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The Mode of Regulation of Pyruvate Dehydrogenase of Lactating Rat Mammary Gland

EFFECTS OF STARVATION AND INSULIN

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1. The 'initial activity' of the pyruvate dehydrogenase enzyme complex in whole tissue or mitochondrial extracts of lactating rat mammary glands was greatly decreased by 24 or 48h starvation of the rats. Injection of insulin and glucose into starved rats 60min before removal of the glands abolished this difference in 'initial activities'. 2. The 'total activity' of the enzyme complex in such extracts was revealed by incubation in the presence of free Mg^{2+} and Ca^{2+} ions (more than 10 and 0.1mM respectively) and a crude preparation of pig heart pyruvate dehydrogenase phosphatase. Starvation did not alter this 'total activity'. It is assumed that the decline in 'initial activity' of the enzyme complex derived from the glands of starved animals was due to increased phosphorylation of its α-subunit by intrinsic pyruvate dehydrogenase kinase. 3. Starvation led to an increase in intrinsic pyruvate dehydrogenase kinase activity in both whole tissue and mitochondrial extracts. Injection of insulin into starved animals 30min before removal of the lactating mammary glands abolished the increase in pyruvate dehydrogenase kinase activity in whole-tissue extracts. 4. Pyruvate (1mM) prevented ATP-induced inactivation of the enzyme complex in mitochondrial extracts from glands of fed animals. In similar extracts from starved animals pyruvate was ineffective. 5. Starvation led to a decline in activity of pyruvate dehydrogenase phosphatase in mitochondrial extracts, but not in whole-tissue extracts. 6. These changes in activity of the intrinsic kinase and phosphatase of the pyruvate dehydrogenase complex of lactating rat mammary gland are not explicable by current theories of regulation of the complex.

Starvation for 24 or 48h has been reported to cause inactivation of the mitochondrial enzyme pyruvate dehydrogenase (EC 1.2.4.1) in rat heart, kidney, adipose tissue and lactating mammary tissue (Wieland *et al.*, 1971*a*; Stansbie *et al.*, 1976; Kankel & Reinauer, 1976). This inactivation can be regarded as part of a glucose-sparing adaptation. Its mechanism involves phosphorylation of the α-subunit of the pyruvate dehydrogenase complex. The metabolic signals that are responsible seem likely to include the increased plasma non-esterified long-chain fatty acid and acetoacetate and decreased plasma insulin concentration that one expects in starvation.

Experiments *in vitro* indicate that an increased supply of long-chain fatty acids can lead to increased phosphorylation of pyruvate dehydrogenase in heart, liver and kidney (Wieland *et al.*, 1971*b*; Patzelt *et al.*, 1973; Guder & Wieland, 1974). Perfusion of isolated rat heart with acetoacetate had a similar effect (Wieland *et al.*, 1971*b*). Insulin is reported to act directly on adipose tissue (Jungas, 1970; Coore *et al.*, 1971) and on liver (Topping *et al.*, 1977) to stimulate pyruvate dehydrogenase of these tissues by diminishing its degree of phosphorylation.

Experiments with isolated mitochondria from liver, heart and adipose tissue and with purified enzyme from heart and kidney have indicated various mitochondrial factors that could mediate effects of long-chain fatty acids or insulin on the phosphorylation status of pyruvate dehydrogenase (reviewed by Denton *et al.*, 1975). These factors, which are the concentrations of pyruvate, free Mg^{2+}, free Ca^{2+} and the ratios of [acetyl-CoA]/[CoA], [NADH]/[NAD^+] and [ATP]/[ADP], influence the relative activities of the kinase and phosphatase associated with the pyruvate dehydrogenase complex, which in turn control the degree of phosphorylation of the α-subunit responsible for the initial decarboxylation of pyruvate.

It has been implicitly assumed that the above factors act only by altering transiently either the susceptibility of the α-subunit to attack by the

kinase or phosphatase or the catalytic efficiency of the latter enzymes. In neither case would one expect effects of such factors on rates of phosphorylation or dephosphorylation to persist when the complex is liberated from its mitochondrial environment. Insofar as one could dilute out or control the above factors in the new environment of the enzyme complex one would expect a disappearance of initial differences in behaviour of enzyme complexes obtained from animals in different physiological states, e.g. fed versus starved. We have attempted to test this expectation with the pyruvate dehydrogenase complex of lactating rat mammary gland, since previous work showed that the degree of phosphorylation of this enzyme is markedly increased by starvation (Kankel & Reinauer, 1976) as well as by insulin deprivation (Field & Coore, 1976). We have concluded that transient allosteric or competitive binding effects by the known effectors of the pyruvate dehydrogenase kinase and phosphatase cannot explain our results, and an additional mode of regulation of the kinase and phosphatase activity is indicated. A preliminary report of some of this work has appeared (Baxter & Coore, 1978).

Experimental

Materials, preparation of whole-tissue extracts, enzyme assays and units and adenine nucleotide assays were generally as given by Coore & Field (1974). Modifications are indicated in legends to Figures or Tables. The animals used were Wistar rats 11–14 days advanced in their first lactation and with at least 10 pups. Soluble insulin was from Burroughs Wellcome and Co., London N.W.1, U.K., and Lubrol 12 A9 was from I.C.I., Macclesfield, Cheshire, U.K. One unit of insulin represents 40μg of pure hormone.

Mitochondria were prepared as described by Titheradge & Coore (1977). Mitochondria at a concentration of 17mg/ml in 1ml of 30mM-triethanolamine/HCl, 0.1% (w/v) Lubrol, 1mM-dithiothreitol, pH7, 5% (v/v) rat serum, were broken by sonication for 2 × 5s at 4°C. Extracts were centrifuged for 5min at 14000g and the supernatants (which contained 85% of the pyruvate dehydrogenase activity in the lysate) were used in the experiments reported. Protein in mitochondrial and whole-tissue extracts was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

'Total pyruvate dehydrogenase activity' referred to in the text or Tables means that maximum activity observed in a tissue homogenate or mitochondrial lysate incubated at 30°C and to which was added 10mM-$MgCl_2$ and 0.1mM-$CaCl_2$ and pig heart pyruvate dehydrogenase phosphatase (10%, v/v). Serial samples were always taken to establish that maximal activity was in fact achieved by 5min in most cases and invariably by 10min. Pig heart pyruvate dehydrogenase phosphatase was obtained essentially as described by Coore & Field (1974), except that it was found convenient to prepare it at 2-weekly intervals from 10ml batches of the pig heart pyruvate dehydrogenase complex (2–3units/ml) stored in solution at −17°C. After thawing, the sample was centrifuged at 180000g for 90min at 4°C. The supernatant containing the pyruvate dehydrogenase phosphatase but negligible pyruvate dehydrogenase activity was concentrated 10-fold by using Amicon filters (CF25-membrane cones: Amicon Corp., Lexington, MA, U.S.A.). This preparation contained about 40 units of phosphatase activity/ml as defined by Siess & Wieland (1972). This solution of pyruvate dehydrogenase phosphatase was stored at −17°C in small portions each sufficient for 1 day's work.

'Initial pyruvate dehydrogenase activity' of tissue extracts was that activity observed when freeze-clamped tissue was extracted in the presence of EDTA sufficient to bind all Mg^{2+} and Ca^{2+}. Such extracts did not show any change in pyruvate dehydrogenase activity when incubated at 30°C for 10min.

'Percentage total activity' of pyruvate dehydrogenase is the enzymic activity observed at any given time or in any given condition relative to the total pyruvate dehydrogenase activity in the same extract taken as 100%.

Data in text, Tables or Legends to Figures are given as means ± S.E.M., with numbers of observations in parentheses. Statements of statistical significance are based on Student's t test.

Time course of ATP-induced inactivation and Mg^{2+}/Ca^{2+}-dependent activation of mammary-gland pyruvate dehydrogenase

The validity of such time courses depended on the stability of the enzyme preparation during the incubation period and also on stopping the phosphorylation or dephosphorylation of a given enzyme sample at a particular time. Instability of the enzyme during incubation at 30°C, i.e. non-ATP-dependent and irreversible loss of activity, was only marked in mitochondrial lysates. A similar phenomenon has been noted in liver mitochondrial lysates (Portenhauser & Wieland, 1972). Inclusion of rat serum in the incubation medium diminished this loss of activity. However, with our preparation there was still variable instability, amounting to as much as 10% loss of total activity by 1min and 25% by 5min. It was therefore necessary to run parallel controls without ATP for experiments involving ATP inactivation of mitochondrial extracts and to express

the inactivation by ATP in relation to the appropriate time-point control. There was no systematic difference in the stability of pyruvate dehydrogenase in mitochondrial extracts of glands from starved compared with fed animals. Parallel controls without ATP were also run for experiments involving whole-tissue homogenates as in Fig. 1, even though correction for non-ATP-dependent loss of activity was not generally necessary.

With regard to prompt stopping of phosphorylation or dephosphorylation when the sample (25μl or less) was added to cuvettes containing 965–990μl of assay medium, the dilution of effectors of the enzyme complex would be a major factor, especially in respect of Mg^{2+}, where the K_m value of the pyruvate dehydrogenase phosphatase for this ion is reported to be 0.5–1mM for the enzyme from those mammalian tissues where this parameter has been determined (Denton *et al.*, 1975). Since, however, the K_m for ATP of the mammalian pyruvate dehydrogenase kinase is generally rather low (about 0.02mM; Denton *et al.*, 1975), we expected that dilution of the sample in the assay cuvette might be insufficient to halt phosphorylation and inactivation. To diminish degradation and phosphorylation, cuvettes containing assay medium and samples were therefore kept on ice up to 2min before assay. Further, pyruvate and thiamin pyrophosphate were present in the assay medium, and this would be expected to slow phosphorylation (Denton *et al.*, 1975). The assay was begun by addition of CoA. It was checked that these precautions were sufficient by assaying duplicate cuvettes containing samples taken at the same moment from an extract of mammary gland incubated with 5mM-ATP. The duplicate cuvettes were treated as stated above, but the reactions in the cuvettes were initiated at different times with a 10min interval between. No difference in the rates of the pyruvate dehydrogenase reactions in such cuvettes was observed. In other experiments ATP was added to cuvettes during the linear phases of the assays of samples of mammary-gland extracts for pyruvate dehydrogenase activity. The final concentration of ATP in the cuvettes was what would be expected when assaying samples taken during the normal procedure for ATP-induced-inactivation time-course experiments. Under these circumstances no change in the rate of the pyruvate dehydrogenase reaction was observed. Both the above types of experiment were performed with gland extracts from fed and starved animals. We concluded that no phosphorylation of mammary-gland pyruvate dehydrogenase occurred after samples of extracts incubated with ATP were added to cuvettes containing the assay medium.

Results

Whole tissue extracts from freeze-clamped gland

Table 1 shows that in none of the conditions studied was there any change in the total pyruvate dehydrogenase activity of the gland when expressed on a protein basis. However, starvation for 24 or

Table 1. *Total activity and percentage total activity of pyruvate dehydrogenase in lactating mammary glands of rats*

'Total' and 'initial' activities of pyruvate dehydrogenase were determined in freeze-clamped glands as described in the Experimental section. Percentage total activity (column 3) was calculated as 100 × initial/total activities for each animal. Numbers in parentheses are observations on which preceding S.E.M. values were based. ** $P < 0.01$ for difference from data for fed animals. Insulin and 0.9% NaCl were injected intraperitoneally, glucose was injected subcutaneously.

Treatment of animal before freeze-clamping	Total pyruvate dehydrogenase activity (munits/mg of protein)	Percentage total activity of pyruvate dehydrogenase	Plasma glucose (mM) at time of freeze-clamping
Fed animals	10.0 ± 0.4 (6)	40.3 ± 2.4 (6)	6.9 ± 0.7 (3)
24h-starved animals	11.8 ± 0.8 (8)	11.8 ± 0.6 (4)**	—
48h-starved animals	9.2 ± 1.0 (3)	9.2 ± 0.9 (3)**	—
24h-starved animals injected with 0.5ml of 0.9% NaCl 30min before freeze-clamping	10.3 ± 0.7 (4)	9.2 ± 0.5 (4)**	6.3 ± 0.6 (7)
24h-starved animals injected with 10 units of soluble insulin 30min before freeze-clamping	9.90 ± 0.3 (9)	27.3 ± 1.7 (7)**	2.1 ± 0.4 (9)**
24h-starved animals injected with 10 units of soluble insulin and 2ml of 3M-glucose 30min before freeze-clamping	10.0 ± 0.8 (4)	33.9 ± 2.9 (4)	5.6 ± 0.6 (4)
24h-starved animals injected with 10 units of soluble insulin and 2ml of 3M-glucose 60min before freeze-clamping	10.3 ± 1.0 (4)	50.1 ± 5.1 (4)	5.1 ± 0.4 (4)

48h led to a large fall in the 'initial' activity of the enzyme, presumably reflecting an increase in the degree of phosphorylation *in vivo*. This is in agreement with Kankel & Reinauer (1976). Robinson & Williamson (1977) have also concluded that starvation for 24h led to inactivation of pyruvate dehydrogenase of mammary gland *in vivo* as deduced from the increased output of pyruvate and lactate.

Table 1 also shows that a large dose of insulin was able partially to restore 'initial' activity of the enzyme towards normal values, i.e. those observed in fed animals by 30min. Injection of insulin and sufficient glucose to prevent a significant fall in the plasma glucose concentration led, after 60min, to complete restoration of normal 'initial' activity of pyruvate dehydrogenase in the mammary gland.

Activity of pyruvate dehydrogenase kinase in extracts

Fig. 1 compares the time courses of inactivation by ATP of pyruvate dehydrogenase extracted from glands of fed and from 24h-starved animals. Similar data from glands of 48h-starved animals were statistically indistinguishable from those of the 24h-starved animals. Unfortunately, the data do not fit on linear semi-logarithmic plots, so it is not possible to derive half-times for the rates of inactivation. Nevertheless, the effect of starvation to increase the rate and extent of ATP-induced-inactivation is clear-cut and statistically significant at every time point. Further, it appeared that at 30min after injection of insulin into 24h-starved animals the behaviour of mammary-gland pyruvate dehydrogenase kinase had been restored to that of the fed state. Incubation of gland extracts from such animals (after activation by exogenous pyruvate dehydrogenase phosphatase) with 5mM-ATP for 1min led to only a small decrease in pyruvate dehydrogenase activity [15 ± 1.4% (5)], compared with a fall of 22.1 ± 4.8% (8) for extracts from fed animals and 47.1 ± 3% (4) for extracts from starved and 0.9% NaCl-injected animals.

For pyruvate dehydrogenase kinase from other tissues it has been shown that ADP is a competitive but relatively weak inhibitor of ATP-induced inactivation of the α-subunit activity (Hucho *et al.*, 1972). It was therefore important to determine whether the rate of ATP degradation and ADP formation differed in extracts of glands from fed and starved animals. Table 2 shows that there was in fact no significant difference in ATP-degradation rate, and therefore one cannot attribute the differences in rate of pyruvate dehydrogenase inactivation to different [ATP]/[ADP] ratios during incubation. The actual rates of decline of pyruvate dehydrogenase activity in extracts from both fed and starved rats are slower than observed for semi-purified enzyme

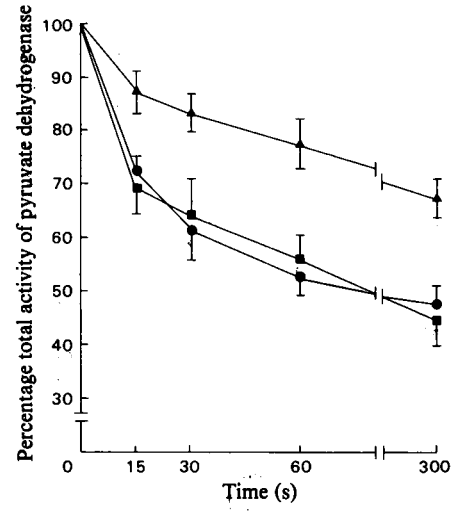

Fig. 1. *Percentage total activity of pyruvate dehydrogenase in extracts of lactating mammary glands of fed or starved rats incubated with 5mM-ATP.*

Glands from eight fed rats (▲), four 24h-starved (●) and four 48h-starved rats (■) were extracted into ice-cold 30mM-triethanolamine/HCl, pH7, 7mM-mercaptoethanol (200–300mg wet wt. into 2ml of above medium). After full activation of pyruvate dehydrogenase with exogenous pyruvate dehydrogenase phosphatase, 5mM-EGTA was added. The time course of inactivation of the enzyme by added 5mM-ATP at 30°C was then determined as described in the Experimental section. The protein concentration of these extracts was 20.1 ± 0.8(10)mg/ml, corresponding to a total pyruvate dehydrogenase activity of 0.215 ± 0.009unit/ml. $P < 0.01$ for differences between data from fed and starved animals at all time points.

(Coore & Field, 1974). Similar slow rates of inactivation of pyruvate dehydrogenase by ATP have been observed in adipose-tissue homogenates (Mukherjee & Jungas, 1975). Since we do not have values for the K_m for ATP of the mammary-gland pyruvate dehydrogenase kinase and also its K_i for ADP, one cannot determine whether the slow rate of ATP-induced inactivation in crude homogenates is entirely or partly explicable by the rapid hydrolysis of ATP in the preparation (0.02μmol/min per mg of protein).

High [Mg^{2+}] and micromolar concentrations of Ca^{2+} are known to be inhibitory to the kinase of the pig heart pyruvate dehydrogenase complex (Cooper *et al.*, 1974). In the experiments of Fig. 1 and Table 2 all extracts were exposed to the same high Mg^{2+} concentrations and effectively zero free Ca^{2+} concentrations owing to the presence of 5mM-EGTA. Omission of EGTA diminished the rate and extent

Table 2. *Adenine nucleotides in incubation media of extracts of lactating rat mammary glands*
Extracts used for data of Fig. 1 were also incubated with 5mM-ATP at 30°C, acidified with $HClO_4$ (0.18M) after 5min and adenine nucleotide concentrations determined. Values are expressed as mmol/litre, ±S.E.M. with numbers of extracts in parentheses.

Treatment of animal	[ATP]	[ADP]	[AMP]
Fed	3.27 ± 0.09 (3)	0.70 ± 0.05 (3)	0.56 ± 0.08 (3)
24h-starved	2.74 ± 0.34 (3)	0.70 ± 0.11 (3)	0.37 ± 0.05 (3)
48h-starved	2.50 ± 0.48 (3)	0.74 ± 0.09 (3)	0.36 ± 0.05 (3)

Table 3. *Activation of pyruvate dehydrogenase in homogenates of lactating mammary glands from fed and starved rats*
The experiments were done on three successive days involving on each day a single fed and a single starved animal. Glands were extracted as in the legend to Fig. 1. Total pyruvate dehydrogenase activity was determined for each extract as described in the Experimental section. Since care was taken to extract equal weights from tissue of fed or starved animals the concentration of total pyruvate dehydrogenase activity was the same in both extracts (0.2unit/ml). The percentage total activity of pyruvate dehydrogenase at various times during incubation of the extracts from glands of starved animals was determined in the absence of exogenous pyruvate dehydrogenase phosphatase, but in the presence of added 10mM-$MgCl_2$ and 0.1mM-$CaCl_2$ (column 2). Extracts from fed and starved animals were then centrifuged at 180000*g* for 90min and recombined as indicated for headings of columns 3 and 4; the sediment from the gland of starved animals was divided into two portions to ensure equality in total pyruvate dehydrogenase activity in the recombined suspensions. The time course of activation of pyruvate dehydrogenase in these recombined suspensions was then determined. There was no pyruvate dehydrogenase activity in these high-speed supernatants nor was there pyruvate dehydrogenase phosphatase activity in the sediment. Results are means ± S.E.M. of three observations in each case, each observation involving a different pair of fed and starved animals.

		Percentage total activity of pyruvate dehydrogenase	
Time of incubation (min)	Homogenate of glands from starved rats	High-speed supernatant of mammary-gland homogenates from starved rats and high-speed sediment of same homogenates	High-speed supernatants of mammary-gland homogenates from fed rats and high-speed sediment of homogenates from starved rats
0	9.4 ± 0.6	23.9 ± 4.4	23.8 ± 4.4
5	52.9 ± 1.8	53.2 ± 2.9	52.7 ± 4.1
10	70.1 ± 1.5	66.1 ± 4.8	66.8 ± 2.5
15	76.3 ± 3.1	71.2 ± 4.4	72.9 ± 3.3

of inactivation by ATP (results not shown). Preliminary experiments established that increase in the concentration of EGTA to 10mM during incubation of extracts from fed or starved animals with 5mM-ATP did not increase the rate of enzyme inactivation. On the other hand, 5mM-EGTA was adequate to prevent significant activity of endogenous or exogenous pyruvate dehydrogenase phosphatase during the 5min periods of these incubations. Extracts of glands from three fed rats were incubated in a medium containing 30mM-triethanolamine, pH7, 7mM-mercaptoethanol, 0.1mM-$CaCl_2$, 10mM-$MgCl_2$, 5mM-EGTA and 10% (v/v) pig heart pyruvate dehydrogenase phosphatase. These are the conditions obtained during experiments involving ATP-induced inactivation. Percentage total activity of pyruvate dehydrogenase in such extracts was 64.6 ± 6.8 (3) at zero time and was 70.1 ± 6 (3) after 5 and 10min at 30°C.

The degree of dilution of the enzyme complex and of any putative bound effectors during these experiments can be estimated as follows. The concentration of total enzyme activity in these crude extracts was about 0.25unit/ml. In mitochondria the total pyruvate dehydrogenase activity was 50units/mg of protein, and it has been shown (Titheradge & Coore, 1977) that the matrix space of those mitochondria is about 0.5μl/mg. Therefore the apparent matrix concentration of pyruvate dehydrogenase activity is about 100units/ml. Consequently the enzyme complex and its mitochondrial effectors had been diluted about 400-fold when it was exposed (in these experiments) to 5mM-ATP. It seems therefore rather unlikely that persisting differences in mitochondrial concentrations of pyruvate or concentration ratios of acetyl-CoA/CoA or NADH/NAD^+ could contribute to our results. Further, preincubation of extracts from the glands of starved rats with 1mM-CoA and 1mM-NAD^+ for 5min did not modify the subsequent time course of ATP-induced inactivation.

Table 4. *Effects on extractable pyruvate dehydrogenase activity of incubation of mitochondria from lactating rat mammary glands*

Mitochondria were incubated for 5min at 30°C in medium containing 30mM-triethanolamine/HCl, pH7, and 7mM-mercaptoethanol with stated additions. The mitochondrial concentration was 17mg of protein/ml. After incubation, the mitochondrial suspension was diluted 40-fold with ice-cold isolation medium and the mitochondria were sedimented by centrifugation at 20000g for 1min and then frozen in liquid N_2. Thawed mitochondrial pellets were extracted as described in the Experimental section and total pyruvate dehydrogenase activity and percentage total activity of the enzyme determined. Numbers in parentheses are numbers of mitochondrial preparations on which preceding S.E.M. values are based. $^{**}P < 0.01$ for difference from data of unincubated mitochondria.

Source of mitochondria ...	Total pyruvate dehydrogenase activity (munits/mg of protein)		Percentage total activity of pyruvate dehydrogenase	
	Fed rats	24h-starved rats	Fed rats	24h-starved rats
No incubation	50.0 ± 1.9 (8)	50.0 ± 2.6 (8)	89.0 ± 3.5 (3)	8.5 ± 0.4 (3)
Incubation for 5min with 10mM-pyruvate and oligomycin (0.5μg/ml)	50.8 ± 2.0 (4)	47.0 ± 3.6 (5)	100.0 ± 0.0 (4)**	9.2 ± 1.3 (5)
Incubations for 5min with 5mM-ATP and oligomycin (0.5μg/ml)	49.1 ± 3.4 (4)	52.7 ± 3.6 (6)	32.5 ± 2.2 (4)**	0.4 ± 0.2 (6)**

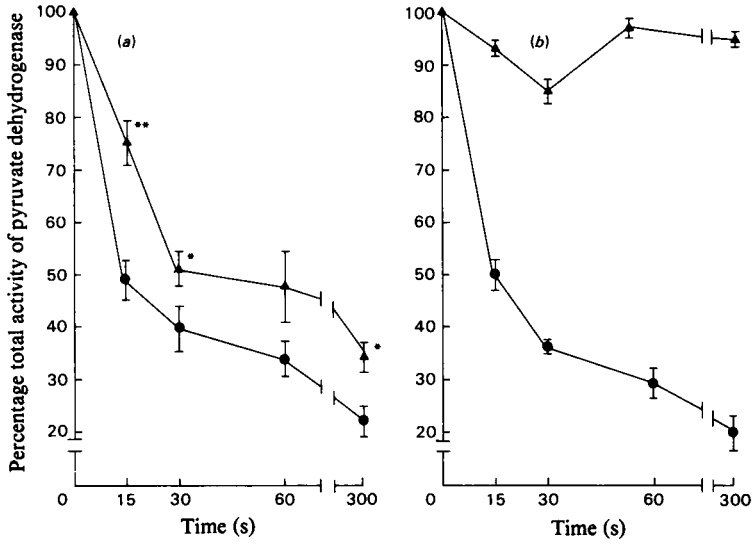

Fig. 2. *Percentage total activity of pyruvate dehydrogenase in lysates of mitochondria from lactating mammary glands of fed or starved rats incubated with 5mM-ATP (a) and with 5mM-ATP and 1mM-pyruvate (b)*

Mitochondria were prepared and extracted as described in the Experimental section and the extracts treated similarly to whole-tissue extracts as described in the legend to Fig. 1 for experiments of Fig. 2(*a*) and with the addition of 1mM-pyruvate for experiments of Fig. 2(*b*). The protein concentration of these extracts was 17 ± 0.6 (16)mg/ml and the total pyruvate dehydrogenase activity was 0.85 ± 0.07 (16)units/ml. ●, Data from 24h-starved rats; ▲, Data from fed rats. Vertical bars indicate S.E.M. Each point was based on four to six observations each involving a separate mitochondrial preparation. (*a*) $^{*}P < 0.05$ for difference from data of starved rats at corresponding time points; $^{**}P < 0.01$ for difference from data of starved rats at corresponding time points. (*b*) Differences between data from starved and fed rats were significant ($P < 0.001$) at all time points.

Pyruvate dehydrogenase phosphatase activity in extracts

Comparing pyruvate dehydrogenase phosphatase activity in whole-tissue extracts from fed with that from starved animals was complicated by our inability to inactivate pyruvate dehydrogenase from fed animals to the same extent as that from starved, and therefore the concentration of substrate (pyruvate dehydrogenase phosphate) relative to the concentration of the complex would not be the same in both

Table 5. *Adenine nucleotides in incubation media of lysates of mitochondria of lactating rat mammary gland*
Other portions of the lysate used for data of Fig. 2(*a*) were incubated with 5mM-ATP at 30°C, acidified with $HClO_4$ (0.18M) after 5min and adenine nucleotide concentrations determined. Values are expressed as mmol/litre, ±S.E.M., with numbers of extracts in parentheses.

Treatment of animal	[ATP]	[ADP]	[AMP]
Fed	2.48 ± 0.14 (12)	0.68 ± 0.15 (3)	1.46 ± 0.09 (3)
24h-starved	2.34 ± 0.22 (8)	0.72 ± 0.04 (3)	1.34 ± 0.06 (3)

Table 6. *Activation of pyruvate dehydrogenase in lysates of mitochondria of lactating rat mammary gland*
The design of the experiment was the same as described in the legend to Table 3, except that instead of whole-tissue extracts we used mitochondrial lysates prepared as described in the Experimental section. Numbers in parentheses are numbers of animals or pairs of animals involved, on which preceding S.E.M. values are based. $**P < 0.01$ for difference of data from corresponding data in column 3.

	Percentage total activity of pyruvate dehydrogenase		
Time of incubation (min)	Lysate of mitochondria from glands of starved rats	High-speed supernatants of mitochondrial lysates from glands of starved rats + high-speed sediments of same lysates	High-speed supernatants of mitochondrial lysates from glands of fed rats + high-speed sediments of lysates from glands of starved rats
0	10.6 ± 0.4 (4)**	31.4 ± 4.6 (4)	30.0 ± 6.0 (3)
5	32.4 ± 3.5 (4)	37.5 ± 4.8 (4)	67.0 ± 3.2 (3)**
10	35.6 ± 3.2 (4)	41.3 ± 3.1 (4)	77.8 ± 3.0 (3)**
15	38.1 ± 2.9 (4)	41.8 ± 3.9 (4)	80.0 ± 1.8 (3)**

cases. However, since one can separate the phosphatase from the rest of the complex by high-speed centrifugation (Linn *et al.*, 1972), it was possible to compare the phosphatase activities in high-speed supernatants from extracts of glands from fed with that from starved animals acting on the pyruvate dehydrogenase complex in the high-speed sediment of the extracts of glands from starved animals. Columns 3 and 4 of Table 3 show that there was no difference in the rate of rise of pyruvate dehydrogenase activity induced by either supernatant. Column 2 compared with column 3 suggests that there was probably no significant loss of phosphatase activity during the high-speed centrifugation, since the pyruvate dehydrogenase activity reached in 15min was the same when the extracts from glands of starved animals were incubated with Mg^{2+} and Ca^{2+} before or after centrifugation. EGTA (5mM) or EDTA (5mM) prevented the rise in pyruvate dehydrogenase activity during incubation of extracts at 30°C for 10min. We conclude that there appeared to be no significant difference in Mg^{2+}/Ca^{2+}-dependent pyruvate dehydrogenase phosphatase activity in extracts of glands from fed compared with extracts from starved animals.

Extracts of lysed mitochondria

Table 4 shows that the total activity of pyruvate dehydrogenase in extracts of mitochondria was the same whether they were derived from the glands of fed or starved animals. However, whereas during the preparation of the mitochondria the percentage total activity of extracted pyruvate dehydrogenase increased from 40 to 89% in the fed animals, there was in fact a slight fall in percentage total activity of the enzyme from starved animals (11.8 to 8.5%). Another difference in enzymic behaviour appeared when both sets of mitochondria were incubated for 5min in hypo-osmotic medium containing 10mM-pyruvate and oligomycin (0.5μg/ml). The mitochondria were uncoupled by this treatment, and other reports suggest that they would have been permeable to coenzymes and small molecules (Ernster & Kuylenstierna, 1969).

There was evidently no loss of pyruvate dehydrogenase from mitochondria during this treatment. The enzyme was fully activated when mitochondria were derived from glands of fed rats, whereas in contrast the enzyme in mitochondria from glands of starved rats remained largely inactivated.

In atempting to minimize or control intramitochondrial effectors that might contribute to this striking difference, we have examined the behaviour of the pyruvate dehydrogenase kinase in lysates of both mitochondrial preparations in a manner analogous to the treatment of whole-tissue extracts. Fig. 2 shows the results. Again we observe faster and more extensive inactivation by ATP of the enzyme from glands of starved rats. Even more striking in the latter case was the complete loss of protection

by pyruvate against ATP-induced inactivation. We have again checked that differences in rates of ATP hydrolysis do not contribute to these results (Table 5).

We have compared the pyruvate dehydrogenase phosphatase activity in mitochondrial lysates in a similar way to that used for whole-tissue extracts. Table 6 shows that there was a significant decline in the Mg^{2+}/Ca^{2+}-dependent phosphatase activity of these mitochondrial lysates from the glands of starved compared with fed animals when tested on the sedimented pyruvate dehydrogenase in the lysates of mitochondria from glands of starved animals. Comparison of columns 2 and 3 indicates that the maximum activity achieved when the mitochondrial lysates from the glands of starved animals were incubated with Mg^{2+} and Ca^{2+} was the same before as after high-speed centrifugation. The data suggest that in the starved animals there may be a decline in affinity of the phosphatase for the α-subunit of the complex such that only limited dephosphorylation is possible. These data contrast with data from whole-tissue homogenates (Table 3), where no difference was found in phosphatase activity in high-speed supernatants of gland extracts from fed and starved animals. This is reminiscent of the report by Stansbie *et al.* (1976) that in adipose tissue there is significant extramitochondrial Mg^{2+}/Ca^{2+}-dependent pyruvate dehydrogenase phosphatase activity. The physiological significance of such activity is unknown, as is its identity or otherwise with the intramitochondrial pyruvate dehydrogenase phosphatase activity. The reported instability of pyruvate dehydrogenase phosphatase when separated from the rest of the complex (Randle *et al.*, 1974) hampers a direct approach to this problem.

Discussion

Experiments with crude enzyme preparations are always liable to interference from unknown factors, and we are well aware of the risks inherent in constructing a new hypothetical scheme of regulation of pyruvate dehydrogenase kinase and phosphatase. We do not propose to do this. Further experiments with the enzyme complex purified to different extents are clearly necessary, and for this improved methods are required, since existing techniques have an extremely low yield. Our main conclusion at this stage is that variation in intramitochondrial concentrations of the known effectors of the enzyme complex cannot explain our results. Starvation (and perhaps diabetes) involves some more permanent change in the regulatory system as well as the phosphorylation status of the α-subunit of pyruvate dehydrogenase. There are two other reports in the literature that hint at this. Robinson & Williamson (1977) observed that the restraint on oxidation of pyruvate shown *in vivo* by lactating mammary gland of starved rats was fully maintained even after preparation of tiny glandular acini and incubation of these acini for 60min. One would expect large changes in the putative mitochondrial effectors during these procedures. Also, Kerbey *et al.* (1977) have observed that mitochondria from hearts of alloxan-diabetic rats, when incubated with pyruvate, did not show activation of pyruvate dehydrogenase to the same extent as did the enzyme in mitochondria from hearts of normal rats. Further, the measured ratios of [ATP]/[ADP], [NADH]/[NAD^+] and [acetyl-CoA]/[CoA] in mitochondria from hearts of diabetic rats were an inadequate explanation of the low activity of pyruvate dehydrogenase actually observed.

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