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Active and Inactive Forms of Pyruvate Dehydrogenase in Rat Heart and Kidney: Effect of Diabetes, Fasting, and Refeeding on Pyruvate Dehydrogenase Interconversion

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Pyruvate dehydrogenase (PDH) activity was measured in homogenates from rat heart and kidney. The activity before addition of Mg^{2+} to homogenate was used as a measure of the active form (PDH_a) originally present. The activity after incubation in presence of Mg^{2+} is then representative for the total PDH activity present in the tissue. This assay is justified if it is assumed that Mg^{2+} stimulates PDH phosphatase which catalyzes conversion of the inactive (PDH_b) to the active form of PDH. Marked changes in the amount of PDH_a without significant changes in total PDH activity were observed in heart and kidney from rats subjected to a variety of metabolic conditions and treatments: on fasting PDH_a levels decreased to less than 15% of total activity. On refeeding glucose or fructose PDH_a levels rose to normal (70% of total activity). Similarly PDH_a levels fell to less than 15% of total activity in heart and kidney of alloxan diabetic rats on withdrawal of insulin and were likewise restored to normal on insulin treatment. Treatment of fasted rats with nicotinic acid—an antilipolytic agent—brought back to normal the PDH_a levels lowered by fasting. PDH_a activity but not total PDH activity was inversely related to serum free fatty acid levels. These and other findings point to a possible role of the PDH interconversion for the regulation of pyruvate metabolism. The observations discussed in this report could explain the well known inhibition of pyruvate oxidation in diabetes and in other metabolic conditions where fatty acids are preferentially oxidized.

The pyruvate dehydrogenase complex (PDH[1]) from heart muscle, kidney, liver, and brain is interconvertible. The active form (PDH_a) is converted to an inactive form (PDH_b) by a Mg^{2+}-ATP dependent kinase. Reactivation, i.e., dephosphorylation of PDH_b is catalyzed by phosphatase (1–5). Working with purified PDH preparations from pig heart we observed that the isolated enzyme exists as a mixture of active and inactive forms (4). In order to appraise the physiological significance of PDH interconversion for the regulation of pyruvate metabolism, it seemed of interest to study changes in the ratio of PDH_a and PDH_b in tissues from rats at various metabolic conditions. A preliminary report of these studies has appeared (6).

[1] Abbreviations: PDH, pyruvate dehydrogenase; LDH, lactate dehydrogenase; PTA, phosphotransacetylase; FFA, free fatty acids; TPP, thiamine pyrophosphate.

MATERIALS AND METHODS

Male Sprague-Dawley rats (Wiga, München-Ottobrunn) weighing 200–300 g were fed on a standard laboratory chow (Labortierfutter 57Z, J. Zahn II, Hockenheim, Germany) and water, unless stated otherwise.

Rats were made diabetic by intravenous injection of alloxan (100 mg/kg body wt). The rats were

fasted overnight and anesthetized with ether. Alloxan solutions were freshly prepared with physiological saline and injected into tail veins. During injection blood flow through the kidneys was hindered by gently pressing the kidneys between thumb and forefinger. This helps to minimize kidney lesions by alloxan (R. Koreč, personal communication). Usually fourth-fifths of the rats treated with alloxan became severely diabetic 12–18 hr after alloxan injections. The diabetic rats were injected twice daily subcutaneously with Depot-Insulin (Farbwerke Hoechst, Germany) for 12–14 days (2 units in the morning, 4 units in the evening). On withdrawal of insulin for 2 days severe diabetes had developed.

Adrenalectomy was performed by the dorsal approach. The adrenalectomized animals were fed *ad libitum* and 0.9% NaCl was added to the drinking water. Sham-operated rats served as controls.

Preparation of tissue homogenates. Rats were anesthetized with ether and heart and kidney were excised as rapidly as possible and frozen immediately with a Wollenberger clamp precooled in liquid nitrogen. No more than 5 sec elapsed between opening the thorax and freezing the heart. Removal of the kidneys took no longer than 20 sec.

The frozen tissues were weighed and pulverized in a mortar cooled with liquid nitrogen. The frozen powder was transferred to centrifuge tubes and reweighed. The samples were kept in liquid nitrogen until used. Homogenates were prepared at 2–4° with a high-speed mechanical tissue disintegrator (Ultra-Turrax TP 18/2; Jahnke & Kunkel, Staufen, Germany). A sixfold (heart) or a threefold (kidney) amount (v/w) of ice-cold 20 mM potassium phosphate buffer pH 7.0 containing 40% (v/v) glycerol was added. [A similar technique was used by Danforth *et al.* for measurements of muscle phosphorylase b and a *in vivo* (7).] The blender was driven at top speed for 90 sec. Extraction was completed in this time. The temperature of the homogenate did not rise above −5°.

Pyruvate dehydrogenase assay. PDH activity was measured in crude tissue homogenates by an assay system (8) involving the following reactions:

$$\text{Pyruvate} + \text{NAD}^+ + \text{CoA-SH} \xrightarrow{\text{PDH}} \text{Acetyl-CoA} + \text{NADH} + \text{H}^+ + \text{CO}_2 \quad (1)$$

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \xrightleftharpoons{\text{LDH}} \text{Lactate} + \text{NAD}^+ \quad (2)$$

$$\text{Acetyl-CoA} + \text{P}_i \xrightleftharpoons{\text{PTA}} \text{Acetylphosphate} + \text{CoA-SH} \quad (3)$$

$$2\ \text{Pyruvate} + \text{P}_i \rightarrow \text{Acetylphosphate} + \text{Lactate} + \text{CO}_2 \quad (4)$$

Acetylphosphate formed in the reaction was converted with hydroxylamine to the hydroxamate and the latter determined as Fe(III) complex colorimetrically. The reaction mixture contained potassium phosphate buffer, pH 8.0, 100 mM; $MgCl_2$, 2 mM; mercaptoethanol, 10 mM; NAD^+, 6 mM; thiamine pyrophosphate, 2 mM; sodium pyruvate, 40 mM; crystalline lactate dehydrogenase, 20 μg/ml; and phosphotransacetylase, 10 μg/ml. The reaction mixtures were freshly prepared for each series of experiments. The assay was carried out in sealed microcups (Eppendorf Apparatebau, Netheler & Hinz, Hamburg-Eppendorf, Germany). Into each cup were pipetted 50 μl of the reaction mixture, 5–30 μl of tissue homogenate, and water to a final volume of 100 μl. The reaction was started by the addition of 10 μl of a 10 mM CoA-SH solution (pH 6.5), which was replaced by water for the blanks. Incubation was at 37° for 30 min. The reaction was stopped by adding 100 μl of a freshly prepared mixture (1:1) of 3.5 N NaOH and 2 M $NH_2OH \cdot HCl$. The samples were kept for 10 min at room temperature. The Fe complex was developed by adding 300 μl of the Fe reagent containing equal parts of a solution of 10% $FeCl_3$ in 0.1 N HCl, of 12% trichloroacetic acid, and of 3 N HCl. After centrif-

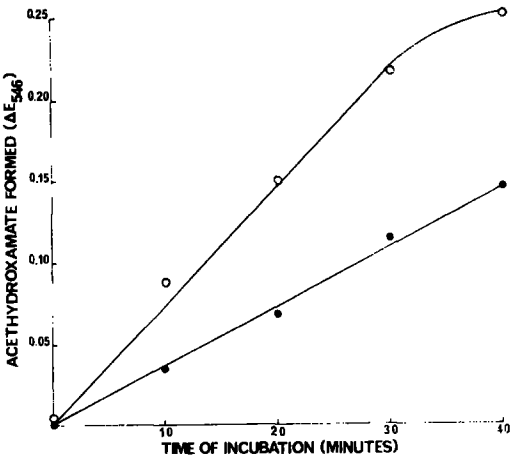

FIG. 1. Time course of hydroxamate formation. Heart muscle homogenate from a 24-hr fasted rat was used. Undiluted homogenate before activation ●——● and 1:4 diluted homogenate after activation ○——○. The linearity of hydroxamate formation with time shows that no activation occurs under assay conditions (pH 8.0).

ugation the extinction was read at 546 nm on the supernatant solution. Acetylhydroxamate formation was linear up to 30 min (Fig. 1), and proportional to protein concentration (Fig. 2). All assays were run at least in duplicate with two or three different homogenate concentrations. When necessary, the homogenates were diluted with 20 mM potassium phosphate buffer, pH 7.0. For standardization of the assay, known quantities of acetylcholine instead of homogenate were assayed using the complete reaction mixture. PDH activity is expressed in units: 1 unit corresponds to formation of 1 μmole acethydroxamate in 30 min at 37° under standard assay conditions.

Table I shows the influence of various components of the system on acethydroxamate formation. Serum FFA were measured by a modified Duncombe procedure (9) as described elsewhere (10). Blood glucose was determined enzymatically with an autoanalyzer (Technicon) (11).

All chemical reagents were analytical grade and purchased from Merck, Darmstadt. Enzymes and coenzymes were products of Boehringer and Sons, Mannheim. Acetylcholine·HCl was a product of Hoffmann La Roche, Grenzach, or Schuchardt, Munich.

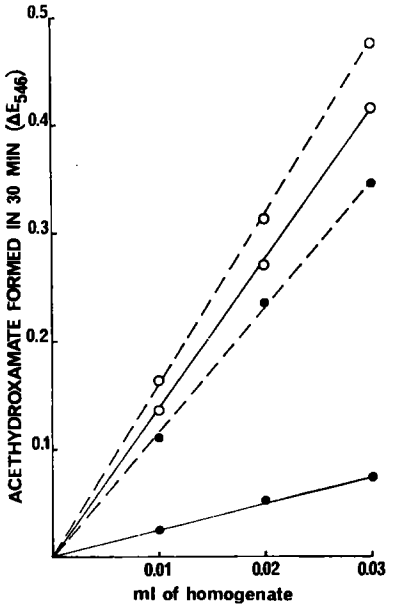

FIG. 2. Dependence of acethydroxamate formation upon the amount of homogenate added. Heart muscle homogenate from 24-hr fasted rat before ●——● and after activation ○——○. Heart muscle homogenate from normal fed rat before ●------● and after ○------○ activation.

TABLE I

COMPONENTS REQUIRED IN THE ASSAY

Component omitted	Acethydroxamate formed in 30 min ΔE_{546}
None	0.079
Pyruvate	0.005
CoA-SH	0
NAD^+	0.004
TPP[a]	0.079
Mg^{2+}	0.068
PTA	0.011
LDH	0.060

[a] Although PDH-bound TPP is usually sufficient for maximal rate of reaction, exogenous TPP was always added to the assay.

TABLE II

DEPENDENCE OF PDH ACTIVATION IN A HEART MUSCLE HOMOGENATE FROM 24-HR FASTED RAT UPON Mg^{2+} CONCENTRATION[a]

Mg^{2+} (mM)	PDH activity (units × 1000)
0	46.2
1.25	70.8
2.5	126.2
5.0	258.5
7.5	215.4
10.0	221.5
12.5	193.8

[a] Aliquots, 10 μl each containing Mg^{2+} as indicated, were incubated as described in Methods.

RESULTS

Active (PDH_a) and inactive (PDH_b) forms of pyruvate dehydrogenase. The original activity of a freshly prepared homogenate is defined as the activity of the active form of pyruvate dehydrogenase (PDH_a). Incubation of the homogenate with Mg^{2+} results in the activation of PDH-phosphatase present in the homogenates. As shown in Table II the rate of activation of the phosphatase was maximal at 7.5–10 mM Mg^{2+}. Hence for the conversion of PDH_b to PDH_a Mg^{2+} in final concentration of 10 mM was added to homogenates which had been diluted 1:4 with 20 mM potassium phosphate buffer pH 7.0. The mixture was incubated for 60 min at 25°. In homogenates fortified with Mg^{2+} all PDH_b present was converted to PDH_a under these conditions. Neither activation nor assay of PDH activity was affected by

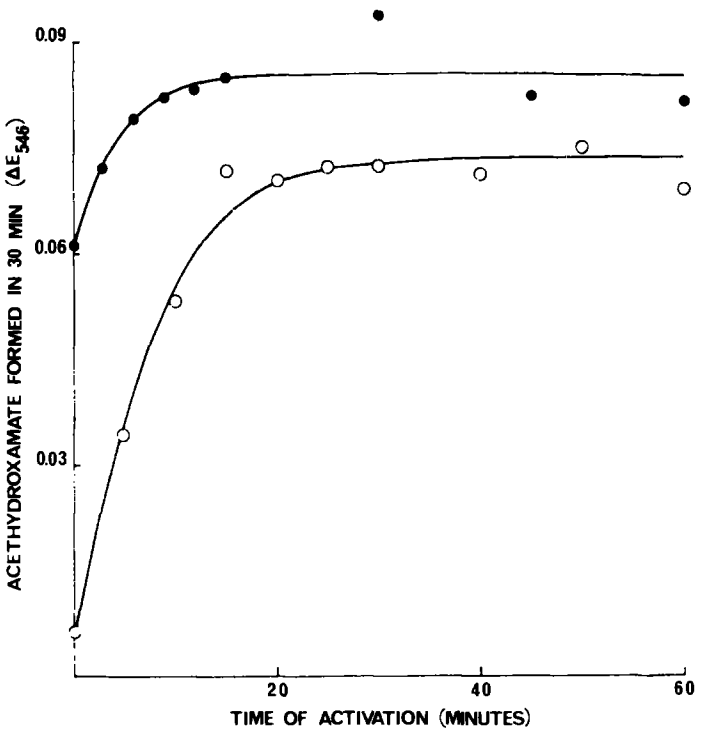

FIG. 3. Time course of Mg^{2+}-dependent activation of PDH in heart muscle homogenates. ●——● homogenate from normal fed rat; ○-- --○ homogenate from overnight fasted rat. The ordinate represents the hydroxamate formed in 30 min of incubation under the assay conditions described in Methods.

TABLE III

INHIBITION OF PDH ACTIVATION BY FLUORIDE[a]

	PDH activity (units × 1000)	
	− NaF	+ NaF
Before activation	98.5	76.9
After activation	356.8	80.0

[a] Aliquots, 20 μl each of a heart muscle homogenate from a 48-hr fasted rat, were activated in the presence of NaF, 80 mM, for 30 min as described in Methods. In the controls NaF was added after activation was complete. Prior to assay NaF was removed by dialysis.

the glycerol present in the 1:4 diluted homogenates. The time course of the PDH_a formation (i.e., activation) is shown in Fig. 3.

Purified pig heart PDH-phosphatase is inhibited by fluoride (E. Siess, unpublished experiments). As shown in Table III NaF also completely abolished the Mg^{2+}-dependent activation of PDH in the crude homogenate. It needs to be shown whether the fluoride inhibition is Mg^{2+}-dependent.

Changes in PDH activity in heart and kidney from normal fed, fasted, and refed rats. As shown in Table IV, heart and kidney from normal fed animals contain about 70% PDH_a. Following a 24-hr fast the active form of PDH is less than 15% in both tissues, but total activity (after conversion to PDH_a with Mg^{2+}) is much less decreased in heart muscle and not at all in kidney homogenates. Longer periods of fasting did not decrease further PDH_a (Fig. 4). When starved rats were refed by stomach tube with glucose, PDH_a activity was fully recovered in 2 hr (Fig. 5). Fructose feeding was as effective as glucose feeding in raising the PDH_a level in kidney of starved rats but somewhat less effective in the heart. Refeeding rats with fat in form of olive oil instead of sugar did not raise PDH_a levels in heart and kidney (see Fig. 6).

PDH activities in heart and kidney from

TABLE IV

PDH ACTIVITY IN HEART AND KIDNEY FROM FED AND FASTED RATS[a]

	Heart muscle		Kidney	
	Fed (n = 35)	24 Hr fasted (n = 34)	Fed (n = 21)	24 Hr fasted (n = 21)
Before activation (PDH_a)	139.2 ± 9.2	23.0 ± 3.1	48.9 ± 3.3	10.9 ± 1.5
	($p < 0.001$)		($p < 0.001$)	
PDH after activation	200.3 ± 11.6	162.7 ± 10.4	72.3 ± 5.9	85.1 ± 5.7
	($p = 0.02$)		(n.s.)	
PDH_a in % of total	69.5	14.2	67.6	12.8

[a] Mean values ± SEM are given. n = Number of experiments. p = statistical significance according to Student's t test.

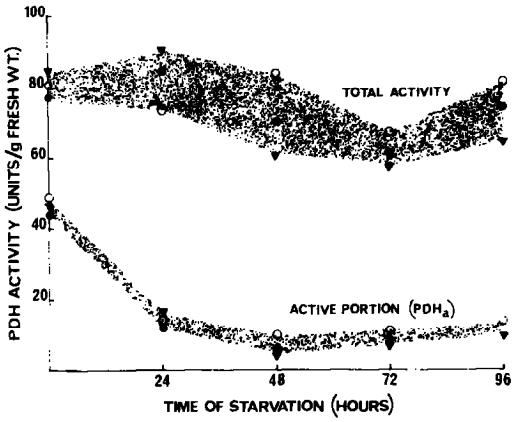

FIG. 4. Changes in PDH activities in rat kidney on fasting. Three animals (○, ●, ▲) were fasted for the times indicated and sacrificed. Identical results were obtained with heart muscle homogenates.

diabetic rats. A decline in PDH_a also occurred in heart and kidney from diabetic rats fed on a standard diet after withdrawal of insulin (see Fig. 7). Insulin treatment of the alloxan diabetic rats raised the PDH_a levels towards normal.

The effect of treatment of fasted rats with nicotinic acid on PDH activities. On starvation and in diabetes plasma FFA are elevated. Nicotinic acid, an antilipolytic agent, lowers plasma FFA (12). It seemed therefore of interest to see what effect treatment with nicotinic acid may have on PDH activity. The results are presented in Fig. 8. Nicotinic acid treatment caused a drop in serum FFA levels in starved rats and PDH_a levels were raised to normal in kidney and heart.

In Fig. 9 serum FFA levels are plotted against the percentage of PDH_a present in heart muscle. Results indicate that the higher serum FFA concentrations, the lower the PDH_a level in the muscle.

Effect of adrenalectomy on PDH activity. From the data in Table V it is apparent that adrenalectomy neither altered the level of PDH_a present in kidney and heart of starved rats nor the conversion (i.e., activation) by Mg^{2+}.

DISCUSSION

Our studies allow for the conclusion that PDH interconversion occurs *in vivo* in rat heart and kidney and that PDH interconversion is under metabolic control. Although the final proof will require the isolation of the [^{32}P]-labeled PDH protein following addition of γ-[^{32}P]-labeled ATP, Mg^{2+} dependency (Table II), time course of the activation (Fig. 3), and inhibition by fluoride (Table III) make it likely that the activation which takes place in the homogenate and *in vivo* is the consequence of a phosphatase catalyzed dephosphorylation of PDH_b.

As to the metabolic controls that are responsible for the conversion of PDH_a to PDH_b little is as yet known conclusively. The data in Fig. 9 suggest that an increased supply of FFA may play a role. Recent work with the perfused isolated rat heart in our laboratory supports this notion (13). Perfusion with palmitate caused a marked lowering of PDH_a similar to that observed here after fasting and in the diabetic state. Consistent with this idea is the relationship

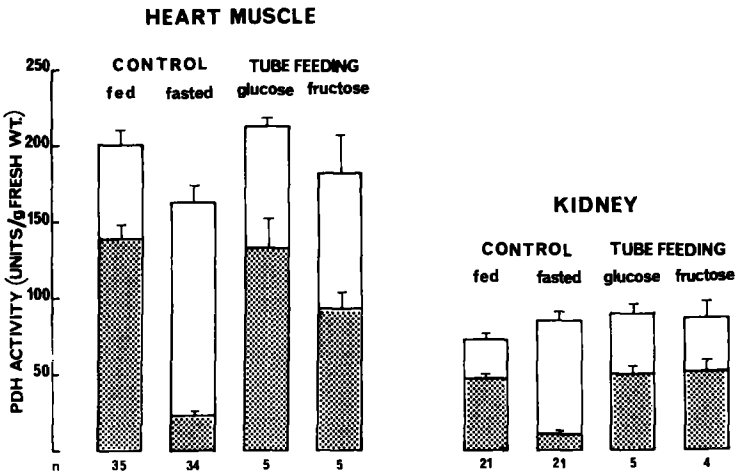

FIG. 5. Changes in PDH_a levels on fasting and refeeding. Total height of the bars represents the total PDH activity (after activation) and the height of the dotted areas the PDH_a activity, in the same units. Mean values ±SEM are given. Rats fasted for 24 hr received 3 ml of 7.5% glucose or fructose solution, respectively, by stomach tube and were sacrificed 2 hr later. For comparison the values for 24-hr fasted rats and for rats fed on standard laboratory diet are given.

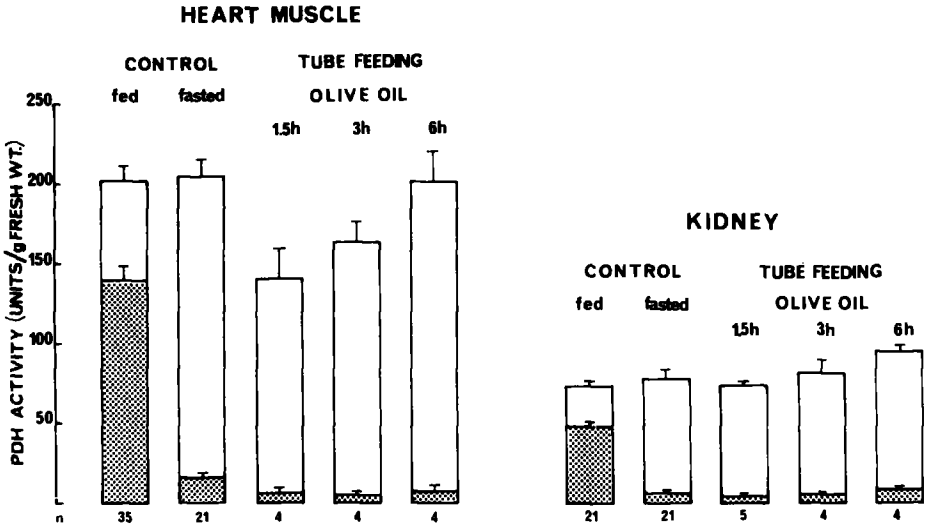

FIG. 6. Changes in PDH_a levels on fasting and refeeding olive oil. Rats fasted for 48 hr received 3.5 ml of olive oil by stomach tube and were sacrificed at the times indicated. Feeding of a 0.9% NaCl solution had no effect. For comparison the values for 48-hr fasted rats and for rats fed on a standard laboratory diet are given. Symbols are those in Fig. 5.

of FFA levels and PDH interconversion in the nicotinic acid-treated rat (see Fig. 8).

In the glycogen synthetase system the b form is converted to the a form by enzymatic mechanisms which seem to involve, as in the case of the PDH system, removal of a covalently bound phosphate (14). Mersmann and Segal (15) showed that the enzyme system that converts hepatic glycogen synthetase b to synthetase a is inactive in the liver of fasted adrenalectomized rats. This is different from the PDH system of heart

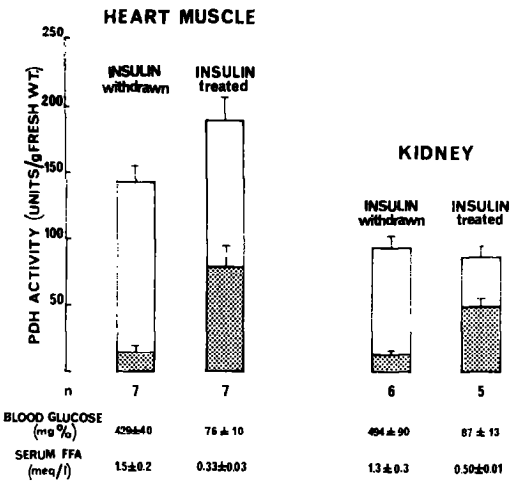

FIG. 7. Changes in the levels of PDH_a in diabetic rats on withdrawal of insulin. For details see Methods. Symbols are those in Fig. 5.

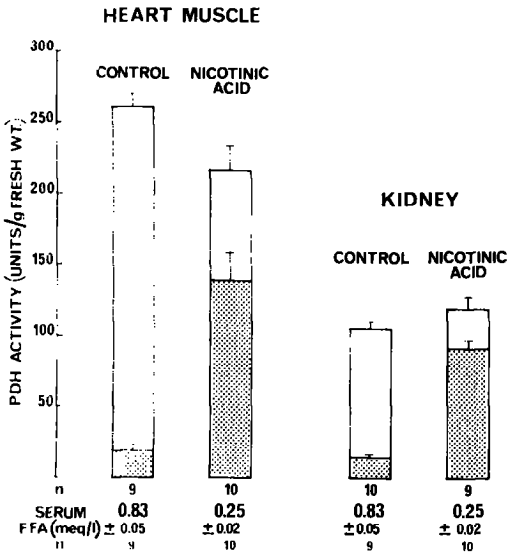

FIG. 8. Changes in levels of PDH_a on treatment with nicotinic acid. Nicotinic acid, 250 mg/kg body wt was injected sc as a neutral solution to 24-hr starved rats 2 hr before sacrifice. Controls are 24-hr fasted rats treated with saline. Symbols are those in Fig. 5.

and kidney since PDH can undergo activation by conversion in adrenalectomized, 48-hr fasted rats (see Table V). Pyruvate oxidation is suppressed in heart and diaphragm of diabetic rats (16, 17). Moreover the amount of glucose or pyruvate that is oxidized to CO_2 is markedly reduced in the diabetic heart or in the normal heart perfused with fatty acids or ketone bodies (18–21). From these and other experiments it was concluded that PDH activity is inhibited by acetyl-CoA (22, 23). Whether acetyl-CoA which increases under these conditions also affects directly or indirectly active and inactive PDH or whether the acetyl-CoA inhibition is a separate mechanism for the control of pyruvate oxidation in starvation and diabetes is not known yet. Perhaps both mechanisms—interconversion and regulation of activity by acetyl CoA—act in concert in the control of this important metabolic reaction. Hormones with lipolytic or antilipolytic action that control the release of FFA might then indirectly control pyruvate oxidation by producing control metabolites.

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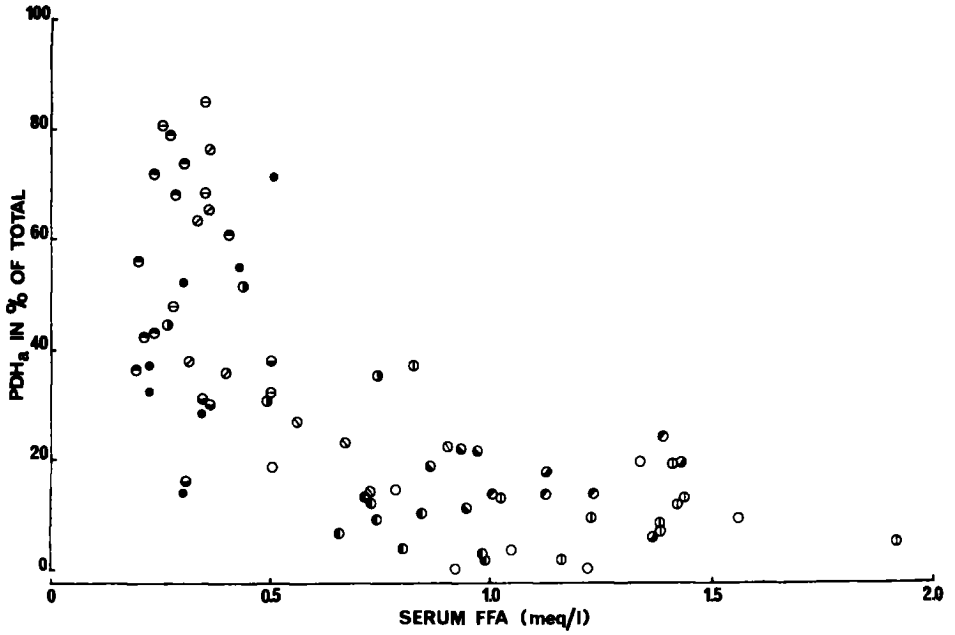

FIG. 9. Relationship between serum FFA and PDH$_a$ levels in rat heart.
● alloxan diabetic, insulin treated
○ alloxan diabetic, insulin withdrawn
◐ 24 hr fasting
◫ 48 hr fasting
◓ 24 hr fasting, nicotinic acid treated
⊖ refed on glucose
⊘ refed on fructose
⦸ 48 hr fasted, 1.5 hr refed on rusk
◒ 48 hr fasted, 3 hr refed on rusk
◑ 48 hr fasted, 6 hr refed on rusk
◔ 48 hr fasted, 1.5 hr refed on egg white
◪ 48 hr fasted, 3 hr refed on egg white
◩ 48 hr fasted 6 hr refed on egg white

TABLE V

PDH ACTIVITY IN HEART AND KIDNEY FROM ADRENALECTOMIZED RATS[a]

Organ	PDH activity before activation		PDH activity after activation	
	Sham-operated	Adrenalectomized	Sham-operated	Adrenalectomized
Heart muscle	(n = 6)	(n = 7)	(n = 6)	
	10.5 ± 2.1	9.0 ± 1.4	212.0 ± 11.7	250.6 ± 9.3
	(n.s.)		($p < 0.05$)	
Kidney	(n = 6)	(n = 7)	(n = 6)	(n = 7)
	4.8 ± 1.3	4.6 ± 1.0	80.6 ± 12.5	56.6 ± 1.3
	(n.s.)		($p < 0.1$)	

[a] Mean values ± SEM are given. n = Number of determinations; p = significance according to Student's t test. The animals were fasted for 48 hr and sacrificed 11 days after the operation.

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