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Properties of Pyruvate Dehydrogenase of Rat Mammary Tissue and its Changes During Pregnancy, Lactation and Weaning

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Pyruvate dehydrogenase of rat mammary tissue showed many of the regulatory properties of the analogous enzyme in other mammalian tissues. It was inactivated in the presence of low concentrations of ATP and this rate of inactivation was slowed if pyruvate or PP_i was also present. Reactivation by Mg^{2+} in the presence of low concentrations of Ca^{2+} occurred over a similar time-course. The K_m value for Mg^{2+} in this process was about 2mM. The enzyme was assayed in extracts of freeze-clamped mammary glands removed from pregnant, lactating or recently weaned rats under halothane anaesthesia. Both the initial activity and the activity after full activation ('total enzyme activity') were determined. The former parameter, when expressed on a DNA basis, varied within a range of 40 times its lowest value. Maximum total enzyme activity was about 1 unit/g wet wt. The total enzyme activity and the fraction in the active form increased in step from pregnancy to mid-lactation, remained elevated until the end of lactation and then fell steeply within 3 days after weaning. The correlation of these two parameters of enzyme activity may indicate a common regulatory factor or else an interdependence arising from inherent properties of the multi-enzyme complex.

The multi-enzyme complex pyruvate dehydrogenase (EC 1.2.4.1) catalyses the co-ordinated series of reactions summarized as:

$$CH_3\text{–}CO\text{–}CO_2H + CoA\text{-}SH + NAD^+ \rightarrow$$
$$CH_3\text{–}CO\text{–}S\text{-}CoA + NADH + H^+ + CO_2$$

Linn *et al.* (1969) first reported that the activity of the purified enzyme from bovine kidney and of the partially purified enzyme from pig liver could be regulated by a cycle of phosphorylation and dephosphorylation catalysed respectively by an ATP-specific kinase and an Mg^{2+}- dependent phosphatase both closely associated with the multi-enzyme complex. Phosphorylation of the first component of the complex completely abolished overall enzyme activity, which was restored after dephosphorylation. Similar findings were reported for the enzyme complex from pig heart (Wieland & von Jagow-Westermann, 1969), pig brain (Siess *et al.*, 1971) and rat adipose tissue (Jungas, 1971; Coore *et al.*, 1971).

Studies with isolated tissues have indicated certain physiological factors which can alter the degree of phosphorylation and therefore the activity of pyruvate dehydrogenase. Such factors are the fatty acids in the perfusion medium of heart and liver (Wieland *et al.*, 1971; Patzelt *et al.*, 1973) and hormones and carbohydrates in the incubation medium of adipose tissue (Jungas, 1971; Coore *et al.*, 1971).

Wieland and co-workers (summarized by Wieland *et al.*, 1972) demonstrated that starvation and diabetes significantly increased the degree of phosphorylation of pyruvate dehydrogenase extracted from heart, kidney and liver of rats. Re-feeding glucose to starved animals restored enzyme activity (decreased phosphorylation) in the enzyme from these tissues. These observations were interpreted in terms of the relative supply of fatty acids and glucose to the tissues.

In all the experiments *in vitro* and *in vivo* mentioned above, total enzyme activity (exhibited after complete dephosphorylation) has remained constant. A contrary report by Sica & Cuatrecasas (1973) states that insulin increased both the initial and total activity of the enzyme from incubated rat adipose tissue. It was decided therefore to examine the enzyme in rat mammary tissue during lactation. It was expected that pyruvate dehydrogenase and other enzymes of the lipogenic pathway would increase in activity and indeed Gumma *et al.* (1973) reported an increase in the total pyruvate dehydrogenase activity of mammary gland during lactation in the rat. These authors did not, however, determine changes in the degree of phosphorylation during lactation, nor was it established that a phosphorylation–dephosphorylation cycle was relevant to the control of the mammary enzyme. Even if such a cycle was operating it was possible that their procedure (which involved simply incubation of a mitochondrial extract in the presence of a high concentration of Mg^{2+}) did not reveal total enzyme activity. Portenhauser & Wieland (1972) thought it necessary to add exogenous pyruvate

dehydrogenase phosphatase to reveal the full enzymic activity in liver homogenates. Our general aim was therefore to study the relative importance of total enzyme synthesis and phosphorylation–dephosphorylation (assuming that this occurred) in pyruvate dehydrogenase of the mammary gland during pregnancy, lactation and weaning. The experiments showed that the assumption of a phosphorylation–dephosphorylation cycle was correct, and that there was a surprisingly close correlation between the degree of activation of the enzyme and the total activity detectable after activation by exogenous phosphatase. This raises the question of whether the two parameters have a common regulatory factor or are interdependent in some way. The experiments also suggest that mammary gland may be a favourable tissue to study further the synthesis and degradation of the components of the pyruvate dehydrogenase multi-enzyme complex.

Experimental

Materials

Rats. Wistar rats (4–6 months; 270–350g) in their first pregnancy were kept in individual cages under alternating 12h periods of light and dark and were supplied with food *ad lib.* The litter size ranged from 6 to 12 pups.

Chemicals. The halothane anaesthetic was from I.C.I. Ltd., Pharmaceuticals Division, Macclesfield, Cheshire, U.K. Pyruvate, phosphoenolpyruvate, pyruvate kinase (EC 2.7.1.40), myokinase (EC 2.7.4.3) and lactate dehydrogenase (EC 1.1.1.27) were from Boehringer Corp. (London) Ltd., London W.5, U.K. Coenzyme A, NADH, ADP and bovine serum albumin were from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K., NAD, thiamin pyrophosphate and DNA (calf thymus) were from BDH Chemicals Ltd., Poole, Dorset, U.K. Pyrophosphate (tetrasodium salt) and diphenylamine were from Fisons Scientific Apparatus, Loughborough, Leics., U.K. ATP was from Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan. Acetyl-CoA was synthesized by the method of Simon & Shemin (1953). Pyruvate dehydrogenase free from phosphatase and pyruvate dehydrogenase phosphatase were made from pig or pigeon hearts by a procedure based on that of Wieland & Siess (1970) up to the stage of isoelectric precipitation, which was followed by the separation recommended by Denton *et al.* (1972).

Arylamine acetyltransferase (EC 2.3.1.5) was made by the method of Tabor *et al.* (1953) except that the acetone-dried powder was extracted with EDTA (1mM)–dithiothreitol (1mM), pH7.0, instead of water. Also the use of alumina after the acetone fractionation was omitted. The enzyme was assayed by the method of Jacobson (1961).

Methods

Tissue samples. (a) Mammary gland. Immediately after removal from her pups if lactating or after the appropriate period if 'weaned', the rat was anaesthetized by confinement in a large desiccator flask through which was passed 7% (v/v) halothane in a stream of O_2 at 1 litre/min from an anaesthetic apparatus (Medical and Industrial Equipment Ltd., London, U.K.). After anaesthetic induction, the animal was maintained in 3–4% halothane in a stream of O_2 at 0.5 litre/min passed through a funnel with a polythene diaphragm enclosing the animal's head.

A portion of the abdominal mammary gland was dissected out, care being taken to preserve its main blood supply. Freeze-clamping (Wollenberger *et al.*, 1960) was then performed *in situ*, the clamped portion of the gland was severed and the aluminium blocks with the enclosed tissue were plunged into liquid N_2. The total time from the beginning of anaesthetic induction was 10–15min. Tissue not properly within the blocks was discarded and the remainder powdered under liquid N_2. The powder was kept in bottles on solid CO_2 until the time for extraction, which was invariably within 2h.

(b) Liver and blood. While the animal was under halothane anaesthesia the abdomen was opened and a portion of liver freeze-clamped. Subsequent treatment was similar to that described for mammary tissue. After freeze-clamping the liver venepuncture of the inferior vena cava was performed and a sample of blood was removed and transferred to a cooled tube containing EDTA.

Extraction of enzymes. A weighed amount (400–600mg) of powdered tissue was homogenized with 2ml of ice-cold 20mM-triethanolamine buffer, pH7.0, containing 7mM-mercaptoethanol, with or without 2mM-EDTA, for 1min by a motor-driven close-fitting Teflon pestle in a glass homogenizer driven at high speed. The homogenate was centrifuged for 10min in a bench centrifuge and the infranatant below the fat layer was clarified by centrifuging for 1min at $14500g_{av.}$ in a micro-centrifuge (Jobling Laboratory Division, Stone, Staffs., U.K.).

Over 90% of the total enzyme activity of the homogenate was retained in the supernatant of this second centrifugation. Enzyme assays followed within 60min of extraction, the extracts being kept on ice until then.

Phosphate or imidazole buffers of equal ionic strength did not extract a greater amount of enzyme than did the triethanolamine buffer, which was preferred because of its weaker cation-binding properties.

Generally, the pyruvate dehydrogenase activity in the medium containing EDTA was lower than that in the medium without the chelator, but increasing the EDTA concentration to greater than 2mM had no further effect.

Extraction and assay of DNA. The method described below is based on the discussion by Munro & Fleck (1966) and the recommendations of Giles & Myers (1965).

Three samples (50–100mg) of the frozen powdered mammary tissue were homogenized with 3ml of 0.25M-$HClO_4$ for 90s by a motor-driven Teflon pestle in a glass homogenizer. DNA standards (accompanied by bovine serum albumin, 5mg/ml) were treated similarly in this and all subsequent procedures. The precipitates were left for 1h at 4°C and then centrifuged in a bench centrifuge at maximum speed for 5min. The precipitates were resuspended in 0.5ml of 0.25M-$HClO_4$ and then a further 3ml of the same medium was added. The precipitates were washed by resuspension and centrifugation in the same medium and the final precipitates resuspended in 0.5ml of 0.5M-$HClO_4$. A further 2ml of 0.5M-$HClO_4$ was added and the suspensions were heated for 20min at 70°C and shaken every 5min. The tubes were immediately plunged into ice and then centrifuged. The supernatants were filtered and the pellets re-extracted with 0.5M-$HClO_4$ as above. Diphenylamine (1ml; 4%, w/v) in acetic acid containing concentrated H_2SO_4 (4%, v/v) was added to 1ml of the pooled supernatants. They were mixed, then 50μl of aq. acetaldehyde (1.6mg/ml) was added and the tubes were incubated at 30°C overnight. After 18h the difference in absorbance at 595 and 700nm was noted and the unknown DNA contents were determined from a standard curve.

Preparation of mammary pyruvate dehydrogenase. Abdominal mammary glands (10; total of 94g) were taken from 21-day lactating rats and stored at −20°C for 2 weeks. The frozen tissue was homogenized for 5min with 450ml of 20mM-triethanolamine buffer, pH7.0, containing $MgCl_2$ (0.5mM) and mercaptoethanol (2mM) at 4°C in an Ato-Mix blender. The homogenate was centrifuged at 18000$g_{av.}$ for 30min at 4°C, then filtered through gauze and the filtrate assayed (100 units). The filtrate was kept at 4°C and stirred vigorously while $CaCl_2$ (0.2M) was added to a final concentration of 0.07M. The pH was restored to 7.0 with 1% (v/v) acetic acid and the precipitate of casein removed by centrifugation (25% of initial enzyme activity remained in the supernatant). Excess of Ca^{2+} was removed by the addition of potassium oxalate to a final concentration of 0.15M. The precipitate was collected by centrifugation and 16% of the initial enzyme activity remained in the supernatant. Saturated $(NH_4)_2SO_4$ was added to the cooled stirred supernatant up to a final concentration of 35% saturation and the precipitate collected by centrifugation. The precipitate was suspended in 25ml of 20mM-potassium phosphate buffer, pH7.0, containing $MgCl_2$ (0.5mM) and mercaptoethanol (2mM) and dialysed against 10 litres of the same buffer for 18h at 4°C, with one change of outer medium after 1.5h. The dialysis residue was clarified by centrifugation and contained 7% of initial enzyme activity. The solution was used for experiments shown in Figs. 2 and 3.

Enzyme and metabolite assays. Pyruvate dehydrogenase was assayed as described by Coore *et al.* (1971) by using arylamine acetyltransferase (EC 2.3.1.5), except that CoA was present at 0.05mM instead of 0.1mM because no greater reaction rate was observed at the higher concentration.

Glutamate dehydrogenase (EC 1.4.1.2) was assayed as described by Martin & Denton (1970), except that NADH was present at 0.2mM instead of 0.1mM because a maximum reaction rate required the higher concentration.

It was confirmed that other components of both assays were present in amounts adequate for maximum rates.

Pyruvate dehydrogenase phosphatase was assayed by the method of Siess & Wieland (1972). ATP and glucose were determined by the method of Lamprecht & Trautschold (1965). ADP, AMP and pyruvate were determined by the method of Adam (1965) and lactate was determined by the method of Hohorst (1965).

Units of enzyme activity. One unit implies 1μmol of product formed/min at 30°C, except for pyruvate dehydrogenase phosphatase, where 1 unit of phosphatase activity formed 1 unit of pyruvate dehydrogenase activity in 5min at 30°C (Siess & Wieland, 1972).

Results

Metabolites in freeze-clamped tissues

Table 1 shows the results of two experiments involving nine animals in which adenine nucleotides, lactate and pyruvate were determined in extracts of freeze-clamped liver and mammary tissue. In one experiment samples of blood were taken for blood glucose determination. The technique of anaesthesia and the time taken to remove mammary tissue were the same as in those experiments in which pyruvate dehydrogenase activity of the tissue was determined.

Activation–inactivation cycle of mammary pyruvate dehydrogenase

Fig. 1 shows that addition of $MgCl_2$ to final concentration of 10mM to a mammary tissue extract (see under 'Methods' and legend to Fig. 1) led to a substantial increase in activity over 30min. A much greater increase in activity was often achieved by the addition of exogenous phosphatase, as shown in

Table 1. *Concentration of metabolites and the metabolite ratios in tissues of rats under halothane anaesthesia*

For details of determinations see the Experimental section. Concentrations of nucleotides are expressed as nmol/g wet wt. of tissue and of glucose as mM. Values are means ± S.E.M. with number of animals used in parentheses.

$$\text{Energy charge} = \frac{[ATP]+\frac{1}{2}[ADP]}{[ATP]+[ADP]+[AMP]}$$

The data in the first three rows were from the same group of rats in which mammary tissue and then livers were freeze-clamped in succession with the animals under anaesthesia throughout.

Source	[Glucose]	[ATP]	[ADP]	[AMP]	Energy charge	$\frac{[\text{Lactate}]}{[\text{pyruvate}]}$
Plasma	4.7±0.7 (4)	—	—	—	—	—
Freeze-clamped mammary tissue	—	1254±89 (4)	475±15 (4)	204±19 (4)	0.77±0.02 (4)	29±4.5 (4)
Freeze-clamped liver of lactating rat	—	1916±99 (4)	1110±77 (4)	235±8 (4)	0.76±0.01 (4)	13±2 (4)
Freeze-clamped livers of non-lactating rats	—	2860±157 (5)	1062±96 (5)	281±28 (5)	0.81±0.01 (5)	—

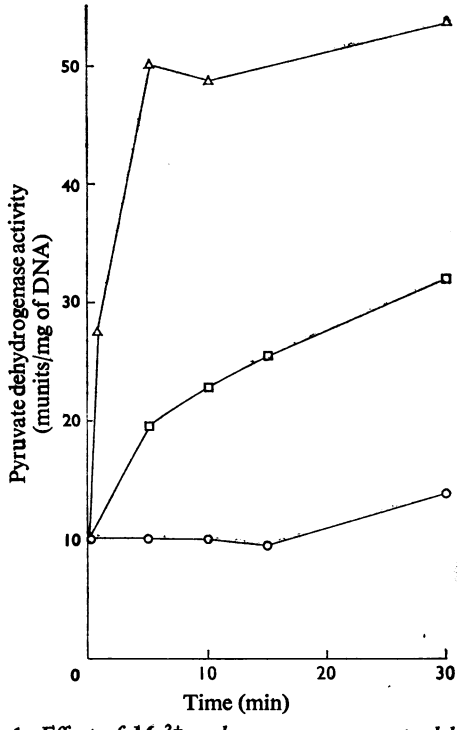

Fig. 1. *Effect of Mg^{2+} and exogenous pyruvate dehydrogenase phosphatase on the activity of pyruvate dehydrogenase in mammary-gland extracts*

Powdered frozen mammary gland (411 mg) from a 3-day-weaned rat was extracted into 2 ml of 20 mM-triethanolamine buffer, pH 7.0, containing mercaptoethanol (7 mM) as described under 'Methods'. Portions (0.3–0.6 ml) of the extract were incubated at 30°C with the following additions: □, 10 mM-$MgCl_2$; △, 10 mM-$MgCl_2$ and pyruvate dehydrogenase phosphatase (0.4 unit/ml); ○, no addition. Samples (0.025–0.1 ml) were taken at intervals for assay of pyruvate dehydrogenase activity.

the upper curve of Fig. 1, and we have assumed that this reveals total enzyme activity. Addition of Ca^{2+} ions did not increase the rate of activation of pyruvate dehydrogenase nor its final activity in the presence or absence of exogenous phosphatase. This contrasts with the behaviour of partially purified pyruvate dehydrogenase from mammary tissue (see below) and is presumably due to sufficient Ca^{2+} already being present in the crude extracts.

It was not possible to demonstrate a consistent effect of ATP on the pyruvate dehydrogenase activity of whole tissue extract. This may have been partly due to other ATP-utilizing reactions, which were estimated to destroy ATP at a rate of 2 μmol/min per g wet wt. of lactating gland.

A clear effect of incubation with low concentrations of ATP was shown on a partly purified sample of pyruvate dehydrogenase from mammary gland (preparation described under 'Methods'). Fig. 2 shows that 0.25 mM-ATP caused 85% inactivation in 5 min at 30°C and even at 0.1 mM-ATP substantial inactivation was obtained by 15 min. Linn *et al.* (1969) reported that pyruvate protects against ATP inhibition, as does PP_i (Wieland *et al.*, 1972). ATP inhibited pyruvate dehydrogenase in a mammary mitochondrial extract (a 60% decrease in activity in 5 min was caused by 0.1 mM-ATP), but this experiment was complicated by the spontaneous decline of activity in the control sample without ATP at 30°C. A similar phenomenon has been noted by Portenhauser & Wieland (1972) for liver mitochondrial extracts.

Fig. 3 shows that reactivation of inactivated enzyme by Mg^{2+} was assisted by low concentrations of Ca^{2+}. [A requirement for Ca^{2+} in this process was first reported by Denton *et al.* (1972) for heart, kidney and adipocyte enzymes.] In other experiments (not shown) the apparent K_m value for the Mg^{2+} effect was 2 mM in the presence of 0.1 mM-$CaCl_2$. The

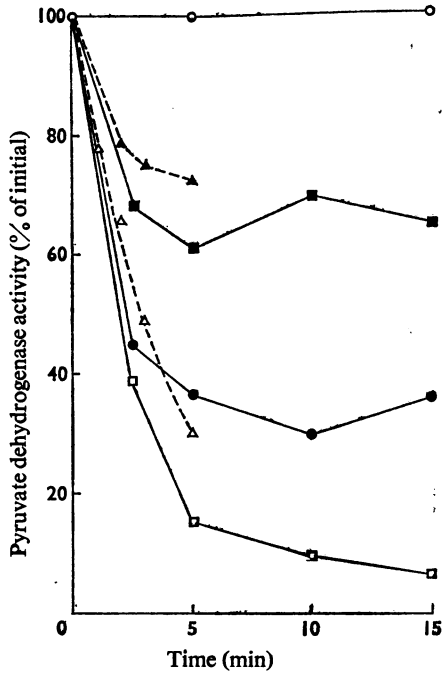

Fig. 2. *Effect of pyruvate and PP_i on the rate of inactivation by ATP of partially purified mammary pyruvate dehydrogenase*

Pyruvate dehydrogenase (0.14 or 0.25 unit/ml) was incubated at 30°C in 20mM-potassium phosphate buffer, pH 7.0, containing mercaptoethanol (2 mM) and EGTA [ethanedioxybis(ethylamine)tetra-acetate] (5mM). Additions were made as follows: ●, 5.5mM-$MgCl_2$ and 0.1mM-ATP; ■, 5.5 mM-$MgCl_2$, 0.1mM-ATP and 0.5mM-sodium pyrophosphate; △, 0.5mM-$MgCl_2$ and 0.1mM-ATP; ▲, 0.5mM-$MgCl_2$, 0.1mM-ATP and 0.3mM-pyruvate; ○, 0.5mM-$MgCl_2$ or 5.5 mM-$MgCl_2$; □, 5.5mM-$MgCl_2$ and 0.25mM-ATP. Samples were removed at the time-intervals indicated and assayed as described under 'Methods'.

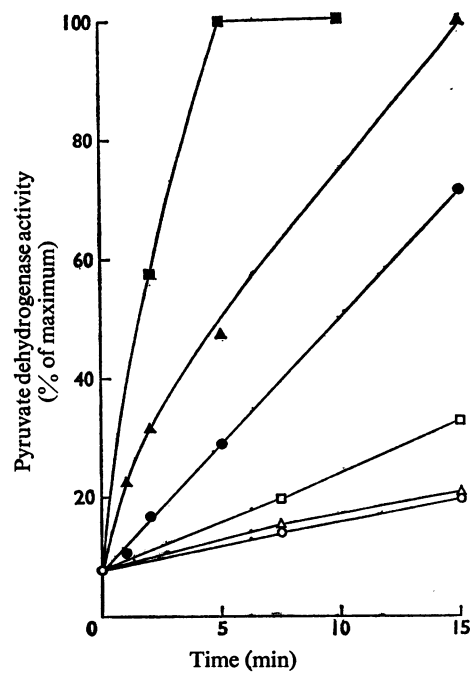

Fig 3. *Activation by Mg^{2+} and Ca^{2+} of partially purified mammary pyruvate dehydrogenase*

Pyruvate dehydrogenase (0.25 unit/ml) in 20mM-potassium phosphate buffer, pH 7.0, containing mercaptoethanol (2mM), $MgCl_2$ (0.5mM) and EGTA (5mM) was inactivated by incubation at 30°C for 15min with 1mM-ATP. The inactive enzyme was dialysed against a 1000-fold volume of 20mM-potassium phosphate buffer, pH 7.0, containing mercaptoethanol (2mM) for 2h at 4°C. Portions (0.4ml) of this enzyme were incubated at 30°C together with the following additions: △, 1μM-$CaCl_2$; □, 100μM-$CaCl_2$; ●, 10mM-$MgCl_2$; ▲, 10mM-$MgCl_2$ and 1μM-$CaCl_2$; ■, 10mM-$MgCl_2$ and 0.1mM-$CaCl_2$; ○, no addition. Samples (0.05ml) were taken at intervals for assay of pyruvate dehydrogenase activity as described under 'Methods.'

apparent effects of $CaCl_2$ by itself at a concentration of 0.1mM may have been due to activation of the enzyme sample while in the cuvette, since it was generally necessary to wait for 1–2min before the maximum linear rate of enzymic activity was observed. No greater rate of apparent activation was seen when 1mM-$CaCl_2$ was added to the incubation medium (in the absence of Mg^{2+}).

The concentration of pyruvate required for half-maximum velocity of reaction due to fully activated enzyme was found to be 13μM.

Similar behaviour with respect to ATP-induced inactivation and Mg^{2+}-induced reactivation was displayed by another preparation of partially purified pyruvate dehydrogenase from mammary gland.

Changes in enzyme activity of mammary pyruvate dehydrogenase during pregnancy, lactation and weaning

There has been controversy over the best method of expressing enzyme activities in lactating mammary gland (Carlsson *et al.*, 1973). Expression of activity on a wet-weight basis might involve a correction for contained milk or adipose tissue. We have therefore thought it preferable to express our results on a DNA basis. We assume that an increase of enzyme activity per unit of DNA indicates an increase in enzyme per cell. We have also measured another mitochondrial enzyme, glutamate dehydrogenase, which, according to Gumma *et al.* (1973), changes

Table 2. *Changes in DNA concentration and glutamate dehydrogenase activity of mammary gland during pregnancy, lactation and weaning*

For details of determinations see the Experimental section. The DNA concentration is expressed as mg/g wet wt. of mammary tissue and glutamate dehydrogenase activity is expressed as units/mg of DNA. Values are means ± S.E.M.

	Pregnancy			Lactation						Weaning		
Time (days) ...	16	19	22	1	2	3	4	12	21	1	2	3
No. of animals ...	3	6	4	3	3	3	5	5	5	4	3	4
Concn. of DNA	5.49 ±1.02	5.25 ±0.3	4.24 ±0.3	5.24 ±1.38	6.79 ±0.47	7.19 ±0.39	7.04 ±0.59	6.82 ±0.21	4.92 ±0.38	3.19 ±0.47	4.1 ±0.45	6.22 ±0.78
Glutamate dehydrogenase activity	0.125 ±0.058	0.125 ±0.02	0.151 ±0.012	0.145 ±0.061	0.153 ±0.01	0.135 ±0.064	0.130 ±0.015	0.208 ±0.041	0.225 ±0.053	0.190 ±0.079	0.151 ±0.035	0.152 ±0.053

relatively little after the first day of lactation. We have found negligible activity of this enzyme in the mitochondrial supernatant of mammary tissue. If it is assumed that the activity of glutamate dehydrogenase per mitochondrion does not decline during lactation, then increases in pyruvate dehydrogenase activity relative to glutamate dehydrogenase indicate increases in the activity of the former per mitochondrion. Table 2 shows the changes in DNA per unit wet wt. of the gland and in glutamate dehydrogenase activity on a DNA basis. During involution of the gland it is clearly conceivable that the concentration of glutamate dehydrogenase per mitochondrion may decline, but we have provisionally assumed that this change is relatively small and that the ratio of pyruvate dehydrogenase activity to glutamate dehydrogenase activity still gives an index of the mitochondrial concentration of the former enzyme.

Fig. 4(*a*) shows that total pyruvate dehydrogenase activity on a DNA basis increases from the sixteenth day of pregnancy to mid-lactation by a factor of about 10, remains steady during the second half of lactation and then declines rapidly during 3 days after weaning. A similar picture is presented in Fig. 4(*b*) for the ratio of pyruvate dehydrogenase activity to glutamate dehydrogenase activity, except that here the apparent increase from pregnancy to mid-lactation is fivefold.

The percentage activation of the total enzyme present at any time followed a very similar time-course (Fig. 4*b*). In fact, there is a good correlation (correlation coefficient, +0.865) between the total pyruvate dehydrogenase activity per unit of DNA for any gland and its percentage activation.

Discussion

Validity of tissue-sampling procedure

It is probable that all methods of anaesthesia disturb tissue metabolism to some extent. Seitz *et al.* (1973) indict all forms of narcosis and neck fracture for giving concentrations of liver adenine nucleotides different from those values yielded by their 'double hatchet' method, which they consider to be the best approximation to the situation *in vivo*. In their data 30min halothane narcosis of rats caused a slight rise in the concentration of ADP, a marked rise in the concentration of AMP and a slight increase in the ratio [lactate]/[pyruvate]. Our results (row 4, Table 1) for freeze-clamped liver of non-lactating rats are intermediate between those of Brosnan *et al.* (1970) (in which neck fracture was used) and those of Start & Newsholme (1968) (in which ether anaesthesia was used) in respect of absolute nucleotide concentrations and also the ratios [ATP]/[ADP], [ATP]/[AMP] and [lactate]/[pyruvate].

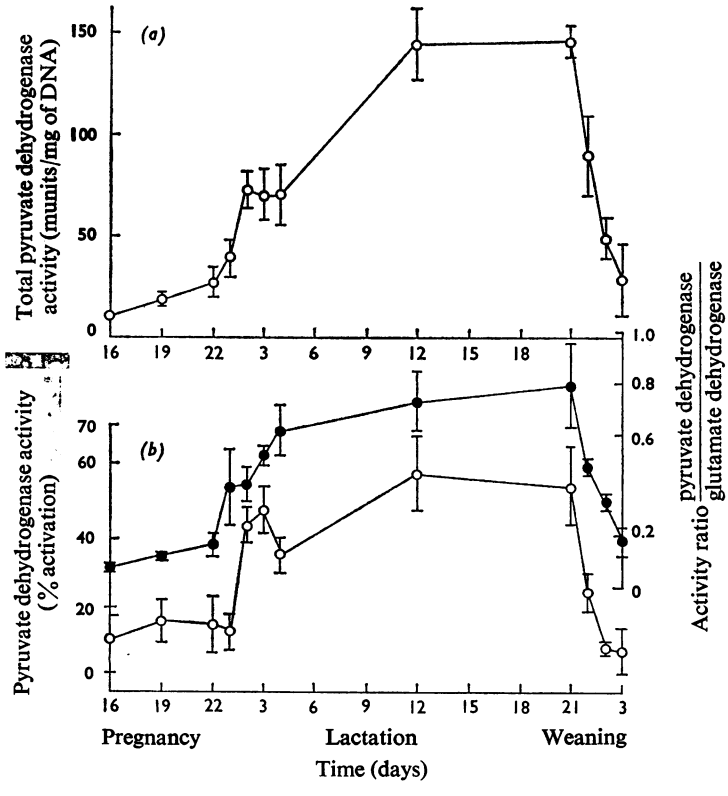

Fig. 4. (*a*) *Changes in total pyruvate dehydrogenase activity of mammary gland during pregnancy, lactation and weaning and* (*b*) *changes in the activity ratio of pyruvate dehydrogenase/glutamate dehydrogenase* (●) *and the percentage activation of pyruvate dehydrogenase* (○) *of mammary gland during pregnancy, lactation and weaning*

(*a*) Samples (400–600mg) of powdered frozen mammary tissue were extracted into 2ml of 20mM-triethanolamine buffer, pH7.0, containing mercaptoethanol (7mM), as described under 'Methods'. The extracts were incubated at 30°C, together with $MgCl_2$ (10mM) and pyruvate dehydrogenase phosphatase (2 units/ml) and samples were taken at intervals for assay of pyruvate dehydrogenase activity. Values shown are the means of results obtained from between three and six animals (see Table 2); vertical bars indicate S.E.M. The addition of Ca^{2+} did not alter the rate of activation of pyruvate dehydrogenase in the extracts nor the maximum activity achieved. Sufficient samples were taken to ensure that maximum activity was achieved for each extract and this occurred between 5–15 min incubation. (*b*) Samples (400–600mg) of powdered, frozen mammary tissue were extracted and assayed for glutamate dehydrogenase and pyruvate dehydrogenase activities as described under 'Methods'. Initial pyruvate dehydrogenase activity was determined on samples of extracts containing EDTA (2mM). Total pyruvate dehydrogenase activity was determined as described in the legend to Fig. 4(*a*) and the percentage activation is expressed as 100×initial activity/total activity. Values shown are the means of results obtained from between three and six animals (see Table 2). Vertical bars indicate S.E.M.

From this we concluded that halothane narcosis was no more deleterious than available alternatives and clearly the 'double hatchet' method was only applicable to liver. Halothane narcosis was much more convenient and easier to regulate than that induced by ether or nembutal during the time-period required to expose and dissect the mammary gland.

One might expect that mammary gland would be rather less favourable for freeze-clamping than liver, since preliminary dissection is required and this may damage the blood supply to some of the tissue. However, the ratio of [ATP]/[ADP] was not less than that of liver (compare rows 2 and 4 of Table 1). The lower [ATP]/[AMP] ratio in the lactating gland compared with liver may not only reflect anoxia during sampling but possibly displacement from equilibrium of the adenylate kinase reaction (Murphy *et al.*, 1973).

Our values for the absolute concentrations of ATP and the [ATP]/[ADP] and [ATP]/[AMP] ratios for lactating rat mammary gland are all considerably higher than those of Baldwin & Cheng (1969) and

this may indicate the merit of freeze-clamping compared with the slower procedure used by those authors. We conclude that our tissue-sampling procedure for mammary gland is probably the best available, though it may well induce a small systematic alteration in adenine nucleotide balance in the tissue. This would be expected to increase the apparent degree of activation of pyruvate dehydrogenase. It may be noted, however, that on many occasions it was possible to extract tissue in which the enzyme was virtually completely inactivated.

The lower ATP concentration and [ATP]/[ADP] and [ATP]/[AMP] ratios in livers freeze-clamped subsequent to freeze-clamping mammary tissue (row 3, Table 1) compared with livers freeze-clamped immediately (row 4) may reflect metabolic disturbance caused by freeze-clamping, longer time under narcosis or possibly the different physiological state of the animals (lactating versus non-lactating).

Aynsley-Green *et al.* (1973) reported that 30min halothane narcosis caused hyperglycaemia in 48h-starved rats, but it is apparent (row 1, Table 1) that for the short period of exposure in our experiments this was not the case.

Changes in pyruvate dehydrogenase activity in relation to lipogenesis

Gumma *et al.* (1973) report a value for total pyruvate dehydrogenase activity of rat mammary gland at 14 days lactation of 0.71 unit (measured at 25°C)/g wet wt. corrected for retained milk and fat. Our value of 1 unit (measured at 30°C)/g wet wt. uncorrected is not greatly different, but an exact comparison is not possible.

Over the period from the 20th day of pregnancy to the 15th day of lactation we observed a sevenfold increase in total pyruvate dehydrogenase activity and a threefold increase in the percentage activation. This suggests that in this period, owing to the co-operation of both mechanisms, a flux change of 21-fold could be accommodated. This may be compared with relative changes in activity of other enzymes in the pathway for conversion of pyruvate into fatty acid, i.e. 17-fold increase in citrate cleavage enzyme, 38-fold increase in fatty acid synthetase (Baldwin & Milligan, 1966) and 20–30-fold increase in acetyl-CoA carboxylase (Howanitz & Levy, 1965).

Properties of isolated mammary pyruvate dehydrogenase

The inactivation–activation cycle of mammary pyruvate dehydrogenase is broadly similar to that displayed by analogous mammalian enzymes and is presumably due to phosphorylation and dephosphorylation. Quantitative characteristics of the enzyme, e.g. its K_m value for pyruvate, the K_m value for Mg^{2+} in the activation process and the time-course of the inactivation–activation cycle seem similar to that of other tissues (see the references in the introduction). More purified enzyme preparations would be required to determine other quantitative characteristics, for example the K_i value for ATP and the minimum concentration of Ca^{2+} required for activation.

Correlation of total pyruvate dehydrogenase activity and the percentage activation of the enzyme

Essentially what we measured was pyruvate dehydrogenase activity in different physiological states on which was superimposed a 'constant' stress of anaesthesia and operation. It may be that we do not thereby exhibit the full range of activation of the enzyme in the unanaesthetized animal. However, in all the reports of the activity of the enzyme in tissues removed from animals after various treatments and by various procedures, in no case was the enzyme extracted with full activity (see the references in the introduction). Nevertheless, the correlation of total enzyme present with degree of activation is still sufficiently marked to require an explanation. One possibility is that the same factor (hormonal, metabolite, ionic) that stimulates synthesis (or assembly) of the components of the complex also regulates the relative activity of the associated kinase and phosphatase. It is possible, though perhaps less likely, that an actual relative increase in the phosphatase protein is involved. Another alternative is that the increase in the degree of activation somehow arises from the very nature of the components of the system. Thus if one assumed Michaelis–Menten relationships for the kinase and phosphatase towards their respective substrates (dephosphorylated and phosphorylated α-subunits of the complex; Roche & Reed, 1972) and assigned reasonable values for their K_m and $V_{max.}$ parameters, it is possible to predict that an increase in concentration of the whole complex would be associated with an increase in the degree of activation. It may be thought unlikely that Michaelis–Menten relationships derived from a concept of 'free' enzyme and substrate molecules in solution would hold for interactions within a multi-enzyme complex. We (H. G. Coore & B. Field, unpublished work) have found with pyruvate dehydrogenase from pigeon heart that tenfold dilution of the entire complex does not affect the rate of inactivation by the kinase, whereas the phosphatase-catalysed activation is severely decreased by dilution even in the presence of the optimum Ca^{2+} concentration. It may therefore not be too fanciful to suggest that an increase in concentration of the total enzyme complex within the mitochondrial matrix would inevitably favour the activity of the phosphatase relative to that of the kinase. Consequently, in the steady state, the con-

centration of the dephosphorylated substrate might exceed that of the phosphorylated substrate (assuming that neither enzyme was saturated by its substrate). Of course, effectors of the kinase, the phosphatase and of the pyruvate dehydrogenase proper would still operate, but the average 'setting' of the system would have increased along with the increase in the concentration of the total complex.

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