

Effect of Streptozotocin-Induced Diabetes Mellitus on the Turnover of Rat Liver Pyruvate Carboxylase and Pyruvate Dehydrogenase

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Immunochemical techniques were used to study the effect of streptozotocin-induced diabetes on the amounts of pyruvate carboxylase and pyruvate dehydrogenase and on their rates of synthesis and degradation. Livers from diabetic rats had twice the pyruvate carboxylase activity of livers from normal rats when expressed in terms of DNA or body weight. The changes in catalytic activity closely paralleled changes in immunoprecipitable enzyme protein. Relative rates of synthesis determined by pulse-labelling studies showed that the ratio of synthesis of pyruvate carboxylase to that of average mitochondrial protein was increased 2.0–2.5 times in diabetic animals over that of control animals. Other radioisotopic studies indicated that the rate of degradation of this enzyme was not altered significantly in diabetic rats, suggesting that the increase in this enzyme was due to an increased rate of synthesis. Similar experiments with pyruvate dehydrogenase, the first component of the pyruvate dehydrogenase complex, showed that livers from diabetic rats had approximately the same amount of immunoprecipitable enzyme protein as the control animals, but a larger proportion of the enzyme was in its inactive state. The rates of synthesis and degradation of pyruvate dehydrogenase were not affected significantly by diabetes.

Hepatic gluconeogenesis from pyruvate or lactate has been shown to be increased significantly in experimental diabetes. Several investigations have shown accompanying enhanced activities of specific gluconeogenic enzymes, including cytosolic phosphoenolpyruvate carboxykinase (EC 4.1.1.32), fructose 1,6-bisphosphatase (EC 3.1.3.11) and glucose 6-phosphatase (EC 3.1.3.9) (Filsell *et al.*, 1969; Wimhurst & Manchester, 1970). It would seem consistent with these findings that another gluconeogenic enzyme, pyruvate carboxylase (EC 6.4.1.1), should also increase under these circumstances, but the previous reports on changes in activity have been contradictory. There have been several reports of increased activity (Prinz & Seubert, 1964; Wagle, 1964; Freedman & Kohn, 1964; Struck *et al.*, 1966; Wimhurst & Manchester, 1970; Chang & Schneider, 1971; Weiss *et al.*, 1971), one of decreased activity (Mehlman *et al.*, 1971), and still others of unchanged activity (Krebs, 1966; R. K. Yamazaki & M. F. Utter, unpublished work; Schrago & Lardy, 1966; Brech *et al.*, 1970). The bases of these discrepancies are not clear, but one

possible interpretation may involve technical difficulties encountered in the assay of this mitochondrial enzyme. In any event there has been no convincing evidence that the activity of this enzyme changes significantly in response to diabetes. The effect of diabetes on the regulation of hepatic pyruvate carboxylase should be clarified by determining the rates of synthesis and degradation of the enzyme, since this provides a more sensitive and a more definitive assay than activity measurements alone. Accordingly, we have investigated by immunochemical methods the effect of diabetes on the content and the rates of synthesis and degradation of the liver enzyme.

The increased demand for net pyruvate utilization for gluconeogenesis that is seen in the diabetic state suggests that a decreased demand for net pyruvate oxidation may be seen in this condition. Inhibition of hepatic pyruvate oxidation in experimental diabetes has been observed by Wieland *et al.* (1972). The activity of pyruvate dehydrogenase complex is regulated by product inhibition (Randle *et al.*, 1966; Wieland *et al.*, 1969) and by a phosphorylation/dephosphorylation cycle (Linn *et al.*, 1969). Although these mechanisms may be

Abbreviation used: SDS, sodium dodecyl sulphate.

sufficient to account for the decreased activity noted in diabetes, there have been no previous measurements of the actual amount of this enzyme in diabetic liver. Accordingly, parallel immunochemical investigations were conducted on the effect of the diabetic state on amount and rates of synthesis and degradation of pyruvate dehydrogenase (EC 1.2.4.1), the first component, which is thought to catalyse the rate-limiting step in the reaction complex. A preliminary report of this work has appeared (Weinberg & Utter, 1979a).

Experimental

Materials

Bovine kidney pyruvate dehydrogenase, a generous gift of Dr. Tracy Linn, Dallas Veterans Hospital, Dallas, TX, U.S.A., and rat liver pyruvate carboxylase were purified and prepared as previously described (Leiter *et al.*, 1978; Weinberg & Utter, 1979b). The ionophore A23187 was kindly supplied by Dr. Robert Hammill from Eli Lilly and Co., Indianapolis, IN, U.S.A. Streptozotocin was a gift of the Upjohn Co., Kalamazoo, MI, U.S.A. Zinc insulin was purchased from Squibb Pharmaceuticals, New Brunswick, NJ, U.S.A.; calf thymus DNA, cytochrome *c* and phosphotransacetylase were from Sigma Chemical Co., St. Louis, MO, U.S.A.; sodium [$1\text{-}^{14}\text{C}$]pyruvate, Hyamine hydroxide, Protosol and Econofluor were from New England Nuclear Corp., Boston, MA, U.S.A. $\text{NaH}^{14}\text{CO}_3$ (0.1 mCi/mmol), L-[4,5- ^3H]leucine (40–60 Ci/mmol) and aqueous counting scintillant were purchased from Amersham-Searle Co., Arlington Heights, IL, U.S.A. All chemicals used for electrophoresis were purchased from Bio-Rad Laboratories, Richmond, CA, U.S.A., and were of electrophoresis grade. Other reagents were obtained from Boehringer-Mannheim Co., Indianapolis, IN, U.S.A., unless otherwise noted.

Animals and their maintenance

Sprague-Dawley male rats (Zivic Miller Laboratories, Pittsburgh, PA, U.S.A.), weighing 160–210 g and maintained on Purina Lab Chow and water *ad libitum*, were used in all experiments. They were housed in a constant-temperature environment (22°C) with a uniform light cycle (07:00–19:00 h daily). The animals were killed at essentially the same time each day.

Inducing diabetes

A single dose of streptozotocin (200 mg/kg body wt.) administered intraperitoneally in 0.1 M-sodium citrate buffer, pH 4.5, was found to be sufficient to induce diabetes in rats (160–180 g). Control rats were injected with citrate only. Diabetes was evident 3 days after streptozotocin administration by the

following criteria: weight loss and glucose (greater than 1 g/100 ml) present in the urine, as determined by Labstic (Ames). Studies were performed after the rats had been diabetic for 12 days. Blood-sugar values were determined for these animals at the time of death.

Rats that had been diabetic for 7 days were injected with 3–5 I.U. of Lente insulin subcutaneously once per day. After 5 days these animals were designated 'insulin-treated diabetic controls' and studies were begun while continuing the insulin administration.

Blood glucose

Blood was collected in heparinized tubes, deproteinized by the Nelson-Somogyi method (Niejadlik & Adamko, 1973), and the protein-free filtrate was frozen until assay. The filtrate was assayed by a method using glucose oxidase (Glucostat Reagents, Worthington Biochemicals Corp.).

Protein and DNA determinations

All measurements of protein were made by a standard biuret procedure (Layne, 1957) with crystallized bovine serum albumin as a standard. DNA was determined by a modification of the method of Burton (1956), with calf thymus DNA as a standard.

Assay of enzymes

Pyruvate dehydrogenase complex activity was determined on mitochondrial extracts as described by Leiter *et al.* (1978), by measuring the decarboxylation of [$1\text{-}^{14}\text{C}$]pyruvate at 30°C. Pyruvate carboxylase activity was assayed by a radioactive- CO_2 -fixation assay described by Ballard & Hanson (1967), except that the mixture contained 2 units of citrate synthase/ml and the enzyme was preincubated with Triton X-100 before initiation of the assay with pyruvate. Water replaced pyruvate in the blank.

Preparation of liver mitochondria

Rats were killed by decapitation and the liver was quickly removed, blotted and weighed. All subsequent steps were carried out at 0–4°C unless otherwise noted. Livers were homogenized and mitochondria isolated as described by Weinberg & Utter (1979b). The final mitochondria suspension (about 50 mg of protein/ml) was kept in ice until use.

The liver homogenates or mitochondria were incubated under specified conditions and then rapidly centrifuged (12000 g-min) in an Eppendorf Microfuge; the resulting pellets were quickly frozen in a solid- CO_2 /ethanol bath and stored at -78°C . Details are presented in the Figure legends. Homogenate of mitochondrial extracts for enzyme assay and immunochemical isolation were prepared from

the frozen pellets as described in the text or appropriate Figure legends.

Immunochemical and electrophoretic techniques

Antibodies were prepared against highly purified preparations of both rat liver pyruvate carboxylase and bovine kidney pyruvate dehydrogenase. Purification and characterization of these antibodies has also been described (Leiter *et al.*, 1978; Weinberg & Utter, 1979b). SDS/polyacrylamide-gel electrophoresis was performed as described by Weber *et al.* (1972), with some modifications (Weinberg & Utter, 1979b).

Methods of estimating the synthesis and degradation of pyruvate carboxylase and pyruvate dehydrogenase

The relative rates of synthesis of pyruvate carboxylase, pyruvate dehydrogenase and mitochondrial protein were determined by injecting rats intraperitoneally with 2.0 μ Ci of L-[4,5-³H]leucine (40–60 Ci/mmol)/g body wt. The rats were killed 90 min later, and their livers removed, homogenized and fractionated as described above. To isolate each enzyme, a known amount of mitochondrial extract was incubated with antibody to either pyruvate carboxylase or pyruvate dehydrogenase (described in the legends to Tables 2 and 3). The immunoprecipitates were washed and dissociated in 1% SDS (Weber *et al.*, 1972) and subjected to electrophoresis as described above. The gels were then sliced and each slice (1 mm) was incubated for 48 h at 37°C in 10 ml of 3% (v/v) Protosol/Econofluor solution with shaking. After cooling, radioactivity was determined in a Packard Tri-Carb liquid-scintillation spectrometer. A second portion of the immunoprecipitate was simultaneously subjected to electrophoresis, but was stained with Coomassie Blue. The radioactivity was then measured in the gel slices corresponding to the protein peak of either pyruvate carboxylase or pyruvate dehydrogenase as determined from the protein-stained gels.

A portion of the mitochondrial fraction was used to determine the total radioactivity in the mitochondrial protein. The protein was precipitated and washed with 10% (w/v) trichloroacetic acid and collected by centrifugation at 12000 g-min. The precipitate was digested for 3 h at 45°C in 0.5 ml of Protosol; 10 ml of Econofluor was added, and the ³H radioactivity measured in a Packard Tri-Carb liquid-scintillation spectrometer. Similarly treated samples of the final wash, which served as blanks, contained less than 1% of the total radioactivity.

Degradation rates for pyruvate carboxylase and pyruvate dehydrogenase were measured essentially as described above, except that rats received one injection of [³H]leucine on the initial day of the experiment and were killed at various times up to 6 days later. Apparent rate constants for synthesis and degradation were calculated as described by Schimke & Doyle (1970) and Arias *et al.* (1969).

Results

Pyruvate carboxylase activity

Pyruvate carboxylase catalytic activity was approximately twice as much in streptozotocin-diabetic rats (blood glucose concentrations 400–500 mg/100 ml) as in normal rats (blood glucose concentrations 90–100 mg/100 ml) (Table 1). Daily administration of insulin to streptozotocin-diabetic rats for 5 days decreased both pyruvate carboxylase activity and blood glucose concentrations (90–160 mg/100 ml) toward normal. The failure of the 5-day period of insulin administration to lower the activity of pyruvate carboxylase to pre-diabetes values suggests that the turnover time for this enzyme may be relatively slow. This suggestion is supported by later experiments (see Fig. 2 and Table 5). The relative differences in the ratio of pyruvate carboxylase activity in the diabetic to that in the control state were the same whether expressed in terms of wet weight, protein, DNA or 100 g body wt.

Table 1. *Effect of streptozotocin-induced diabetes mellitus on pyruvate carboxylase activity in rat liver homogenates* Each value represents the mean \pm s.d. for five observations. Activities are expressed as μ mol of product formed at 30°C/min per g wet wt. of liver, g of protein, mg of DNA, or 100 g body wt., from liver homogenates assayed as described in the Experimental section. Blood glucose concentrations represent the range of values observed.

Rats	Blood glucose (mg/100 ml)	Pyruvate carboxylase activity			
		(units/g of liver)	(units/g of protein)	(units/mg of DNA)	(total units/ 100 g body wt.)
Normal	90–100	14.2 \pm 1.2	68.4 \pm 7.7	5.5 \pm 0.7	60.0 \pm 10.1
Diabetic	400–500	24.9 \pm 2.0	105.9 \pm 8.5	10.2 \pm 1.4	114.4 \pm 9.5
Insulin-treated diabetic control	90–160	15.4 \pm 1.5	67.0 \pm 6.5	7.5 \pm 0.7	90.8 \pm 8.8

(Table 1). Expression of enzymic activity on the basis of liver DNA will be used hereafter.

Experiments in which equal parts of liver homogenate from diabetic and normal rats were mixed showed no significant deviation in activity recovered from that expected from simple additive

effects, suggesting that the alterations are not due to the presence of activators or inhibitors. Hepatic pyruvate carboxylase activity was also found to be increased 2-fold in rats made diabetic by alloxan (results not shown).

Immunochemical isolation of pyruvate carboxylase and pyruvate dehydrogenase

Intramitochondrial protein concentrations of pyruvate carboxylase and pyruvate dehydrogenase were compared with enzyme activities in the diabetic and control states (Tables 2 and 3) by immunochemical isolation of the two enzymes. The techniques used in these studies for extraction, immunochemical isolation and quantification of immunoreactive pyruvate carboxylase and pyruvate dehydrogenase have been described (Leiter *et al.*, 1978; Weinberg & Utter, 1979b).

A comparison of the amounts of antibody required to neutralize the catalytic activities of pyruvate carboxylase derived from diabetic and normal rats is shown in Fig. 1. Although the specific activities of pyruvate carboxylase in the diabetic and normal preparations differed by almost 2-fold, the precipitin curves were found to be essentially identical. Thus the immunological properties of pyruvate carboxylase were not altered by the diabetic state, suggesting that differences in enzymic activity reflect parallel changes in the amount of pyruvate carboxylase protein. This idea is supported more directly by the experiments summarized in Table 2. The changes in amounts of immunoprecipitable protein in the diabetic, insulin-treated diabetic and untreated rats closely parallel the changes in measurement of pyruvate carboxylase activity and provide strong evidence for an actual increase in the content of the liver enzyme in

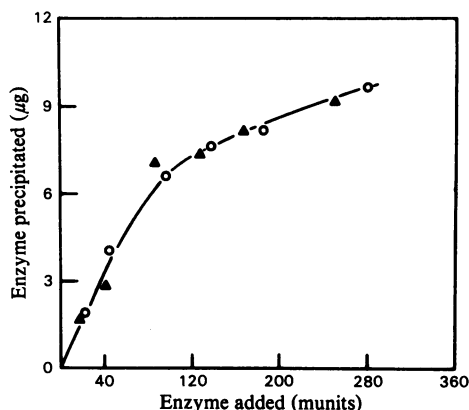


Fig. 1. Immunochemical precipitation of pyruvate carboxylase from mitochondrial extracts of livers of diabetic (▲) and normal (○) rats

Rabbit antibody to rat liver pyruvate carboxylase (2mg) was added to increasing amounts of pyruvate carboxylase activity from clarified mitochondrial extracts. Enzyme-activity determinations and immunoprecipitations were performed as described in the Experimental section and Weinberg & Utter (1979b). Immunoprecipitates were solubilized in SDS, divided into three equal fractions, and subjected to SDS/polyacrylamide-gel electrophoresis as indicated in the text.

Table 2. Effect of streptozotocin-induced diabetes mellitus on the activity and intracellular amounts of immunoprecipitable pyruvate carboxylase

Each value represents the mean \pm S.D. for five observations. Activities are expressed in $\mu\text{mol}/\text{min}$ at 30°C per mg of liver DNA. For determination of enzyme protein, quantitative immunoprecipitation of pyruvate carboxylase was performed on mitochondrial extracts prepared essentially as described by Weinberg & Utter (1979b). Anti-(pyruvate carboxylase) antiserum (1.0mg) was added to 3.7mg of mitochondrial extract from normal livers. In all cases, sufficient antibody was used to precipitate the activity of enzyme present. The immunoprecipitates were washed, solubilized in SDS, divided into three equal fractions and subjected to SDS/polyacrylamide-gel electrophoresis on gels containing 5% acrylamide. The gels were stained with Coomassie Blue; the amount of pyruvate carboxylase present was determined by calculating the area of its peak on spectrophotometric gel scans and comparing these values with those of peaks produced by known amounts of purified rat liver pyruvate carboxylase. Specific activities have been calculated by dividing enzyme activity (column 2) by μg of protein in the immunoprecipitate (column 3).

Rats	Activity (units/mg of liver DNA)	Enzyme protein ($\mu\text{g}/\text{mg}$ of liver DNA)	Specific activity (units/mg of enzyme protein)
Normal	5.5 ± 0.7	225 ± 23	24.4
Diabetic	10.2 ± 1.4	417 ± 25	24.5
Insulin-treated diabetic control	7.5 ± 0.7	327 ± 21	22.9

Table 3. *Effect of streptozotocin-induced diabetes mellitus on the activity of pyruvate dehydrogenase complex and the intracellular amounts of pyruvate dehydrogenase*

Each value represents the mean \pm S.D. for five observations. Activities are expressed in $\mu\text{mol}/\text{min}$ at 30°C per g of mitochondrial protein. Mitochondria in which pyruvate dehydrogenase complex (PDC) was assayed were incubated with either sodium dichloroacetate (5 mM) to inhibit pyruvate dehydrogenase kinase, or NaF (20 mM), EGTA (0.5 mM) and ionophore A23187 (5 $\mu\text{g}/\text{ml}$) to inhibit pyruvate dehydrogenase phosphatase. 'Total activity' represents the difference between those obtained under these two treatment regimens. Mitochondrial incubations and enzyme assays were performed as described in the Experimental section. 'PDHa (%)' represents the set point of 'endogenous' PDC activity and is the activity observed in isolated mitochondria not preincubated with dichloroacetate as a percentage of the activity of a similar portion of mitochondria activated with dichloroacetate. For determination of enzyme protein, quantitative immunoprecipitation of pyruvate dehydrogenase was performed on mitochondrial extracts prepared as described by Weinberg & Utter (1979b). Anti-(pyruvate dehydrogenase) serum (1.0 mg) was added to 5.0 mg of mitochondrial extract from normal livers. In all cases, sufficient antibody was used to precipitate the activity of enzyme present. The immunoprecipitates were washed, solubilized in SDS, divided into three equal fractions and subjected to SDS/polyacrylamide-gel electrophoresis on gels containing 8% acrylamide. The gels were stained with Coomassie Blue, the amount of pyruvate dehydrogenase present was determined by calculating the area of its peak on spectrophotometric gel scans and comparing these values with those of peaks produced by known amounts of purified bovine kidney pyruvate dehydrogenase. The total area was determined by triangulating both α - and β -chain peaks and subtracting any overlap from their sum.

Rats	Pyruvate dehydrogenase activity (units/g of mitochondrial protein)	PDHa (%)	Enzyme protein (mg of pyruvate dehydrogenase/g of mitochondrial protein)
Normal	26.6 \pm 2.7	33 \pm 3	42 \pm 3
Diabetic	25.4 \pm 1.2	8 \pm 0.5	52 \pm 6*
Insulin-treated diabetic control	24.3 \pm 1.1	27 \pm 3	40 \pm 7

* $P < 0.01$ compared with values for controls (according to Student's t test).

the diabetic state. The calculated specific activities of pyruvate carboxylase in the diabetic and control states agree well.

The effects of diabetes on the activity and amounts of immunoprecipitable pyruvate dehydrogenase are shown in Table 3. The maximal catalytic activity of pyruvate dehydrogenase complex, expressed on the basis of mitochondrial protein, was not altered in the diabetic state. Maximal activity was attained by preincubation of the mitochondria with dichloroacetate (Leiter *et al.*, 1978). However, the amount of immunoprecipitable pyruvate dehydrogenase was increased slightly in diabetes (about 25%). The significance of this apparently conflicting observation is not clear, although one possibility is that the maximal catalytic activity of the complex may have been underestimated in the diabetic animals. In contrast with the unchanged maximal activities in the diabetic state, the ratio of endogenous activity to maximal activity was sharply decreased in diabetes (8%) compared with normal (32%) or insulin-treated diabetic rats (27%).

Relative rates of synthesis of pyruvate carboxylase and pyruvate dehydrogenase

The ratios of the amount of L-[4,5- ^3H]leucine incorporated into pyruvate carboxylase and pyruvate dehydrogenase to the amounts incorporated into total mitochondrial protein were determined in

the diabetic and normal states by pulse-labelling experiments (Schimke & Doyle, 1970). The rate of synthesis of pyruvate carboxylase in diabetic rats was 2.4 times that in normal rats, whereas no difference was seen in incorporation into total mitochondrial protein of diabetic rats (Table 4). The results were almost identical when expressed on the basis of total liver protein rather than mitochondrial protein. A similar analysis shows that the synthesis of pyruvate dehydrogenase appears to be lowered slightly in diabetes (Table 4).

Determination of the rates of synthesis and degradation of pyruvate carboxylase and pyruvate dehydrogenase

The pulse-labelling experiments described in the previous section indicate that the alteration in the concentration of pyruvate carboxylase observed in the diabetic state may reflect changes in the rate of synthesis. Absolute rates of synthesis can be calculated from degradation rates during steady-state conditions (Schimke & Doyle, 1970). Under these conditions, $E = k_s/k_d$, where E is the content of enzyme per unit mass, k_s is an apparent zero-order rate constant of synthesis per unit mass, and k_d is an apparent first-order rate constant of degradation expressed in time^{-1} . The requirement for steady-state conditions was met for pyruvate carboxylase

Table 4. *Effect of streptozotocin-induced diabetes on the relative rates of synthesis of pyruvate carboxylase and pyruvate dehydrogenase from rat liver*

Each value represents the mean \pm S.D. for three observations. Animals treated as described in the text were killed (three rats per group) 90 min after the injection of $2\mu\text{Ci}$ of L-[4,5- ^3H]leucine/g body wt. Radioactivity was measured in immunoprecipitable pyruvate carboxylase and pyruvate dehydrogenase and trichloroacetic acid-precipitable mitochondrial protein as described in the Experimental section. Specific radioactivity was expressed per g of liver. Relative synthesis is expressed as the amount (c.p.m.) of [^3H]leucine incorporated into pyruvate carboxylase or pyruvate dehydrogenase per 10^3 c.p.m. incorporated into the total mitochondrial protein.

Rats	[^3H]Leucine incorporated (c.p.m./g of liver)		Total mitochondrial protein ($10^3 \times$ c.p.m./g of liver)	$10^3 \times$ Relative rate of synthesis	
	Pyruvate carboxylase	Pyruvate dehydrogenase		Pyruvate carboxylase	Pyruvate dehydrogenase
Normal	1490 \pm 114	504 \pm 4	8.54 \pm 0.40	1.8	0.59
Diabetic	3560 \pm 207	406 \pm 10	8.50 \pm 0.10	4.2	0.48

Table 5. *Effect of streptozotocin-induced diabetes mellitus on the turnover of pyruvate carboxylase and pyruvate dehydrogenase from rat liver*

The apparent rate constants of degradation (k_d) are derived from the slope of the decay curves presented in Fig. 2, expressed in days $^{-1}$. The apparent rate constants of synthesis (k_s) are derived by multiplying k_d by the amount of enzyme protein present. Half-life is derived from the expression $t_{1/2} = \ln 2/k_d$.

Enzyme	Rats	Protein* (μg)	k_d (days $^{-1}$)	k_s (μg of protein/day)*	Half-life (days)
Pyruvate carboxylase	Normal	225 \pm 23	0.15	34.1	4.6
	Diabetic	417 \pm 25	0.15	64.1	4.5
Pyruvate dehydrogenase	Normal	42 \pm 3	0.085	3.6	8.1
	Diabetic	52 \pm 6	0.093	4.8	7.5

* Expressed as μg of protein/mg of DNA as determined by immunoprecipitation.

in the diabetic and normal states. After administration of L-[4,5- ^3H]leucine to the various groups of rats that had attained steady-state conditions, the rats were killed at intervals up to 6 days. Pyruvate carboxylase was isolated immunochemically, the immunoprecipitates were solubilized and subjected to SDS/polyacrylamide-gel electrophoresis (Weinberg & Utter, 1979b), and the protein representing pyruvate carboxylase was analysed for its ^3H content. The progressive loss of specific radioactivity of hepatic pyruvate carboxylase was plotted semi-logarithmically versus time (Fig. 2) and the slope, k_d , of the straight line was determined. The half-life ($t_{1/2} = \ln 2/k_d$) for pyruvate carboxylase from normal rat liver was 4.6 days and was not significantly affected in the diabetic group (Table 5). The calculated k_s values (Table 5) show an almost 2-fold increase in diabetic rats compared with the untreated rats. The overall results of Table 5 suggest that changes in the catalytic activity of pyruvate carboxylase in the diabetic state are determined

mainly by regulation of enzyme synthesis *de novo* and that alterations in the rate of degradation are of little importance.

The rate of loss of radioactivity from the mitochondrial proteins was also measured. The half-life for the average mitochondrial proteins in the normal rats was 3.8 days, whereas diabetic rats showed a decreased rate of turnover (half-life 5.1 days).

By similar experiments, the half-life of pyruvate dehydrogenase from untreated diabetic-rat liver was 8.1 days, as compared with 7.4 days for the diabetic state (Fig. 2). The calculated apparent rate constant of synthesis for pyruvate dehydrogenase was increased by 35% over the values in the normal state, a finding which mirrors the increase in immunoprecipitable enzyme (Table 5).

Discussion

The rate of hepatic gluconeogenesis from pyru-

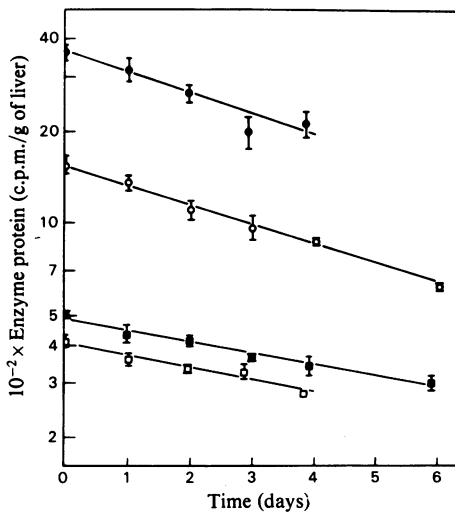


Fig. 2. Rate of degradation of radiolabelled pyruvate carboxylase and pyruvate dehydrogenase from diabetic and normal (untreated) rats

Animals in each group (maintained as described in the Experimental section) were serially killed (three rats per time point) at the time points indicated on the abscissa. The initial time point was taken 90 min after the injection of $2\mu\text{Ci}$ of L-[4,5- ^3H]leucine/g body wt. Radioactivity in immunoprecipitable pyruvate carboxylase and pyruvate dehydrogenase was measured as described in the Experimental section and expressed per g of liver. Linear regression analysis was used to determine the best fit. ●, ^3H -labelled pyruvate carboxylase from diabetic rats; ○, ^3H -labelled pyruvate carboxylase from normal rats; ■, ^3H -labelled pyruvate dehydrogenase from diabetic rats; □, ^3H -labelled pyruvate dehydrogenase from normal rats.

vate is dramatically increased in the diabetic state. This has been proposed to result, at least in part, from increase in the activity of the gluconeogenic enzymes at the level of pyruvate utilization (Williamson *et al.*, 1969a,b) although other interpretations are possible, including a decrease in pyruvate kinase activity (Blair *et al.*, 1976). Metabolite cross-over patterns have implicated the conversion of pyruvate into phosphoenolpyruvate as one of the rate-limiting processes in gluconeogenesis (Williamson *et al.*, 1969a; Exton & Park, 1969). Hanson and co-workers (see Tilghman *et al.*, 1976, for review) have demonstrated increased amounts of phosphoenolpyruvate carboxykinase in the diabetic state.

The present results appear to be consistent with some of the studies that report increased activity in the diabetic state (Freedman & Kohn, 1964; Prinz & Seubert, 1964; Wagle, 1964; Struck *et al.*, 1966;

Wimhurst & Manchester, 1970; Chang & Schneider, 1971; Weiss *et al.*, 1971). However, none of these previous studies reported activities above 25% of the values shown here. In addition, the present studies show that this increase in enzymic activity is due to an increased amount of protein because of an increased rate of synthesis. The administration of insulin to diabetic rats produced a decrease in hepatic concentrations of pyruvate carboxylase. The failure of the 5-day insulin regimen to decrease this enzyme to pre-diabetes amounts can be attributed to its slow turnover rate ($t_{1/2}$ 4.5 days).

In the diabetic state the increased demand for pyruvate utilization for gluconeogenesis might be accompanied by decreased pyruvate oxidation. Decreased endogenous catalytic activity of pyruvate dehydrogenase complex in diabetes has been observed in the present studies and presumably results from changes in the relative degree of phosphorylation of pyruvate dehydrogenase rather than a decrease in enzyme content. The decreased activities observed in the present study are consistent with values reported by Wieland *et al.* (1972), and suggest that circulating insulin may play an important role in the regulation of pyruvate dehydrogenase by phosphorylation, either by some direct interaction on pyruvate dehydrogenase interconversion or by an indirect interaction such as decreasing plasma non-esterified fatty acids (Wieland *et al.*, 1972), but appear to play no significant role in regulation by altered enzyme concentrations.

In the present studies on the regulation of pyruvate dehydrogenase, changes in activity probably reflect changes in the relative degree of phosphorylation of the complex. Totally active complex (dichloroacetate-treated) is assumed to be dephosphorylated, and the endogenous activity is assumed to reflect the relative state of phosphorylation. Although these assumptions may be valid, other causes of changes in complex activity cannot be excluded, e.g. lysosomal proteolysis and inactivation of the enzyme in crude extracts (Wieland, 1975). More definitive analyses of possible changes in total activity are offered by quantitative immunological techniques. We have provided immunological evidence that the amount of the first component of the complex, pyruvate dehydrogenase, thought to catalyse the rate-limiting step in the overall reaction (Roche & Reed, 1972), does not decrease and may actually increase (25%) in the diabetic state.

The rates of degradation of pyruvate carboxylase and pyruvate dehydrogenase were little affected by diabetes. The two enzymes measured in the present study are being replaced at relatively slow rates ($t_{1/2}$ 4.6 and 8.1 days, respectively) compared with

average mitochondrial protein ($t_{1/2}$ 3.8 days). These results are similar to data reported by us about the effects of thyroid status on these enzymes (Weinberg & Utter, 1979b). The interpretation of results obtained by the radioisotope-decay method may be complicated by reutilization of radioisotope (Gan & Jeffay, 1967; Russell & Snyder, 1968). The half-life of these and other mitochondrial enzymes may be underestimated. The extent to which this will occur is difficult to predict and may depend on several factors (Poole, 1971). The use of the radiochemical [*guanido*- ^{14}C]arginine has been shown to be less affected by reutilization (Schimke, 1973), but experimentally it proved to be unsatisfactory for our purposes. Therefore the half-life of these and other matrix enzymes determined by a radioisotope-decay method should be considered as 'apparent'. In any case, these observations suggest that regulation of pyruvate carboxylase and pyruvate dehydrogenase at the catalytic level may be more important when metabolic needs change acutely in diabetes. Over longer periods, the diabetic state may involve regulation of pyruvate carboxylase by altered enzyme concentrations.

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