noted that glucagon stimulation of gluconeogenesis from pyruvate is consistent with activation of pyruvate carboxylase (17). Studies of changes in levels of intermediary metabolites following glucagon perturbation indicate a "crossover" between pyruvate and oxalacetate (18). It has also been reported that glucagon 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 glucagon.

**EXPERIMENTAL PROCEDURES**

**Materials**

Pyruvate carboxylase was purified from chicken liver and stored as described by Scrutton and Pung (22). Beef kidney pyruvate dehydrogenase was purified and crystallized as described by Linn et al. (23) and was stored lyophilized at -70°C. Each of these two enzymes was judged to be at least 95 to 98% pure according to electrophoretic analysis on SDS-acrylamide gels. All measurements of protein concentration were made using a standard biuret procedure. Firefly luciferase was purified from Bombyx mori (23) and was stored lyophilized at -70°C. Each of these two enzymes 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 used.

**Methods**

Preparation of Mitochondria—Rat liver mitochondria were prepared by homogenizing livers from fed male Sprague-Dawley rats (200 to 300 g body weight) in 9 volumes of buffer at 4°C (mannitol, 0.22 M; sucrose, 0.07 M; Mops, 0.005 M; defatted albumin, 0.5 mg/ml; pH 7.4). The homogenate was centrifuged twice at 4°C using a standard rate procedure. Firefly luciferase was obtained from Sigma, Lincoln Science A5197 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 used obtained from Boehringer Mannheim Co. unless noted otherwise.

**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; thiamine 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 x 75 mm) and cutting off the serum stopper with Hamilton syringes fitted with PB-600 repeat dispensers. Mitochondrial extracts were prepared by thawing frozen mitochondria in a 20°C water bath followed by suspension in ice cold 20 mM sodium phosphate, pH 7.0, to obtain a protein concentration of 40 to 20 mg/ml. Within 2 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 11.5 μl pyruvate (Amersham/Searle) to a concentration of about 0.5 mM (specific activity, 150 to 450 cpm/nmol). The assay was terminated by addition 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 at least 30 min incubation 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 (2,5-diphenyloxazole (PPO) 0.3 g/liter, 1,4-bis[2-(4-methyl-5-phenyl)-benzene dithiothreitol, 1 mM; CoASH, 0.5 mM; lactic dehydrogenase, 10 μg/ml) was added. The 14-C-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. Enzyme 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). Pyruvate kinetics with this enzyme were studied with a sensitive radioactive CO₂ fixation assay as described by Ballard and Hanson (26) except that the mix contained 20 μl of citrate synthase. Pyruvate of ATP was omitted from the blanks.

**Preparation of Intramitochondrion Perchloric Acid Extracts for Analysis of Mitochondrial ATP**

Intramitochondrial perchloric acid extracts were prepared by placing 0.1 ml of mitochondrial suspension in one of the following layers: 0.1 ml of incubation medium containing glucose, 0.5 mg/ml, pH 7.4. The homogenate was centrifuged twice at 4°C using a standard rate procedure. Mitochondrial extracts for enzyme assays and immunoelectrophoresis were prepared by suspending the homogenate with rubber stopper to make a protein concentration of about 100 ng/ml/mg of protein. This suspension was kept on ice until use. Typical respiratory control ratios were 6 to 8 with sucinate as substrate and 4 to 5 with glutamate as substrate. This variability could be reduced by presoaking the rubber stopper with Hamilton syringes fitted with PB-600 repeat dispensers.

**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 about 7.5% or 8.0% acrylamide. In any given experiment gels were stained in Coomassie brilliant blue R-250 for 20 h at room temperature and de-stained with methanol/acetic acid overnight. For this reason, triplicate gels were run initially: one was stained for protein and the gels were always run to obtain a more accurate estimate of the amount of protein present. This variability could be reduced by presoaking the gels in several changes of 15% trichloroacetic acid prior to protein staining.

**Immunochemical 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 5 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 about 7.5% or 8.0% acrylamide. In any given experiment gels were stained in Coomassie brilliant blue R-250 for 20 h at room temperature and de-stained with methanol/acetic acid overnight. For this reason, triplicate gels were run initially: one was stained for protein and the gels were always run to obtain a more accurate estimate of the amount of protein present. This variability could be reduced by presoaking the gels in several changes of 15% 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°C. After cooling, 4 ml of tolune-based scintillant (24 g of 2,5-diphenyloxazole, 0.5 g of 1,4-bis[2-(5-phenyloxazolyl)] benzene per gallon) was added and the 32P was determined in a Packard scintillation counter. Mass and weights were always determined as described previously (28). In later experiments, the stained polyamide bands corresponding to either the chain of pyruvate dehydrogenase or to the polypeptide of pyruvate carboxylase were cut out and digested for 4 h at 50°C in 0.2 ml of 30% HCO₃. After complete digestion of the gels, 3.5 ml of Amosol (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 form; 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 dehydrogenase 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 dehydrogenase (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 a 2-mm slice 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 ($M_r = 53,000$) is not seen as a separate peak because of the large amount of γ-globulin heavy chain present. The sum of these observations suggests that the immune complex isolated consists of pyruvate dehydrogenase complex and antibody, and that the phosphorylated protein is confined to the α chain of pyruvate dehydrogenase.

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 immunochemical 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 fraction of the goat antiserum to pyruvate dehydrogenase. The immunoprecipitates were centrifuged, washed, dissociated, and subjected to SDS-gel electrophoresis as described in the legend to Fig. 2. One milliliter of extract was added to 0.1 ml of the clear mitochondrial extract. The immunoprecipitates were processed as described in the legends of Figs. 2, 3, and 5. The upper trace is of a spectrophotometric scan of 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. 4. 32P distribution in SDS-acrylamide gels of pyruvate dehydrogenase immunoprecipitates. Open circles show the distribution from immunoprecipitates of mitochondria treated with 20 mm fluoride. The closed circles are from mitochondria treated with 5 mm sodium dichloroacetate. Other experimental details are as described under "Methods" and the legend of Fig. 3.

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 carboxylase is present in the precipitates in the linear portion of the curve. This calculation assumes 0.16 unit of pyruvate carboxylase per mg of mitochondrial protein at 30° and that the purified rat liver enzyme has a specific activity of 22 units/mg.

Pyruvate carboxylase was isolated by immunoprecipitation under the optimal conditions established above from different extracts of the same frozen mitochondria employed in the preceding phosphorylation experiments on pyruvate dehydrogenase. The precipitates were dissolved and subjected to SDS-gel electrophoresis. The gels were again analyzed for both protein and 32P. Fig. 6 shows the distribution of radioactivity and protein in duplicate gels of a pyruvate carboxylase immunoprecipitate in 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 immunoprecipitates were processed as described in the legends of Figs. 2, 3, and 5. The upper trace is of a 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 immunnoprecipitation of pyruvate dehydrogenase in the pyruvate carboxylase determination and were compared to the areas produced in gels run in the same experiment containing known amounts of highly purified chicken liver pyruvate carboxylase or bovine kidney pyruvate dehydrogenase. However, the lower trace shows no incorporation of $^{32}$P into this polypeptide even though pyruvate dehydrogenase from the same mitochondria was extensively phosphorylated. Furthermore, no $^{32}$P was incorporated into pyruvate carboxylase obtained from mitochondria that had been treated with dichloroacetate or had received no treatment. It is unlikely that a non-immuno-reactive phosphorylated pyruvate carboxylase is present since almost all of the enzyme calculated to be present was precipitated by antibody regardless of the mitochondrial treatment. Closer inspection of Fig. 6 reveals a small peak of $^{32}$P at approximately 5.5 cm. This position approximately corresponds to the mobility on 5% acrylamide gels of the $\gamma$ chain of pyruvate dehydrogenase. If control goat IgG and rabbit antigoat IgG were added to the mitochondrial extracts, this small peak was also observed in the immunoprecipitates. Therefore, we feel that this peak appeared due to non-specific 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 (32, 33). The amount of $^{32}$P present in the terminal phosphate was determined by chromatographic isolation of glucose-6-phosphate formed upon addition of glucose and hexokinase. Fig. 7 shows that $^{32}$P specific activity of the phosphate of ATP approached that of the added $^{32}$P in less than 1 min and remains at that value thereafter. 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 total area of the peaks was estimated by triangulating both $\alpha$ and $\beta$ chain peaks and subtracting any overlap from their sum.

In addition to allowing the intramitochondrial pool of ATP to reach isotopic equilibrium with the added $^{32}$P, it is also necessary to establish the same condition for the phosphate on pyruvate dehydrogenase before meaningful calculations can be made. We have attempted to investigate the conditions necessary to establish isotopic equilibrium with the pyruvate dehydrogenase phosphate in the experiments summarized in Table I. Mitochondria were preincubated for 2, 5, and 12 min with $^{32}$P. The ATP pool reaches isotopic equilibrium in less than 1 min as already shown (Fig. 7).
7). 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 unopposed kinase activity. When the preincubation period is 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 preincubated 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/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) (6), 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</th>
<th>$^{32}$P in pyruvate dehydrogenase</th>
<th>Mol $^{32}$P incorporated per mol enzyme</th>
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<tbody>
<tr>
<td></td>
<td>µg protein</td>
<td>net counts/ min</td>
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</tr>
<tr>
<td>Preincubation with $^{32}$P before addition of NaF for:</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>1 min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>4.6 ± 0.06</td>
<td>12050 ± 380</td>
<td>1.31 ± 0.02</td>
</tr>
<tr>
<td>B</td>
<td>4.93 ± 0.22</td>
<td>7970 ± 159</td>
<td>1.23 ± 0.08</td>
</tr>
<tr>
<td>II 5 min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>4.43 ± 0.29</td>
<td>17590 ± 280</td>
<td>1.88 ± 0.13</td>
</tr>
<tr>
<td>B</td>
<td>5.30 ± 0.37</td>
<td>11090 ± 1085</td>
<td>1.58 ± 0.09</td>
</tr>
<tr>
<td>III 12 min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>4.70 ± 0.35</td>
<td>19200 ± 494</td>
<td>1.94 ± 0.18</td>
</tr>
<tr>
<td>B</td>
<td>5.91 ± 0.07</td>
<td>19230 ± 354</td>
<td>1.78 ± 0.03</td>
</tr>
<tr>
<td>IV 2 min + dichloroacetate(^a)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>2.63 ± 0.10</td>
<td>12050 ± 473</td>
<td>2.27 ± 0.01</td>
</tr>
<tr>
<td>B</td>
<td>4.83 ± 0.24</td>
<td>10980 ± 579</td>
<td>1.58 ± 0.04</td>
</tr>
</tbody>
</table>

\(^a\) 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>Treatment</th>
<th>Pyruvate dehydrogenase complex</th>
<th>Pyruvate carboxylase (PC) from rat liver mitochondria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Activity(^a) mol $^{32}$P/mol PDH</td>
<td>Activity(^a) mol $^{32}$P/mol PC</td>
</tr>
<tr>
<td>Dichloroacetate</td>
<td>25.8 ± 2.7 (N = 3)</td>
<td>0.006 ± 0.004 (N = 3)</td>
</tr>
<tr>
<td>Fluoride + A23187</td>
<td>0.32 ± 0.1 (N = 8)</td>
<td>1.89 ± 0.08 (N = 4)</td>
</tr>
</tbody>
</table>

\(^a\) Milliunits per mg of mitochondrial protein, 30°C.
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 dehydrogenase 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 assy the mitochondrial extracts 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 isotopes 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.2

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 carboxymethylxylation. 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 = 52 units/mg at 30°C 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.

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Pyruvate Dehydrogenase: Pyruvate Carboxylase in Mitochondria

Relationship between phosphorylation and activity of pyruvate dehydrogenase in rat liver mitochondria and the absence of such a relationship for pyruvate carboxylase.

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