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Regulation of Pyruvate Dehydrogenase and Pyruvate Dehydrogenase Phosphate Phosphatase Activity in Rat Epididymal Fat-Pads

EFFECTS OF STARVATION, ALLOXAN-DIABETES AND HIGH-FAT DIET

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1. Pyruvate dehydrogenase phosphate phosphatase activity in rat epididymal fat pads was measured by using pig heart pyruvate dehydrogenase [^{32}P]phosphate. About 80% was found to be extramitochondrial and therefore probably not directly concerned with the regulation of pyruvate dehydrogenase activity. The extramitochondrial activity was sensitive to activation by Ca^{2+}, but perhaps less sensitive than the mitochondrial activity. Prior exposure of fat-pads to insulin did not have any appreciable effects on phosphatase activity measured in either whole tissue or mitochondrial extracts. 2. Alloxan-diabetes and starvation (48 h) markedly decreased the proportion of pyruvate dehydrogenase in the active non-phosphorylated form without greatly decreasing the total activity. This decrease is still evident after incubation of fat-pads with insulin *in vitro*, suggesting that there is some persistent alteration in adipose-tissue metabolism under these conditions that affects interconversion of the two forms of pyruvate dehydrogenase. No evidence for any change in tissue or mitochondrial pyruvate dehydrogenase phosphate phosphatase activity was found. 3. Feeding rats with a balanced diet containing 40% fat for 6 days resulted in changes in pyruvate dehydrogenase activity similar to those observed in alloxan-diabetes and starvation. However, feeding the diet for 14–26 days resulted in a marked decrease in total activity without appreciable change in the proportion in the active form. This appeared to be an adaptive change restricted to adipose tissue; no changes in total activity were found in muscle, liver and kidney. No parallel decrease in fat-pad mitochondrial pyruvate dehydrogenase phosphate phosphatase activity was found. The possibility that components of the mammalian pyruvate dehydrogenase system are under separate genetic control is discussed.

The activity of the mammalian pyruvate dehydrogenase complex (EC 1.2.4.1) may be regulated by two types of control, i.e. end-product inhibition by high ratios of concentrations of acetyl-CoA/CoA and of NADH/NAD^+, which may involve the accumulation of acetyl hydrolipoate (Garland & Randle, 1964; Randle *et al.*, 1966), and interconversion of the phosphorylated (inactive) and non-phosphorylated (active) forms of the complex (Linn *et al.*, 1969*a*,*b*). Inactivation is catalysed by an ATP-requiring kinase which is strongly bound to the complex; pyruvate dehydrogenase phosphate phosphatase is rather loosely associated with the complex and re-activates the enzyme by removing the covalently bound phosphate (for recent reviews, see Reed, 1974; Denton *et al.*, 1975).

The activity of pyruvate dehydrogenase in the rat epididymal fat-pad is markedly increased after brief (10 min) exposure of the tissue to insulin either *in vitro* or *in vivo* (Coore *et al.*, 1971; Jungas, 1971; Weiss *et al.*, 1971). The increase in activity appears to be solely the result of an increase in the proportion of the complex in the active non-phosphorylated form, since the effect of insulin is lost if the extracts are treated with pyruvate dehydrogenase phosphate phosphatase under conditions that allow conversion of all the complex in the inactive form into active form (Weiss *et al.*, 1971; Severson *et al.*, 1974). The mechanism of activation by insulin is not fully understood, but it does not seem to be primarily due to increased rates of glucose uptake or the inhibition of lipolysis (Martin *et al.*, 1972; Denton *et al.*, 1975). The effects of insulin could involve either activation of phosphatase or inhibition of the kinase or possibly both. The kinase from a number of sources including adipose tissue is inhibited by ADP and pyruvate (Hucho *et al.*, 1972; Cooper *et al.*, 1974; Martin

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et al., 1972). More recent studies with the pig heart enzyme have shown that the regulation is complex and that the activity of the kinase may be altered by thiamin pyrophosphate (TPP), Ca^{2+}, acetoin and the [NADH]/[NAD^+] and [acetyl-CoA]/[CoA] ratios (Cooper *et al.*, 1974; Kerbey *et al.*, 1976), but it is not known to what extent this applies to the adipose-tissue kinase. The phosphatase, including that from adipose tissue, requires both Mg^{2+} (Linn *et al.*, 1969*a*,*b*) and Ca^{2+} (Denton *et al.*, 1972; Randle *et al.*, 1974; Severson *et al.*, 1974) for full activity. The effects of insulin persist during the preparation of mitochondria from fat-pads and are still evident after 10–20min incubation of fat-pad mitochondria with respiratory substrates other than pyruvate (Denton *et al.*, 1975). This would appear to rule out changes in pyruvate and adenine nucleotides as being important in the effect of insulin, as there is no pyruvate under these conditions and the ATP concentration in mitochondria from both insulin-treated and control tissues is the same. Some evidence has been obtained with inhibitors of Ca^{2+} transport, which suggests that insulin may act through changes in the mitochondrial Ca^{2+} concentration (Severson *et al.*, 1974), but efforts to show changes in mitocondrial calcium content directly have not been successful (Severson *et al.*, 1976). Severson *et al.* (1974) could find no evidence for any change in the activity of pyruvate dehydrogenase phosphate phosphatase in extracts of adipose tissue previously exposed to insulin, but Mukherjee & Jungas (1975) have presented evidence for a persistent increase in activity in extracts of fat-pads. The reason for this discrepancy is not clear, but it has become apparent from the present studies that only a small fraction of the phosphatase activity present in adipose-tissue extracts is associated with the mitochondrial fraction and therefore directly concerned with the regulation of pyruvate dehydrogenase activity.

In this paper, we present results of investigations focused on the possible long-term regulation of the pyruvate dehydrogenase complex in adipose tissue. Studies have been made on the effects of alloxan-diabetes and starvation, which are both conditions associated with marked decreases in plasma insulin concentrations and with greatly decreased rates of fatty acid synthesis from glucose both *in vitro* and *in vivo*. We have also studied the effects of fat feeding, which results in greatly decreased rates of fatty acid synthesis but not necessarily in a large diminution in plasma insulin concentrations (Hausberger & Milstein, 1955; Griglio *et al.*, 1969; Saggerson & Greenbaum, 1970; Zaragoza & Felber, 1970; Zaragoza-Hermans & Felber, 1972; Malaisse *et al.*, 1969; D. Stansbie, & R. M. Denton, unpublished work). All three conditions have been shown to result in a marked depression of the activities of many other enzymes involved in fatty acid synthesis in adipose tissue (for recent review see Romsos & Leveille, 1974).

Starvation for 24–36h has been reported to result in a decreased proportion of active pyruvate dehydrogenase in adipose tissue (Wieland *et al.*, 1973). In the present investigation we confirm this finding and also report that alloxan-diabetes results in a decreased proportion of the complex in its active form in adipose tissue without marked changes in the total amount of the complex. Decreases in the proportion of active pyruvate dehydrogenase in alloxan-diabetes have been demonstrated in heart and kidney (Wieland *et al.*, 1971, 1973; Kerbey *et al.*, 1976). The decreases in adipose tissue in both starvation and alloxan-diabetes do not appear to be directly linked to the low insulin concentrations, since we have found in the present study that the decreases persist after prolonged incubation *in vitro* in the presence or the absence of insulin. No evidence was found for any decrease in pyruvate dehydrogenase phosphate phosphatase activity in alloxan-diabetes.

The effects of feeding a diet containing about 40% by weight of fat for 6 days were changes in the proportion of pyruvate dehydrogenase in its active form rather similar to those of alloxan-diabetes and of starvation. However, feeding this high-fat diet for 14–26 days resulted in a marked decrease in the total activity of pyruvate dehydrogenase and little change in the proportion of the enzyme in its active form. Pyruvate dehydrogenase phosphate phosphatase activity appeared not to be decreased in the mitochondria of fat-pads under these conditions.

Experimental

Materials

Rats. Epididymal fat-pads were obtained from male albino Wistar rats (150–210g, 6–7 weeks old) allowed free access to either a stock laboratory diet (modified 41B; Oxoid, London S.E.1, U.K.) or to a diet based on the stock diet but including 40% (w/w) of suet (Atora, Hugon and Co., Greatham, Hartlepool TS25 2HD, U.K.), together with an amount of casein calculated to maintain the total protein content of the diet at 16%. Supplementary vitamins were added, principally DL-tocopheryl acetate (700 units/kg of diet) (Supplevite; Beecham Animal Health Products, Manor Royal, Crawley, Sussex, U.K.), and animals were allowed access to the diet from weaning for 20–26 days. Diabetes was induced 48h before use by the intravenous injection of alloxan (60mg/kg) under diethyl ether anaesthesia; the animals had blood glucose concentrations in excess of 15mM before use. Animals were killed after the administration of Nembutal (100mg/kg), except in the incubation experiments, when they were stunned and decapitated.

Chemicals. Biochemicals and enzymes were

purchased from Boehringer Corp. (London) Ltd., London W.5, U.K., with the following exceptions. Crystalline insulin was a gift from Boots Pure Drug Co. Ltd., Nottingham, Notts., U.K., fructose was from BDH Chemicals, Poole, Dorset, U.K., and [U-^{14}C]glucose and $KH^{14}CO_3$ were from The Radiochemical Centre, Amersham, Bucks., U.K. Acetyl-CoA and arylamine acetyltransferase were prepared as described by Coore *et al.* (1971).

Pyruvate dehydrogenase phosphate phosphatase was prepared from pig heart as described by Severson *et al.* (1974). Pig heart pyruvate dehydrogenase free of phosphatase activity was prepared by a modification of the method of Linn *et al.* (1972) as described by Cooper *et al.* (1974), and converted into pyruvate dehydrogenase [^{32}P]phosphate as described by Denton *et al.* (1972).

Media. Fat-pads were incubated in bicarbonate-buffered medium (Krebs & Henseleit, 1932) containing one-half the recommended $CaCl_2$ concentration and gassed with O_2+CO_2 (95:5). Isolated fat-cells were prepared by collagenase digestion as described by Severson *et al.* (1976).

Methods

Assay of enzyme activities in extracts of fat-pads, liver, kidney, heart and skeletal muscle. After onset of Nembutal anaesthesia, fat-pads and liver pieces were rapidly removed and frozen in liquid N_2 and extracted at 0°C with 100mM-potassium phosphate buffer, 2mM-EDTA (pH7.0) containing either 5mM-2-mercaptoethanol or 1mM-dithiothreitol in a Polytron PT10 tissue homogenizer (position 5) for 30s. Kidney was frozen in liquid N_2, and heart and psoas were freeze-clamped at the temperature of liquid N_2 and extracted at 0°C with 100mM-potassium phosphate buffer, 5mM-EDTA, 10mM-sodium pyruvate, 1mM-dithiothreitol (pH7.0) with a Polytron PT10 tissue homogenizer as described above. Tissue extracts were centrifuged for 15s in a Quickfit micro-centrifuge before assay of the enzyme activities. Initial pyruvate dehydrogenase and glutamate dehydrogenase (EC 1.4.1.2) were assayed in extracts by the methods described by Martin *et al.* (1972). It is assumed that the rapid removal and prompt freezing of tissues from anaesthetized animals followed by extraction in a buffer containing EDTA assures that the initial pyruvate dehydrogenase activity in the extracts accurately reflects the proportion of active form in the tissue *in vivo*. Total pyruvate dehydrogenase activity was assayed after incubation of extract with pig heart pyruvate dehydrogenase phosphate phosphatase (0.5 unit/ml), 25mM-$MgCl_2$ and 1–2mM-$CaCl_2$ for 10min at 30°C. Citrate synthase (EC 4.1.3.7) was assayed by the method of Coore *et al.* (1971). Total acetyl-CoA carboxylase (EC 6.4.1.2) activity was assayed after a 30min incubation at 30°C in the presence of 20mM-magnesium citrate, by the method of Halestrap & Denton (1973). $NADP^+$–malate dehydrogenase (EC 1.1.1.40) was assayed in 2ml of 100mM-triethanolamine buffer containing 2mM-$MgCl_2$, 0.5mM-sodium malate and 20μM-$NADP^+$, pH7.4. All enzyme assays were conducted at 30°C.

Pyruvate dehydrogenase phosphate phosphatase activity in tissue extracts was assayed as the release of [^{32}P]phosphate from pig heart pyruvate dehydrogenase [^{32}P]phosphate (Denton *et al.*, 1972). Details were as given by Severson *et al.* (1974), except that frozen samples of fat-pads and of fat-cells were extracted by using the Polytron PT20 homogenizer with 20mM-potassium phosphate buffer, pH7.0, containing 2mM-EDTA and 1mM-dithiothreitol. After centrifugation, 10μl samples were used for the assay of phosphatase activity in a total volume of 20μl in either the absence or the presence of Ca^{2+}. Final concentrations present in the assay were as follows: potassium phosphate (20mM); $MgCl_2$ (25mM); 2-mercaptoethanol (2.5mM); dithiothreitol (0.5mM); pyruvate dehydrogenase [^{32}P]phosphate (1nmol of protein-bound phosphate/ml); EGTA* (10mM) and either 0 or 9.75mM-$CaCl_2$, giving approx. 8.2mM-Mg^{2+} and either 0 or 7.2μM-Ca^{2+}.

Assay of enzyme activities in fat-pad and fat-cell mitochondrial fractions. Mitochondrial fractions were prepared as described by Severson *et al.* (1976), except that dinonyl phthalate was not used to separate the fat-pads and the fat-cells from the incubation medium and Ruthenium Red was not added to the sucrose-based extraction medium.

Extracts were prepared by freezing and thawing pellets (about 0.5mg of mitochondrial protein) in 250μl of 20mM-potassium phosphate buffer, pH7.0, containing 2-mercaptoethanol (5mM) and EDTA (2mM). Initial and total activity of pyruvate dehydrogenase and the activities of glutamate dehydrogenase, citrate synthase and pyruvate dehydrogenase phosphate phosphatase were assayed in appropriate samples of the extracts by the methods given above.

DNA assay. Powdered frozen fat-pad (300mg wet wt.) was extracted with 3ml of 5% (w/v) $HClO_4$ and 3ml of diethyl ether in a motor-driven Kontes tissue grinder, and centrifuged at 2000$g_{av.}$ for 3min. The ether was aspirated off and most of the remaining fat removed by a further extraction with 3ml of ether. Traces of ether in the aqueous phase were evaporated under a stream of N_2 before the precipitate was compacted by centrifugation. The precipitate was analysed for DNA content by the method of Burton (1956).

Protein assay. Tissue extracts were assayed for protein content by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

* Abbreviation: EGTA, ethanedioxybis(ethylamine)-tetra-acetate.

Analysis of incubation media and metabolism of [^{14}C]glucose. Glucose, pyruvate, lactate and glycerol were measured spectrophotometrically in neutralized $HClO_4$ extracts of incubation medium as described by Coore *et al.* (1971). The rate of conversion of [^{14}C]glucose into glyceride glycerol and fatty acids in epididymal fat-pads was determined as described by Denton & Randle (1967). The rate of conversion into CO_2 was measured by collecting $^{14}CO_2$ into 0.5ml of 50% (v/v) 2-phenethylamine in methanol, and ^{14}C was assayed after the addition of 5ml of toluene/methoxyethanol-based scintillator (Severson *et al.*, 1974).

Expression of results. A unit of enzyme activity catalyses the disappearance of substrate at a rate of 1μmol/min at 30°C. For pyruvate dehydrogenase phosphate phosphatase, units were calculated in terms of μmol of P_i released/min.

Results

Effects of starvation and alloxan-diabetes on the pyruvate dehydrogenase activity of rat epididymal fat-pad

Starvation for 48h resulted in an 80% decrease in the proportion of pyruvate dehydrogenase present in its active form, but the total activity was only slightly decreased (Table 1). Wieland *et al.* (1973) found that 24h starvation resulted in a 50% decrease in the active form without appreciable effect on the total activity. Alloxan-diabetes resulted in very similar changes as starvation for 48h (Table 1). The expression of measurements of enzyme activities presents particular problems in adipose tissue because of the large and variable triglyceride content. In Table 1, the results have been expressed in a wide variety of ways: in terms of tissue wet weight, tissue DNA concentration, tissue protein concentration, per fat-pad pair and as an activity ratio with glutamate dehydrogenase. Similar conclusions on the effects of starvation and alloxan-diabetes on pyruvate dehydrogenase can be drawn no matter which means of expression of results is chosen.

The total pyruvate dehydrogenase activity of fat-pad extracts varies quite considerably from batch to batch of animals no matter how results are expressed. Some variations in age, weight and feeding patterns of animals may be responsible; in all experiments particular care has been taken that the control and experimental animals were carefully matched for age and weight at the beginning of the experiments.

Table 2 shows the results of experiments in which fat-pads from normal, starved and alloxan-diabetic rats were incubated *in vitro* with fructose in the presence and the absence of insulin. In fat-pads from control animals, insulin increased the proportion of pyruvate dehydrogenase in its active form from about 37 to 68% of the total activity. This closely agrees with findings in previous studies (Severson *et al.*, 1974). After incubation of fat-pads from starved or alloxan-diabetic rats in the absence of insulin, the proportion of pyruvate dehydrogenase in its active form was 16–18% (i.e. much the same as *in vivo*; Table 1) or about one-half the percentage in the active form in control tissue incubated under the same conditions. Incubation in the presence of insulin increased the proportion to about 37 and 45% respectively for fat-pads from starved and alloxan-diabetic rats. The pyruvate dehydrogenase system is still clearly able to respond to the presence of insulin in the tissue. However, these proportions in the active form were less than that present (nearly 70%) in tissue

Table 1. *Effects of 48h starvation and alloxan-diabetes on the activity of pyruvate dehydrogenase in rat epididymal fat-pads*

Rats were anaesthetized with Nembutal (100mg/kg). Epididymal fat-pads (three or four per observation) were rapidly removed and frozen in liquid N_2 and extracts assayed for initial and total pyruvate dehydrogenase as described in the Experimental section. Diabetes was induced by a single injection of alloxan (60mg/kg) 48h before use; starved animals were deprived of food but not water for 48h before use. Results are given as means±S.E.M. of the numbers of observations in parentheses.

		Pyruvate dehydrogenase						
			Total activity as munits per					
Expt. no.	Animal	Initial activity (munits/g wet wt.)	g wet wt.	mg of protein	munit of glutamate dehydrogenase	fat-pad pair	mg of DNA	$\frac{\text{Initial activity}}{\text{Total activity}} \times 100$
1	Normal	125±9.1	289±31	20.6±2.10	0.31±0.03	199±20	936±87	46±4.1 (10)
	48h-starved	29±5.2**	249±14	14.2±1.04*	0.32±0.01	139±19*	698±85	12±2.1** (10)
2	Normal	137±11.4	246±9.3	16.0±0.87	0.24±0.02	193±17	783±41	56±5.4 (8)
	Alloxan-diabetic	40±8.6**	250±5.1	10.7±0.67**	0.26±0.03	161±18	729±60	18±3.4** (8)

* $P<0.05$; ** $P<0.001$ versus appropriate control.

Table 2. *Effects of insulin on the activity of adipose-tissue pyruvate dehydrogenase and glycerol release in 48h-starved and alloxan-diabetic rats*

Animals were killed by decapitation and fat-pads (in paired groups of two or three) preincubated in bicarbonate-buffered medium (Krebs & Henseleit, 1932) containing fructose (2mg/ml) for 30min at 37°C. This was followed by incubation in fresh medium containing fructose (2mg/ml) with or without insulin (10munits/ml) for 60min. Fat-pads were removed, lightly blotted and frozen in liquid N_2 before extraction as described in the Experimental section. Pyruvate dehydrogenase assays and the treatment of alloxan-diabetic and starved animals were as given in the legend to Table 1. After incubation, media were acidified with $HClO_4$ and glycerol was assayed as described in the Experimental section. Results are given as means±S.E.M. of the numbers of observations in parentheses.

Expt no.	Animal	Additions to incubation media	Pyruvate dehydrogenase: Initial activity (munits/g wet wt.)	Pyruvate dehydrogenase: Total activity (munits/g wet wt.)	Pyruvate dehydrogenase: Initial activity / Total activity ×100	Glycerol output (μmol/h per g)
1	Normal	None	81.7±5.7	206±16.1	36.6±5.9 (12)	1.83±0.10 (4)
		Insulin	168±8.6**	243±14.0	67.3±3.8**† (12)	0.73±0.11** (4)
	48h-starved	None	20.1±6.8	144±12.4	18.4±3.1 (12)	1.78±0.27 (4)
		Insulin	47.3±5.9*	133±13.3	37.0±5.1**† (12)	1.15±0.06 (4)
2	Normal	None	116±12	328±18	36.5±4.9 (6)	1.48±0.13 (6)
		Insulin	225±25*	344±35	69.3±9.6**† (6)	0.41±0.07** (6)
	Alloxan-diabetic	None	49.0±4.1	305±21	16.0±1.0 (7)	3.00±0.06 (7)
		Insulin	131±10**	298±19	45.5±5.4**† (7)	1.28±0.09** (7)

* $P<0.01$; ** $P<0.001$ versus appropriate control incubated without insulin.
† Calculated on basis of paired differences.

from control animals incubated in the presence of insulin. There was some alteration in the tissues from alloxan-diabetic and starved tissues, which resulted in a lowering in pyruvate dehydrogenase activity which persisted after 1 h incubation in the presence of insulin. In agreement with studies of fat-pads from normal rats (Weiss *et al.*, 1971; Severson *et al.*, 1974), the total activity of pyruvate dehydrogenase was not altered after incubation with insulin in fat-pads from normal, starved or alloxan-diabetic rats.

Effects of feeding a high-fat diet on pyruvate dehydrogenase activity of rat epididymal fat-pad

Table 3 gives the results of experiments in which rats were allowed free access for 6, 14 and 22 days to a diet in which, by the addition of beef suet, the fat content was increased from about 3 to 40% by wt. In each group animals matched for age and weight were killed 20–22 days after weaning. Those animals receiving the diet for 22 days were weaned on to the high-fat diet and gained weight at the same rate as the control animals. The fat-pad weights of this group were appreciably larger than those of the control animals. Zaragoza & Felber (1970) and Griglio *et al.* (1969), using high-fat diets with much less carbohydrate, have observed similar increases. Animals receiving the high-fat diet for 6 or 14 days tended not to reach the same weight as their controls at the time of death; this may be attributed in part to an initial reluctance of the rats to accept fully a new dietary regime.

The initial activity of pyruvate dehydrogenase was greatly decreased in the fat-pads of the animals on the high-fat diet for 6, 14 and 22 days. In the fat-pads of animals on the diet for 6 days the decrease could largely be attributed to a lowering of the proportion of pyruvate dehydrogenase in its active form. In contrast, the decrease of activity in fat-pads of animals on the diet for 14 and 22 days was the result of a marked decrease of the total activity of pyruvate dehydrogenase. The proportion of enzyme in the active form was not appreciably different from that in controls. The total activity of pyruvate dehydrogenase was significantly decreased by 60–80% if results were expressed as an activity ratio with glutamate dehydrogenase or on a fat-pad DNA or protein basis or as activity per fat-pad pair. No evidence was found from mixing experiments for the presence of any substance in extracts of fat-pads from fat-fed rats which inhibited pyruvate dehydrogenase activity in extracts of fat-pads from control animals.

Other experiments indicated that the administration of a diet containing about 10% beef suet for 20 days from weaning resulted in a smaller but still significant decrease of total pyruvate dehydrogenase activity from 311±44 to 166±47 munits/g wet wt. of tissue (mean±S.E.M. for four observations). It was also found that the administration of the normal diet for 4 days to rats previously fed on the 40%-suet diet for 20 days increased the total pyruvate dehydrogenase activity from 42.8±7.7 to 202±12.8munits/g wet wt. of tissue (mean±S.E.M. for five observations).

The high-fat diet fed for 20–23 days also resulted in

Table 3. *Effects of feeding a high-fat diet on pyruvate dehydrogenase and NADP–malate dehydrogenase activity in rat epididymal fat-pad*

Animals received the control diet 41B (Oxoid) or the high-fat diet (40% beef suet, 60% diet 41B plus protein and vitamin supplement) *ad libitum* for the number of days indicated. All animals were matched for age and weight at the beginning of the experiment and were killed 20–22 days from weaning. Fat-pads (three or four per observation) were removed from animals under Nembutal-induced anaesthesia, rapidly frozen in liquid N_2 and initial and total pyruvate dehydrogenase and $NADP^+$–malate dehydrogenase activities measured in extracts as detailed in the Experimental section. Results are given as means ± S.E.M. of the numbers of observations in parentheses.

Diet	No. of days	Rat wt. (g)	Fat-pad wt. (g)	Pyruvate dehydrogenase: Initial activity (munits/g wet wt.)	Pyruvate dehydrogenase: Total activity as munits per g wet wt.	Pyruvate dehydrogenase: Total activity as munit of glutamate dehydrogenase	Initial activity / Total activity ×100	NADP–malate dehydrogenase activity (munits/g wet wt.)
Normal	6	204±2.6 (8)	0.40±0.031	117±7.5	292±40	0.263±0.038	42±4.6	2392±292 (5)
High-fat		175±4.1** (8)	0.43±0.035	49.4±6.2**	378±42	0.337±0.053	13±1.5**	812±68.0* (5)
Normal	14	195±3.4 (8)	0.39±0.016	74.6±20	214±21	0.211±0.022	33±4.8	1742±127 (5)
High-fat		160±6.6** (8)	0.37±0.007	49.0±6.9**	124±15*	0.128±0.013*	39±2.4	830±47.0** (5)
Normal	22	164±7.1 (16)	0.34±0.020	190±17	442±29	0.348±0.037	45±5.1	1902±135 (11)
High-fat		150±4.1 (16)	0.46±0.010**	49.8±5.7**	138±5.7**	0.171±0.011**	37±3.9	196±13.4** (11)

* $P<0.01$; ** $P<0.001$ versus appropriate control.

a very marked decrease in total acetyl-CoA carboxylase and NADP–malate dehydrogenase activities but little or no change in the activities of either glutamate dehydrogenase or citrate synthase (Tables 3 and 4). Both these latter enzymes are, like pyruvate dehydrogenase, mitochondrial enzymes.

Table 5 summarizes the effects of the high-fat diet on glucose metabolism and pyruvate dehydrogenase activity in fat-pads incubated *in vitro* in the presence and the absence of insulin. The most marked change in glucose metabolism with fat feeding was a decrease in the rate of fatty acid synthesis and in the associated CO_2 formation especially in the presence of insulin (Table 5*a*). The rates of synthesis of glyceride glycerol and the output of lactate plus pyruvate were not greatly changed. In fact, in fat-pads from fat-fed rats incubated with insulin the output of lactate and pyruvate approached the rate of fatty acid synthesis and accounts for some 20% of the total glucose metabolism; in fat-pads from control animals the output was about 10% of the rate of fatty acid synthesis and accounted for only about 5% of the total glucose metabolism. This relative diversion of glucose carbon to lactate and pyruvate and away from fatty acid synthesis is consistent with the inhibition of pyruvate metabolism at pyruvate dehydrogenase. Insulin increased the rate of conversion of glucose into fatty acids, CO_2, glyceride glycerol, lactate and pyruvate and decreased glycerol output in fat-pads from fat-fed rats, but the effects on a percentage basis tended to be less than in fat-pads from control animals.

Insulin increased the proportion of pyruvate dehydrogenase in its active form in fat-pads from the rats fed on the high-fat diet; the total activity of pyruvate dehydrogenase was again markedly less than in the control tissues (Table 5*b*).

Table 4. *Effects of feeding the high-fat diet on enzyme activities in rat epididymal fat-pads*

Weight- and age-matched animals received the control or the high-fat diet for between 20 and 23 days from weaning. Fat-pads were removed from animals under Nembutal-induced anaesthesia and frozen rapidly in liquid N_2. Extracts were prepared and enzymes assayed as described in the Experimental section. Results were calculated as activity/fat-pad pair and are given for the tissue from fat-fed animals expressed as a percentage ± S.E.M. of the values observed in their controls. The numbers of observations are given in parentheses.

Enzyme	Activity (% of control)
Total pyruvate dehydrogenase	38.5±3.0 (11)
Total acetyl-CoA carboxylase	9.2±1.3 (11)
NADP–malate dehydrogenase	13.5±1.3 (11)
Glutamate dehydrogenase	84±7.0 (11)
Citrate synthase	103±8.8 (10)

Table 5. *Effects of feeding the high-fat diet on (a) glucose metabolism and (b) pyruvate dehydrogenase activity in rat epididymal fat-pads incubated in the presence and absence of glucose*

Weight- and age-matched animals received control or high-fat diet for between 19 and 23 days from weaning before death by decapitation. (*a*) Fat-pads (in paired groups of two) were preincubated in bicarbonate-buffered medium containing glucose (1.5mg/ml) for 30min and then transferred to fresh medium containing [U-^{14}C]glucose (1.5mg/ml, 0.2μCi/ml) with or without insulin (10munits/ml) and incubated in Marie flasks for 60min. Radioactivity in CO_2, fatty acids and glyceride glycerol and outputs of lactate, pyruvate and glycerol were measured as described in the Experimental section. (*b*) Fat-pads (in paired groups of two) were preincubated and incubated with or without insulin (10munits/ml) as described in the legend to Table 2. All results are expressed as means±S.E.M. of the numbers of observations in parentheses.

Additions to incubation medium ...	Fat-pads from rats fed on normal diet		Fat-pads from rats fed on high-fat diet		
	None	Insulin	None	Insulin	
(*a*) Incorporation of ^{14}C from [U-^{14}C]glucose (as μg-atoms of C/h per g) into:					
CO_2	3.00±0.70	23.9±1.25	1.33±0.16	6.31±0.46**	(4)
Fatty acid	4.76±0.68	36.3±1.11	1.63±0.35*	6.29±1.37**	(4)
Glyceride glycerol	1.19±0.21	3.58±0.19	1.18±0.17	2.41±0.06*	(4)
Output (as μg-atoms of C/h per g)					
Lactate+pyruvate	1.40±0.41	3.69±0.33	1.92±0.19	3.74±0.42	(4)
Glycerol	1.84±0.35	0.99±0.09	2.04±0.07	1.41±0.11	(4)
(*b*) Pyruvate dehydrogenase activity (as munits/g wet wt.)					
Initial	90.4±6.7	173±6.6**	24.4±3.7	53.5±4*	(5)
Total	294±43.3	343±25.5	120±36.8	132±34.5	(5)
$\frac{\text{Initial}}{\text{Total}} \times 100$	32.6±3.4	51.2±3.5*	20.6±6.7	41.8±8.1	(5)

* $P<0.01$; ** $P<0.001$ versus appropriate control (normal diet).

Effects of feeding the high-fat diet on the pyruvate dehydrogenase activity of rat heart, psoas, kidney and liver

Feeding rats a high-fat diet for 19–26 days had no appreciable effect on the total activity of pyruvate dehydrogenase in rat heart, psoas, kidney and liver (Table 6). In heart and psoas, the proportion of pyruvate dehydrogenase in the active form was decreased. This may reflect the increased availability of lipid fuels in the fat-fed group. Decreased rates of oxidation of pyruvate in diaphragms from fat-fed rats has been shown by Bringolf *et al.* (1972).

Effects of insulin, alloxan-diabetes and feeding the high-fat diet on pyruvate dehydrogenase phosphate phosphatase activity in rat epididymal fat-pads

Pyruvate dehydrogenase phosphate phosphatase activity was assayed as the release of [^{32}P]phosphate from added pig heart pyruvate dehydrogenase [^{32}P]phosphate (Tables 7 and 8). The amount of fat-pad pyruvate dehydrogenase phosphate present in the assays varied, but was always less than 10% of the added labelled substrate and has been neglected in all the calculations. Assays were conducted with saturating concentrations of Mg^{2+} (Severson *et al.*, 1974) and either with near-zero Ca^{2+} (10mM-EGTA) or about 7.2μM-Ca^{2+} (obtained by using an EGTA/Ca^{2+} buffer). Measurements have been made both in extracts of fat-pads and fat-pad mitochondria, since it became clear that the phosphatase activity as a ratio to the total activity of pyruvate dehydrogenase or glutamate dehydrogenase was much greater in extracts of fat-pads than in extracts of mitochondria prepared from the fat-pads (Table 8). Mixing experiments revealed no inhibitory substance in mitochondrial extracts; rather it appeared that about 80% of the phosphatase activity in whole tissue extracts was extramitochondrial. This was confirmed by fractionation of isolated fat-cells into mitochondrial and supernatant fractions (Martin & Denton, 1970; Severson *et al.*, 1976). Over 80% of the phosphatase activity was found in the supernatant fraction that contained no detectable pyruvate dehydrogenase activity and less than 10 and 20% of the cell content of citrate synthase and glutamate dehydrogenase respectively. Phosphatase activity in the supernatant fraction was activated by Ca^{2+} but to a lesser extent than the activity extracted from fat-cell mitochondria. The calcium sensitivity of phosphatase measured in whole-tissue extracts of fat-pads also tended to be less than the sensitivity of phosphatase in mitochondrial extracts (Tables 7 and 8).

Exposure of fat-pads from normal animals to

Table 6. *Effects of feeding the high-fat diet on the initial and total activities of pyruvate dehydrogenase in rat epididymal fat-pads, heart, psoas muscle, kidney and liver*

The details of the animals were as given in the legend to Table 5. In one series of experiments after Nembutal anaesthesia fat-pads were first removed and rapidly frozen in liquid N_2, then immediately the heart and a sample of psoas muscle were excised and freeze-clamped at the temperature of liquid N_2. In the other series of experiments after anaesthesia either a kidney or a piece of liver (1 g) was removed and immediately frozen in liquid N_2. Extraction of tissue and assay of enzymes were as given in the Experimental section. Results are expressed as means±S.E.M. for the numbers of observations in parentheses.

		Pyruvate dehydrogenase			
Tissue	Diet	Initial activity (munits/g wet wt.)	Total activity (munits/g wet wt.)	$\frac{\text{Initial activity}}{\text{Total activity}} \times 100$	
Fat-pad	Normal	92.8±19.0	339±35.3	23.8±3.44	(5)
	High-fat	31.0±4.65*	169±22.6*	18.3±2.10	(5)
Heart	Normal	1037±171	2897±66.6	36.2±6.30	(5)
	High-fat	469±108*	3405±292	15.5±5.54*	(5)
Psoas muscle	Normal	538±37.6	1092±48.1	49.2±2.17	(5)
	High-fat	213±33.4**	1207±168	18.5±2.42**	
Kidney	Normal	331±30.4	1857±201	19.1±3.38	(5)
	High-fat	359±9.90	2099±148	17.4±1.17	(5)
Liver	Normal	81.0±4.10	1015±64.0	8.1±0.94	(4)
	High-fat	81.0±14.0	1443±107	5.6±0.79	(4)

* $P<0.02$; ** $P<0.001$ versus appropriate control fed on normal diet.

insulin *in vitro* had no significant effect on phosphatase activity in either extracts of fat-pads or fat-pad mitochondria, although there was a clear increase in the proportion of pyruvate dehydrogenase in its active form (Table 7). Similarly the decrease in the proportion of pyruvate dehydrogenase in the active form observed in extracts prepared from tissue of alloxan-diabetic animals was not associated with any decrease in phosphatase activity. Indeed there may be some increase in the ratio of phosphatase activity to that of total pyruvate dehydrogenase in extracts of mitochondria of pads from alloxan-diabetic animals. The marked diminution in total pyruvate dehydrogenase activity in extracts from fat-pads of fat-fed rats was not matched by equal decreases in phosphatase activity. In both whole tissue and mitochondrial extracts the ratio of phosphatase activity to that of total pyruvate dehydrogenase was more than doubled. In all conditions phosphatase activities in mitochondrial extracts were much less than those in whole-tissue extracts. Insulin, alloxan-diabetes and fat-feeding did not have any clear effects on the calcium sensitivity of phosphatase activity in either whole tissue or mitochondrial extracts.

Discussion

Pyruvate dehydrogenase phosphate phosphatase activity in rat epididymal fat-pad: effects of insulin

These studies have shown that only about 20% of the phosphatase activity capable of removing phosphate and activating pyruvate dehydrogenase in fat-pads is associated with mitochondria. The physiological role of the 80% outside mitochondria is not clear. This activity is still activated by Ca^{2+}, but perhaps to a lesser extent than the phosphatase activity associated with the mitochondrial fraction. Rat heart muscle may also contain some extramitochondrial pyruvate dehydrogenase phosphate phosphatase activity, but the activity probably accounts for less than 30% of the total tissue activity (Kerbey *et al.*, 1976). It seems unlikely that the extramitochondrial activity in adipose tissue is directly concerned with the regulation of pyruvate dehydrogenase activity, since previous studies indicate that the whole pyruvate dehydrogenase system, including phosphatase sensitive to Mg^{2+} and Ca^{2+}, is located within the inner mitochondrial membrane (Severson *et al.*, 1974; Denton *et al.*, 1975). Preliminary studies have shown that a number of phosphoprotein phosphatase preparations of differing molecular weights from rabbit skeletal muscle, which are capable of the rapid dephosphorylation of histones, troponin and phosphorylase *a*, do not catalyse the release of P_i from pyruvate dehydrogenase phosphate (K. P. Ray, H. T. Pask, R. M. Denton & P. J. England, unpublished work). The identity and physiological role of the extramitochondrial pyruvate dehydrogenase phosphate phosphatase activity in adipose tissue is an intriguing problem which remains to be established.

It now appears that the previous conflicting findings on the effects of insulin on pyruvate dehydrogenase phosphate phosphatase activity in whole

Table 7. *Effects of insulin, alloxan-diabetes and feeding the high-fat diet on pyruvate dehydrogenase phosphate phosphatase activity in extracts of intact fat-pads and fat-pad mitochondria*

For the measurement of enzyme activities in fat-pad extracts, fat-pads were removed from decapitated animals and incubated in the presence or the absence of insulin (10munits/ml) in bicarbonate-buffered medium containing fructose (2mg/ml) for 20min at 37°C (Expt. 1) or (Expts. 2 and 3) removed after induction of Nembutal anaesthesia. Fat-pads were then frozen in liquid N_2 and extracted as given in the Experimental section. For the measurement of enzyme activities in fat-pad mitochondrial extracts, fat-pads were removed from decapitated animals and incubated with or without insulin for 30min in bicarbonate-buffered medium containing fructose (2mg/ml) and mitochondrial fractions prepared and extracted as given in the Experimental section. Pyruvate dehydrogenase phosphate phosphatase activity was measured by using pig heart pyruvate dehydrogenase [^{32}P]phosphate in the presence of about 8.2mM-Mg^{2+} and either EGTA (without Ca^{2+}) or an EGTA/Ca^{2+} buffer which gave a [Ca^{2+}] of 7.2μM (further details are given in the Experimental section). Results are given as mean±S.E.M. for the number of observations given in parentheses. Each observation was made on extracts derived from separate groups of two to four fat-pads. Some data from Expt. 1 were given in Severson *et al.* (1974).

			Total pyruvate dehydrogenase activity			Pyruvate dehydrogenase phosphate phosphatase activity measured with Ca^{2+}			
Expt. no.	Animal	Additions to incubation media	(munits/g wet wt.)	(munit/unit of glutamate dehydrogenase activity)	Initial pyruvate dehydrogenase activity (% of total)	(μunit/unit of total pyruvate dehydrogenase)	(μunit/g wet wt.)	(μunit/unit of glutamate dehydrogenase)	(% of activity measured without Ca^{2+})
Activities in fat-pad extract:									
1	Normal	None (4)	330±55	—	23.9±4.0	210±29	69.3±2.0	—	214±10
	Normal	Insulin (4)	353±51	—	54.3±6.9*	176±29	62.0±9.1	—	198±25
2	Normal	— (9)	195±18	—	37.1±4.4	493±54	89.0±2.5	—	385±13
	Alloxan-diabetic	— (9)	154±10*	—	12.2±2.4*	507±41	64.2±5.7	—	399±15
3	Normal	— (6)	306±28	—	—	214±26	63.0±4.3	—	216±14
	High-fat diet	— (6)	65±8.4*	—	—	502±37*	33.0±5.3	—	236±15
Activities in fat-pad mitochondrial extract:									
4	Normal	None (4)	—	536±70	46.1±4.8	67.1±14.8	—	34±6.7	302±39
	Normal	Insulin (4)	—	504±59	81.5±6.3	80.0±17.0	—	39±9.1	322±41
5	Normal	None (4)	—	635±32	39.3±1.7	102.2±7.8	—	64.5±4.8	499±64
	Alloxan-diabetic	None (4)	—	508±45	23.6±1.3	175.0±18.8*	—	83.7±6.0	599±51
6	Normal	None (4)	—	306±17	—	82.7±4.6	—	25.1±0.6	335±5
	High-fat diet	None (4)	—	119±7*	—	186±19.1*	—	31.1±2.2	400±20

* $P < 0.01$ versus appropriate normal control.

Table 8. *Distribution of pyruvate dehydrogenase phosphate phosphatase activity in epididymal fat-pads and isolated fat-cells of normal rats*

Isolated fat-cells were prepared as given in the Experimental section. Cells (approx. 1 g dry wt.) and fat-pads (in groups of two to four) were incubated for 30 min at 37°C in bicarbonate-buffered medium containing fructose (2 mg/ml) before preparation of extracts and assay of enzyme activities as given in the Experimental section and in the legend to Table 7. The supernatant fraction from fat-cells obtained after centrifugation to remove mitochondria contained less than 10% of the cell citrate synthase, less than 15% of cell glutamate dehydrogenase and no detectable pyruvate dehydrogenase. Results are given as means ± S.E.M. for the numbers of observations given in parentheses. Each observation was made on extracts or fractions derived from separate groups of two to four fat-pads.

	Pyruvate dehydrogenase phosphate phosphatase activity measured with Ca^{2+}			
	(μunits/unit of total pyruvate dehydrogenase activity)	(μunit/unit of glutamate dehydrogenase activity)	(% of activity in whole tissue or cell extract)	(% of activity without Ca^{2+})
Fat-pad				
Whole-tissue extract (5)	519 ± 40	256 ± 34	—	221 ± 29
Mitochondrial extract (5)	115 ± 11	45 ± 4.8	22.2 ± 2.2	298 ± 33
Isolated fat-cells				
Whole-cell extract (3)	614 ± 26	151 ± 9.9	—	—
Supernatant fraction (3)	—	—	85.6 ± 0.7	214 ± 20
Mitochondrial extract (3)	130 ± 6	44 ± 1.6	14.1 ± 0.7	477 ± 21

tissue extracts (Sica & Cuatrecasas, 1973; Severson *et al.*, 1974; Mukherjee & Jungas, 1975) have little relevance to the regulation of pyruvate dehydrogenase. Nevertheless, no evidence for any persistent effect of insulin on pyruvate dehydrogenase phosphate phosphatase activity in mitochondrial fractions was found in this study. This of course does not rule out the possibility of insulin acting through increasing phosphatase activity by a mechanism such as a change in concentration of an effector, which would not necessarily persist in extracts.

Effects of starvation and alloxan-diabetes

The pyruvate dehydrogenase activity is markedly diminished in fat-pads of starved and alloxan-diabetic rats. The decreases are very largely the result of a decrease in the proportion of the enzyme in its active, non-phosphorylated form and little evidence was found for any change in total activity. Similar changes are observed in fat-pads of normal animals injected with anti-insulin serum (Coore *et al.*, 1971; D. Stansbie & R. M. Denton, unpublished work), so it could be argued that the effects of starvation and alloxan-diabetes on adipose-tissue pyruvate dehydrogenase activity are the direct result of the low plasma insulin concentrations under these conditions. However, the effects of starvation and alloxan-diabetes persist in fat-pads incubated *in vitro* with insulin, suggesting that there is some alteration in these tissues which affects pyruvate dehydrogenase activity and which is not corrected by incubation with insulin *in vitro*. One possible mechanism would be a decrease in the concentration of pyruvate dehydrogenase phosphate phosphatase (brought about by a decrease in rate of its synthesis or increase in its breakdown), but no evidence for any decrease in activity in alloxan-diabetes was found. Pyruvate dehydrogenase kinase activity is inhibited by pyruvate and incubation of fat-cell or heart mitochondria with pyruvate leads to a progressive increase in the pyruvate dehydrogenase activity (Martin *et al.*, 1972; Cooper *et al.*, 1974). It has been shown that pyruvate dehydrogenase activity in mitochondria prepared from hearts of alloxan-diabetic rats is relatively insensitive to activation by pyruvate (Kerbey *et al.*, 1976), and this finding has been extended to mitochondria from fat-pads (B. J. Bridges, R. M. Denton & P. J. Randle, unpublished work). It seems likely that increases in fatty acid mobilization and oxidation are important in the changes in pyruvate oxidation and pyruvate dehydrogenase activity in heart and kidney in starvation and alloxan-diabetes (Randle *et al.*, 1966; Wieland *et al.*, 1973; Patzelt *et al.*, 1973) and that changes in the activity of pyruvate dehydrogenase kinase brought about by alterations in the mitochondrial acetyl-CoA/CoA and NADH/NAD^+ concentration ratios may be involved (Kerbey *et al.*, 1976).

Effects of feeding a high-fat diet

After 6–26 days of feeding with a diet containing about 40% fat, the initial activity of pyruvate dehydrogenase in rat epididymal fat-pads was very greatly decreased. After 6 days the decrease was largely the result of a diminution in the proportion of enzyme in the active form and there was no

appreciable change in total amount of enzyme. This is similar to the changes seen with alloxan-diabetes and starvation. In contrast, after 14–26 days on the high-fat diet the decrease in activity could be very largely accounted for by a marked diminution in the total amount of pyruvate dehydrogenase, and there was little evidence of any change in the proportion of enzyme in the active form. Studies with rats on the diet for 19–23 days indicate that this repression is not common to all enzymes in fat-cell mitochondria, since no alteration in the activities of citrate synthase and glutamate dehydrogenase was evident. Re-feeding the normal high-carbohydrate–low-fat diet for 4 days largely reversed the effects of the high-fat diet. No decreases in total pyruvate dehydrogenase activity were observed in any other tissue studied. These results point to there being a specific and reversible adaptive change in the pyruvate dehydrogenase content of adipose tissue under these conditions similar to that reported for many other enzymes involved in fatty acid synthesis, including ATP citrate lyase (EC 4.1.3.8), acetyl-CoA carboxylase, fatty acid synthase, the NADP-linked dehydrogenases of the pentose cycle and NADP-linked malate dehydrogenase. One possible difference is that the adaptive changes of pyruvate dehydrogenase may be rather slower than these other enzymes, but this remains to be established. Rather surprisingly there was no parallel change in mitochondrial pyruvate dehydrogenase phosphate phosphatase activity, suggesting that the long-term regulation of the phosphatase and pyruvate dehydrogenase may be independent. This would also explain the fact that the ratio of pyruvate dehydrogenase phosphate phosphatase activity/total pyruvate dehydrogenase activity is considerably less in fat-pad mitochondria than in rat heart mitochondria (Kerbey *et al.*, 1976). The total activity of pyruvate dehydrogenase in mammary gland has also been shown to undergo adaptive changes on lactation (Coore & Field, 1975), but measurements of mitochondrial phosphatase activity have not been reported. It remains to be established whether the adaptive changes in total pyruvate dehydrogenase activity in fat tissue and mammary gland are due to parallel changes in all three catalytic components of the complex or whether only one or two components are affected. If the latter were the case, this would imply that the composition of the complex may be genetically determined.

The very considerable parallel decreases in the enzyme profile of adipose tissue and its capacity for fatty acid synthesis observed in the present studies resulted from administration of a diet that contained approx. 40% by weight each of lipid and carbohydrate and 15% by weight of protein. The composition of the diet is thus close to the typical diet of humans in developed western countries. The very low rates of fatty acid synthesis found by many workers in human adipose tissue (see Patel *et al.*, 1975) may be related to similar metabolic adaptations to diet to those seen in rats.

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