Biochem. J. (1971) **125**, 115–127
Printed in Great Britain

Regulation of Adipose Tissue Pyruvate Dehydrogenase by Insulin and Other Hormones

By H. G. COORE, R. M. DENTON, B. R. MARTIN and P. J. RANDLE
Department of Biochemistry, University of Bristol, Bristol BS8 1TD, U.K.

(*Received* 25 *May* 1971)

1. In epididymal adipose tissue synthesizing fatty acids from fructose *in vitro*, addition of insulin led to a moderate increase in fructose uptake, to a considerable increase in the flow of fructose carbon atoms to fatty acid, to a decrease in the steady-state concentration of lactate and pyruvate in the medium, and to net uptake of lactate and pyruvate from the medium. It is concluded that insulin accelerates a step in the span pyruvate→fatty acid. 2. Mitochondria prepared from fat-cells exposed to insulin put out more citrate than non-insulin-treated controls under conditions where the oxaloacetate moiety of citrate was formed from pyruvate by pyruvate carboxylase and under conditions where it was formed from malate. This suggested that insulin treatment of fat-cells led to persistent activation of pyruvate dehydrogenase. 3. Insulin treatment of epididymal fat-pads *in vitro* increased the activity of pyruvate dehydrogenase measured in extracts of the tissue even in the absence of added substrate; the activities of pyruvate carboxylase, citrate synthase, glutamate dehydrogenase, acetyl-CoA carboxylase, NADP–malate dehydrogenase and NAD–malate dehydrogenase were not changed by insulin. 4. The effect of insulin on pyruvate dehydrogenase activity was inhibited by adrenaline, adrenocorticotrophic hormone and dibutyryl cyclic AMP (6-*N*,2′-*O*-dibutyryladenosine 3′:5′-cyclic monophosphate). The effect of insulin was not reproduced by prostaglandin E_1, which like insulin may lower the tissue concentration of cyclic AMP (adenosine 3′:5′-cyclic monophosphate) and inhibit lipolysis. 5. Adipose tissue pyruvate dehydrogenase in extracts of mitochondria is almost totally inactivated by incubation with ATP and can then be reactivated by incubation with 10mM-Mg^{2+}. In this respect its properties are similar to that of pyruvate dehydrogenase from heart and kidney where evidence has been given that inactivation and activation are catalysed by an ATP-dependent kinase and a Mg^{2+}-dependent phosphatase. Evidence is given that insulin may act by increasing the proportion of active (dephosphorylated) pyruvate dehydrogenase. 6. Cyclic AMP could not be shown to influence the activity of pyruvate dehydrogenase in mitochondria under various conditions of incubation. 7. These results are discussed in relation to the control of fatty acid synthesis in adipose tissue and the role of cyclic AMP in mediating the effects of insulin on pyruvate dehydrogenase.

The increase in fatty acid synthesis from glucose induced by insulin in rat epididymal adipose tissue is often in excess of 500%, whereas the associated increase in pyruvate output is rarely greater than 200% (Denton, Halperin & Randle, 1968; Denton & Halperin, 1968; Saggerson & Greenbaum, 1970*a*; Halperin & Robinson, 1970). This preferential incorporation of pyruvate carbon atoms into fatty acids compared with its release from fat-pads in the presence of insulin led to the suggestion that insulin might specifically activate the conversion of pyruvate carbon atoms into fatty acid in addition to its well-established effect on glucose uptake (Denton *et al.* 1968; Denton & Martin, 1970). Adrenaline, on the other hand, may antagonize an action of insulin on the conversion of pyruvate carbon atoms into fatty acid because it increases the output of lactate and pyruvate from glucose and either inhibits or leaves unchanged the rate of incorporation of glucose carbon atoms into fatty acid (Denton *et al.*

1968; Denton & Halperin, 1968; Saggerson & Greenbaum, 1970*a*).

In this paper we report effects of insulin on fructose metabolism in rat epididymal fat-pads which add further support for an effect of insulin on the conversion of pyruvate into fatty acid. Evidence is presented from studies on isolated fat-cell mitochondria and on enzyme activities measured in extracts of whole tissue and mitochondrial fractions which indicate that these effects of insulin and adrenaline are achieved at least in part by activation or inhibition respectively of pyruvate dehydrogenase (EC 1.2.4.1). Further analysis of the effect of insulin on the activity of pyruvate dehydrogenase indicates that it most probably operates via the phosphorylation–dephosphorylation cycle previously shown to influence the activity of the enzyme complex from heart, liver, kidney and brain (Linn, Pettit & Reed, 1969; Wieland & Siess, 1970; Siess, Wittman & Wieland, 1971).

The activation by adrenaline and the inhibition by insulin of glycogenolysis and lipolysis in adipose tissue have been interpreted in terms of effects of cyclic AMP (adenosine 3′:5′-cyclic monophosphate) (on protein kinase) leading to activation of glycogen phosphorylase and triglyceride lipase (Jungas, 1966; Robison, Butcher & Sutherland, 1968; Huttunen, Steinberg & Mayer, 1970). Adrenaline has been shown to elevate adipose tissue concentrations of cyclic AMP and insulin to lower them. The effects of adrenaline on lipolysis and tissue cyclic AMP concentrations are reproduced by adrenocortiocotrophic hormone and dibutyryl cyclic AMP (6-*N*,2′-*O*-dibutyryl adenosine 3′:5′- cyclic monophosphate) whereas the effects of insulin on these parameters are reproduced by prostaglandin E_1 (Robison *et al.* 1968; Butcher & Baird, 1968). Because of the possibility that insulin and adrenaline might influence the activity of pyruvate dehydrogenase through their effects on adipose tissue cyclic AMP concentration, studies have also been made of the effects of these other agents. In the light of these results and other published work we discuss the possible mechanism of these hormonal effects and interactions, and in particular the extent to which known variations in the tissue concentration of cyclic AMP provide an adequate explanation for these phenomena.

While this work was in progress Jungas (1970, 1971) reported evidence for activation of adipose tissue pyruvate dehydrogenase by insulin and inhibition by adrenaline and for control of enzyme activity by a phosphorylation–dephosphorylation cycle. Some of the present results have been published in preliminary form (Denton, Coore, Martin & Randle, 1971*a*; Coore, Denton, Martin & Randle, 1971; Denton, Martin, Coore & Randle, 1971*b*).

EXPERIMENTAL

Materials

Rats. Epididymal fat-pads were obtained from male albino Wistar rats (150–200 g) with free access to a stock laboratory diet (modified 41B; Oxoid Ltd., London S.E.1, U.K.). The animals were killed by decapitation and in any one experiment were closely matched for age and weight.

Chemicals. Except where otherwise stated, enzymes, adenine nucleotides, coenzymes, pyruvate and 2-oxoglutarate were from Boehringer Corp. (London) Ltd., London W.5, U.K. Crystalline insulin, a gift of Boots Pure Drug Co. Ltd., Nottingham, U.K. was dissolved in 3mM-HCl to yield a stock solution of 10 units/ml. Adrenaline was from Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K.; a concentrated solution was freshly prepared in 10mM-HCl and diluted appropriately in incubation medium. Adrenocorticotrophic hormone was from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. Prostaglandin E_1, a gift from Dr J. Pike, Upjohn Co., Kalamazoo, Mich., U.S.A. to Dr J. G. Schofield of this Department, was diluted from a stock solution in ethanol (1 mg/ml). Bovine plasma albumin (fraction V) was obtained from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K. and freed of fatty acids, citrate and other impurities as described by Denton & Halperin (1968). Fructose was from BDH (Chemicals) Ltd., Poole, Dorset, U.K. and [U-^{14}C]fructose from The Radiochemical Centre, Amersham, Bucks., U.K. Collagenase was from Sigma (London) Chemical Co. Ltd. (lot 70C-0290).

Acetyl-CoA was synthesized by the method of Simon & Shemin (1953); arylamine acetyltransferase (EC 2.3.1.5) was prepared from pigeon livers by the method of Tabor, Mehler & Stedtman (1953).

Anti-insulin sera prepared by the method of Robinson & Wright (1961) were kindly given by Dr A. Cole of this Department. The sera bound approx. 7 μg of ^{125}I-labelled bovine insulin/ml at 4°C.

Media. Fat-pads were incubated in bicarbonate-buffered saline medium (Krebs & Henseleit, 1932) gassed with $O_2 + CO_2$ (95:5). For the preparation and incubation of fat-cells, the same medium plus albumin (40 mg/ml) was dialysed against 20 vol. of albumin-free medium at 4°C by using Visking seamless tubing treated to remove plasticizing substances. The composition of media for incubation of mitochondria is given in the appropriate table legend.

Isolated fat-cells and fat-cell mitochondria. These were prepared as described by Martin & Denton (1971). Because of the extreme sensitivity of fat-cells to insulin, vessels used in their preparation were soaked in 10M-KOH for 12 h to inactivate any insulin and anti-insulin serum (1 μl/ml) was added to control media to neutralize insulin that may contaminate some batches of albumin. Equivalent amounts (1 μl/ml) of non-immune guinea-pig sera were added to media with added insulin.

Methods

Extraction and analysis of enzymes in fat-pads and fat-cells. At the end of incubation, the pads were quickly and lightly blotted and then immediately frozen and ground in liquid N_2. After evaporation of the liquid N_2 a

weighed portion of powder (about 400 mg) was extracted for 1 min in a glass tissue grinder either with 2 ml of ice-cold 100 mM-potassium phosphate buffer, pH 7.0, containing 2 mM-EDTA (for assays of pyruvate dehydrogenase, glutamate dehydrogenase and NAD- and NADP-malate dehydrogenase) or with 2 ml of 50 mM-triethanolamine, pH 7.4, containing 2 mM-EDTA and 0.25 mM-dithiothreitol (for assays of citrate lyase and acetyl-CoA carboxylase). The extracts were then centrifuged for 2 min at approx. 16000*g* in an Eppendorf 3200 centrifuge. Enzyme activities were assayed in samples of the clear infranatant within 4 h of extraction. Control experiments showed no loss of enzyme activity over this period of storage and that a single extraction removed all but negligible activities of the enzymes studied.

At the end of incubation of fat-cells, the cells were separated by centrifugation at room temperature for 1 min at about 2000*g*, and after removal of the infranatant medium by syringe the cells were frozen with liquid N_2 and extracted as described above for fat-pads.

Extraction and analysis of enzymes in mitochondrial pellets. Fat-cell mitochondria were packed by centrifuging for 2 min at about 16000*g* in an Eppendorf 3200 centrifuge and the pellet was frozen by plunging the plastic centrifuge tube in liquid N_2. The pellet was thawed and dispersed on a Vortex mixer in potassium phosphate buffer (details are given in legends to figures and tables). The dispersion was frozen and thawed once and then re-frozen until required for assay. It was found in preliminary experiments that further freezing and thawing failed to extract further activity of pyruvate dehydrogenase and glutamate dehydrogenase from the mitochondrial pellet. Treatments of the extracts to demonstrate effects of ATP and Mg^{2+} on pyruvate dehydrogenase activity are given in legends to appropriate figures and tables.

Enzyme assays. All assays were conducted at 30°C. Control experiments showed in each case that rates of reaction were proportional to the volume of extract used, the concentrations of substrates used gave maximum reaction rates and that the rates were constant over the period of assay (2–5 min). Blanks were included in each case by omission of one substrate.

Pyruvate dehydrogenase was assayed spectrophotometrically by coupling with arylamine acetyltransferase (Tabor *et al.* 1953), and following the change in extinction at 460 nm due to acetylation of *p*-(*p*-aminophenylazo)-benzenesulphonic acid (Jacobson, 1961). The assay was conducted in 100 mM-tris chloride buffer, pH 7.8, containing EDTA (0.5 mM), $MgCl_2$ (1 mM), mercaptoethanol (5 mM), thiamine pyrophosphate (1 mM), NAD (0.5 mM), CoA (0.1 mM), pyruvate (1 mM), *p*-(*p*-aminophenylazo)-benzenesulphonic acid (10 μg/ml) and arylamine acetyltransferase (0.1 unit/ml). Glutamate dehydrogenase (EC 1.4.1.2), ATP citrate lyase (EC 4.1.3.8), NAD-malate dehydrogenase (EC 1.1.1.37), NADP-malate dehydrogenase (EC 1.1.1.40) and pyruvate carboxylase (EC 6.4.1.1) were assayed as described by Martin & Denton (1970). Citrate synthase (EC 4.1.3.7) was assayed by a slight modification of the method of Srere, Barzil & Conen (1963). Final concentrations in the cuvette were acetyl-CoA 50 μM, oxaloacetate 0.1 mM and 5,5′-dithiobis-(2-dinitrobenzoic acid) 0.1 mM in tris chloride buffer, pH 7.4 (0.1 M). Acetyl-CoA carboxylase (EC 6.4.1.2) was assayed by a modification of the methods of Martin & Vagelos (1962) and Saggerson & Greenbaum (1970*b*). Fat-pad extract (200 μl) was added to 50 mM-triethanolamine buffer, pH 7.4, containing 80 mM-potassium citrate, 20 mM-$MgCl_2$, 8 mM-$MnCl_2$, 30 mM-$KH^{14}CO_3$ (10 μCi/μmol), 10 mM-ATP, 1 mM-dithiothreitol and 0.15 mM-acetyl-CoA in a total volume of 1.2 ml. After 10 min at 30°C, the reaction was terminated by addition of 200 μl of 5 M-HCl and $^{14}CO_2$ was removed *in vacuo* after addition of 50 μl of saturated $KHCO_3$. A sample (200 μl) was then transferred to a scintillation bottle, heated to dryness at 85°C (about 30 min) and assayed for radioactivity after addition of 200 μl of water and 10 ml of methoxyethanol–toluene-based scintillator. Controls lacking acetyl-CoA were carried through the procedure.

Analysis of incubation media. Glucose, lactate and glycerol were measured spectrophotometrically in neutralized $HClO_4$ extracts of incubation media and uptakes and outputs were calculated as described by Denton & Randle (1967). For assay of pyruvate, media were acidified with $HClO_4$ immediately after incubation and assays performed without neutralization within 2 h by the method of Bücher, Czok, Lamprecht & Latzko (1963). Fructose was assayed by the method of Klotzsch & Bergmeyer (1963). Citrate and malate outputs of fat-cell mitochondria were measured as described by Martin & Denton (1971).

RESULTS AND DISCUSSION

Effects of insulin on glucose and fructose metabolism in vitro. Fig 1(*a*) shows that insulin stimulates the rate of glucose uptake by incubated epididymal fat-pad by more than tenfold whereas the rate of fructose uptake was little changed by the hormone in this particular experiment. By pooling results from several experiments, it was, however, possible to show that insulin accelerates fructose uptake by $42 \pm 7.7\%$ of the control rate in paired fat-pads ($P < 0.02$; data of Table 1). The effect of insulin on the incorporation of ^{14}C from [U-^{14}C]fructose into glyceride fatty acids appeared to be somewhat larger (Table 1). Further, this increase of incorporation into fatty acids was accompanied by a definite fall in the steady-state concentrations of pyruvate and lactate in the medium achieved after 30 min incubation, i.e. insulin caused net uptake of lactate and pyruvate (Figs. 1*b* and 1*c*). The relationship of transient changes in medium pyruvate concentration to its intracellular concentration may be doubtful but it seems reasonable to assume that a fall in the medium concentration to a new steady-state value may be associated with a fall in the intracellular concentration of pyruvate to a new steady-state value. If this assumption is accepted then the rate of flow of fructose to fatty acid through an obligatory intermediate (pyruvate) was increased whereas the intracellular concentration of the intermediate (pyruvate) fell. This would suggest

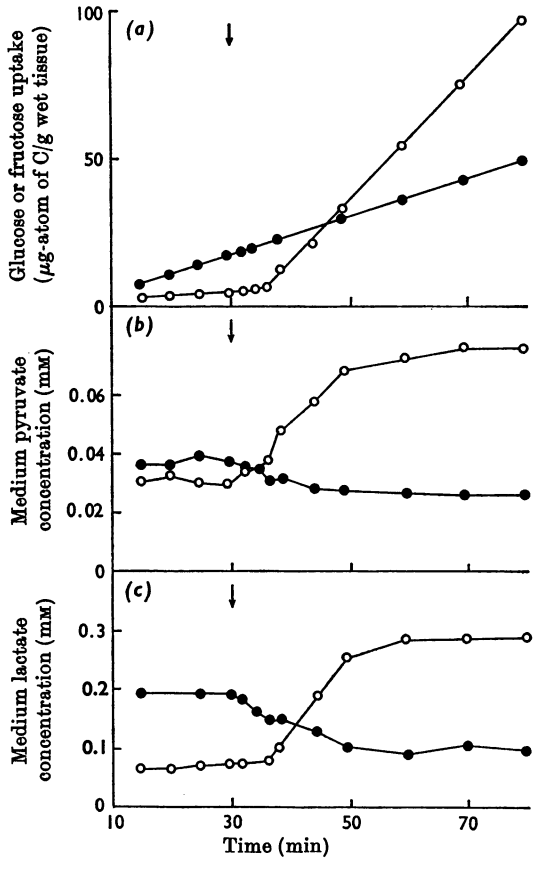

Fig. 1. Effect of insulin on the time-course of glucose or fructose uptake by incubated epididymal fat-pads and the medium pyruvate and lactate concentrations. Pads (six) were incubated in 10ml of bicarbonate-buffered medium containing intially either glucose (2mg/ml) (○) or fructose (2mg/ml) (●). Insulin (10m-units/ml) was added at 30min (indicated by an arrow). Samples of media for analysis of glucose, fructose, pyruvate and lactate were taken by syringe through a serum stopper.

acceleration by insulin of a step in the pathway of fatty acid synthesis in the span pyruvate→fatty acid. Presumably this effect was most clearly demonstrable with fructose as substrate because of the very small effect of insulin on membrane transport of fructose. Halperin (1970) has reported stimulation by insulin of the utilization of extracellular pyruvate for fatty acid synthesis and this effect may be compared with the present results for pyruvate formed intracellularly from fructose. A further point of difference in behaviour of glucose and fructose is that whereas insulin increases the lactate/pyruvate ratio in the medium when glucose is the substrate (Denton, Yorke & Randle, 1966; Halperin & Denton, 1969) the ratio tends to fall when fructose is present instead. This may indicate a fall in the cytoplasmic NADH/NAD^+ ratio with insulin in the presence of fructose.

Effects of insulin on mitochondrial metabolism of pyruvate. Fat-cell mitochondria incubated in potassium chloride medium containing pyruvate (1.5mM), potassium bicarbonate (12.5mM), ADP (1mM) and potassium phosphate (2mM) transform at least 90% of the pyruvate taken up into citrate and malate (Martin & Denton, 1971). Table 2 shows that the rate of this mitochondrial conversion of pyruvate into citrate and malate is increased when fat-cells are exposed to insulin before isolation of the mitochondria. Apparently an insulin-sensitive step in the mitochondrial metabolism of pyruvate had retained increased activity in spite of the drastic environmental alterations involved in the isolation and incubation of mitochondria. As shown in Table 2 mitochondria prepared from fat-cells exposed to insulin also showed increased rates of citrate formation on incubation with pyruvate and malate in the presence of uncoupler *m*-chlorocarbonylcyanide phenylhydrazone. Under these conditions (low ATP and bicarbonate, presence of malate) pyruvate carboxylase is non-functional and the oxaloacetate utilized in citrate formation was

Table. 1. *Effect of insulin on the metabolism of fructose by epididymal fat-pad.*

Fat-pads, paired in groups of two to six pads, were preincubated for 30min in medium containing fructose (2mg/ml) and then incubated for a further 30min in fresh medium containing [U-^{14}C]fructose (2mg/ml and 0.1μCi/ml); one flask of each pair contained 10mU of insulin/ml. Results are given as mean values ±S.E.M. (number of paired groups given in parentheses).

Parameter	Control	With insulin
Fructose uptake (μg-atom of C/30min per g of wet tissue)	12.3 ± 0.83 (15)	17.7 ±1.63* (15)
Incorporation of [U-^{14}C]fructose into glyceride glycerol (μg atom of C/30min per g of wet tissue)	2.94± 0.36 (7)	3.19±0.39 (7)
Incorporation of [U-^{14}C]fructose into glyceride fatty acids (μg atom of C/30min per g of wet tissue)	2.45± 0.34 (7)	4.30±0.50* (7)
Medium pyruvate concn. (μM) after 30min incubation	29 ± 0.8 (15)	19.8 ±0.8* (15)
Medium lactate concn. (μM) after 30min incubation	209 ±20 (15)	121 ±18* (15)
Medium lactate/pyruvate concn. ratio	7.37± 0.99 (15)	6.04±0.80*† (15)

* $P<0.02$ versus no-insulin control.

† Calculated on the basis of paired tissues.

Table 2. *Rates of citrate and malate output from mitochondria prepared from cells incubated in the presence or absence of insulin*

Fat-pads in paired groups of four (number of groups given in parentheses) were incubated for 30min in Krebs bicarbonate buffer containing fructose (2mg/ml) with or without insulin (10mU/ml). Fat-cells were then prepared from each group of pads, insulin again being present in the same member of each matched pair during the preparation and washing of the cells. Mitochondria were prepared from each group of cells and incubated in 130mM-KCl, 2mM-$MgCl_2$, 2mM-ethanedioxybis(ethylamine)tetra-acetate, 20mM-tris–HCl, pH7.4, plus stated additions.

	Additions to incubation media	Cell source of mitochondria	Citrate output (μmol/unit of glutamate dehydrogenase per 5min)	Malate output (μmol/unit of glutamate dehydrogenase per 5min)
Expt. 1	Pyruvate (0.25mM), $KHCO_3$ (12.5mM), ADP (1mM)	Normal	0.44±0.03 (8)	0.22±0.02 (8)
	Phosphate (1mM)	Insulin-treated	0.70±0.06** (8)	0.33±0.03* (8)
Expt. 2	Pyruvate (5mM), Malate	Normal	1.80±0.14 (4)	—
	m-Chlorocarbonyl cyanide phenylhydrazone	Insulin-treated	2.57±0.19** (4)	—

* $P<0.05$, ** $P<0.01$ versus no-insulin control.

derived from malate by oxidation. We have concluded from this experiment that pyruvate carboxylase was unlikely to be the insulin-sensitive step. This appeared to leave as likely candidates pyruvate dehydrogenase, citrate synthase and mitochondrial transporting systems for pyruvate, citrate and malate.

Effects of insulin on enzymes involved in the pathway of pyruvate carbon incorporation into fatty acids. In these studies, the maximum activities of the mitochondrial enzymes pyruvate dehydrogenase, pyruvate carboxylase, citrate synthase and glutamate dehydrogenase were measured in homogenates prepared from fat-pads incubated with or without insulin, together with the activities of three extramitochondrial enzymes of importance in fatty acid synthesis from pyruvate (NADP–malate dehydrogenase; ATP citrate lyase; acetyl-CoA carboxylase). The absence of suitable methods prevented accurate quantitative measurements of the activities of mitochondrial transporting systems.

As shown in Tables 3 and 4 only pyruvate dehydrogenase, of the mitochondrial enzymes, was affected by insulin treatment and its activity was increased by 60–140% over the no-insulin controls; and insulin treatment did not affect the activities of the cytoplasmic enzymes NADP–malate dehydrogenase, ATP citrate lyase or acetyl-CoA carboxylase under conditions when a marked effect on pyruvate dehydrogenase was exhibited (Table 4). The activity of the exclusively mitochondrial enzyme, glutamate dehydrogenase (Martin & Denton, 1970, 1971), was measured as a convenient index of recovery of mitochondrial enzymes. Its activity was unaffected by insulin treatment (Tables 3 and 4) and in subsequent experiments the pyruvate dehydrogenase/glutamate dehydrogenase activity ratio has been used as an index of changes in the activity of pyruvate dehydrogenase.

Table 3 shows in addition that the effect of insulin on pyruvate dehydrogenase activity was not dependent on the presence of a substrate such as glucose or fructose in the incubation medium. We conclude from this that the effect of insulin on pyruvate dehydrogenase is not dependent on its effect on the membrane transport of glucose (or fructose). It is notable that in the non-insulin-treated tissue pyruvate dehydrogenase has the lowest maximum activity of all those enzymes concerned with the incorporation of pyruvate carbon atoms into fatty acid whose activity was measured, which is also suggestive that it might be a control point in the process. Further, the enzyme activity after exposure to insulin is in the range 10–20μmol/h per g of wet tissue at 37°C (assuming enzyme activities double for each 10°C rise in temperature). This would be sufficient to account for published rates of carbon atom flow through pyruvate dehydrogenase when fatty acids are synthesized from glucose with insulin (Saggerson & Greenbaum, 1970*a*; Flatt & Ball, 1964; Katz & Rognstad, 1966). It is also noteworthy that the observed increase in pyruvate dehydrogenase activity due to insulin is comparable with the magnitude of the hormonal effect on fatty acid synthesis from fructose (Table 1). Most experiments involved a 30min exposure to insulin but it was possible to detect a significant effect of the hormone on fat-pad pyruvate dehydrogenase after only 10min exposure to the hormone *in vitro* (activity found after insulin exposure was 136±9% of the

Table 3. *Activities of pyruvate dehydrogenase, pyruvate carboxylase, citrate synthase and glutamate dehydrogenase measured in fresh extracts of rat epididymal fat-pads previously incubated in the presence or absence of insulin*

Fat-pads (in paired groups of two or three pads) were preincubated for 30 min in medium containing no substrate, or glucose (2 mg/ml) or fructose (2 mg/ml), and then incubated for a further 30 min in fresh but similar media containing in one flask of each paired group insulin (10 mU/ml). Results are given as means ± S.E.M.

Additions to incubation medium	Enzyme activities (μmol/min per g of wet tissue)					
	None	Insulin	Glucose	Glucose + insulin	Fructose	Fructose + insulin
Number of groups	(8)	(8)	(4)	(4)	(4)	(4)
Pyruvate dehydrogenase	0.087 ± 0.11	0.140 ± 0.018*	0.099 ± 0.015	0.165 ± 0.025**	0.072 ± 0.007	0.176 ± 0.012**
Citrate synthase	3.05 ± 0.24	3.07 ± 0.20	3.63 ± 0.17	3.49 ± 0.20	4.13 ± 0.52	4.03 ± 0.34
Pyruvate carboxylase	1.40 ± 0.25	1.60 ± 0.19	—	—	0.83 ± 0.12	0.83 ± 0.08
Glutamate dehydrogenase	0.65 ± 0.04	0.65 ± 0.04	0.84 ± 0.04	0.75 ± 0.30	0.98 ± 0.14	1.06 ± 0.06

* $P<0.05$, ** $P<0.005$ versus no-insulin control when calculated for paired groups.

control value; mean ± S.E.M. for four observations).

When administered *in vivo* an even greater effect of insulin on fat-pad pyruvate dehydrogenase could be demonstrated. Pentabarbitone-anaesthetized rats (approx. 60 mg/kg by intraperitoneal injection) were injected via tail veins with either 250 μl of guinea-pig anti-serum or 0.3 unit of bovine insulin in 250 μl of non-immune guinea-pig serum. After 15 min the epididymal fat-pads were quickly removed, frozen and ground under liquid N_2. Extraction and assay of pyruvate dehydrogenase and glutamate dehydrogenase were then carried out as detailed in the Experimental section. Blood glucose determinations at time of removal of tissue showed animals treated with anti-insulin serum to have concentrations of 150 mg/100 ml or above and insulin-treated animals to have concentrations of below 90 mg/100 ml. Activity of pyruvate dehydrogenase in extracts of fat-pads from rats treated with anti-insulin serum was 0.053 ± 0.015 μmol/min per g of wet tissue and this was increased in pads from insulin-treated rats to 0.237 ± 0.045 μmol/min per g of wet tissue (means ± S.E.M. for four observations in each case). There was no detectable difference in the activity of glutamate dehydrogenase.

Effects of adrenaline, adrenocorticotrophic hormone, dibutyryl cyclic AMP and prostaglandin E_1 on pyruvate dehydrogenase. Exposure of fat-pads to adrenaline (1 μg/ml) for 30 min almost completely abolished the stimulatory effect of insulin on pyruvate dehydrogenase but adrenaline failed to diminish the activity of the enzyme in the absence of insulin (Table 5). However, in a separate experiment in which adrenaline exposure was limited to 10 min, pyruvate dehydrogenase activity was decreased to 78 ± 1.5% of the control value (mean ± S.E.M. for four observations; $P<0.01$). In a further experiment with fat-cells prepared with collagenase, adrenaline after 30 min incubation markedly decreased the activity of the enzyme and this inhibition was prevented by the β-blocker propanolol at 10 μg/ml (Table 5). Adrenocorticotrophic hormone and dibutyryl cyclic AMP were also found to reverse the stimulatory effect of insulin on pyruvate dehydrogenase (Tables 6 and 7).

Prostaglandin E_1 had no significant effect on pyruvate dehydrogenase although decreasing the rate of lipolysis to an extent similar to that of insulin (Table 6). In a further experiment, prostaglandin E_1 again failed to increase pyruvate dehydrogenase activity but clearly diminished the rate of glycerol output. Combining the results of the two experiments, the activities of pyruvate dehydrogenase after exposure to insulin and prostaglandin E_1 were respectively 153 ± 12% and 98 ± 9% of the control value whereas the rates of glycerol output were respectively 60 ± 6% and 47 ± 5% of control

Table 4. *Effect of insulin on extramitochondrial enzymes concerned in fatty acid synthesis along with the mitochondrial enzymes pyruvate dehydrogenase and glutamate dehydrogenase*

Fat-pads in four paired groups of three pads were preincubated (30min) and incubated (30min) in bicarbonate-buffered medium containing fructose (2mg/ml) and, where added, insulin (10mU/ml).

	Enzyme activity (μmol/min per g of wet tissue)	
Additions to incubation medium ...	None	Insulin
Pyruvate dehydrogenase	0.094±0.012	0.244±0.019*
Glutamate dehydrogenase	1.21 ±0.1	1.23 ±0.07
NAD–malate dehydrogenase	76.7 ±6.1	77.8 ±5.0
NADP–malate dehydrogenase	4.9 ±0.2	5.5 ±0.6
ATP citrate lyase	0.92 ±0.05	0.94 ±0.06
Acetyl-CoA carboxylase	0.22 ±0.03	0.25 ±0.01

* $P < 0.01$ versus control.

(mean±S.E.M. for eight observations). When added in the presence of insulin, prostaglandin E_1 had no significant effect on glycerol output or pyruvate dehydrogenase activity (Table 7).

Thus it would appear that elevation of cyclic AMP concentration in adipose tissue (e.g. with adrenaline, adrenocorticotrophic hormone or dibutyryl cyclic AMP) may be correlated with antagonism to insulin stimulation of pyruvate dehydrogenase; but in contrast lowering of cyclic AMP concentrations (e.g. with prostaglandin E_1) is not necessarily associated with activation of pyruvate dehydrogenase.

Studies on the effects of ATP and magnesium concentration on adipose tissue pyruvate dehydrogenase activity. Pyruvate dehydrogenases in liver, kidney, heart and brain have been shown to exist in phosphorylated (inactive) and non-phosphorylated (active) forms (Linn *et al.* 1969; Wieland & Siess, 1970; Siess *et al.* 1971). The interconversions were found to be mediated by an ATP-dependent kinase and a Mg^{2+}-stimulated phosphatase. Evidence for the operation of a similar system in adipose tissue has been obtained as follows.

Addition of ATP to uncoupled fat-cell mitochondria incubated in the presence of pyruvate and malate led to a marked decrease in citrate output and to a parallel diminution in the activity of pyruvate dehydrogenase subsequently extracted from the mitochondria (Table 8).

Addition of $MgATP^{2-}$ (1mM) or $MgCl_2$ (10mM) to extracts of whole fat-pads incubated at 20°C led to some inhibition and activation respectively of pyruvate dehydrogenase activity but the effects were small and variable. However, clear and consistent evidence for inhibition of fat-cell pyruvate dehydrogenase by ATP and activation by Mg^{2+} was obtained from observations of the effects of additions of ATP and Mg^{2+} to extracts of fat-cell mitochondria. In the experiment shown in Fig. 2, mitochondria were isolated from non-insulin-treated fat-cells and extracted by freezing and thawing in 30mM-potassium phosphate buffer, pH7.0, containing 0.5mM-$MgCl_2$, oligomycin (1μg/ml) and 5mM-mercaptoethanol. Pyruvate dehydrogenase activity in this extract was constant during incubation at 30°C for at least 30min. If, however, $MgCl_2$ (10mM) was added the activity was markedly increased until, after 20–30min, the pyruvate dehydrogenase/glutamate dehydrogenase ratio was about 0.6; on tenfold dilution with magnesium-free phosphate buffer activity declined only very slowly but after addition of ATP (0.1mM) activity was rapidly lost and after 30min the pyruvate dehydrogenase/glutamate dehydrogenase ratio was less than 0.05. When the order of addition of ATP and Mg^{2+} was reversed it was found that the loss of activity seen with ATP was overcome on the subsequent addition of $MgCl_2$ (10mM).

As shown in Table 9, the difference in pyruvate dehydrogenase activity between extracts of mitochondria prepared from insulin-treated and those from non-insulin-treated fat-cells could be eliminated by incubation with 1mM-$MgATP^{2-}$ or with 10mM-Mg^{2+}. This finding suggests that the activating effect of insulin on pyruvate dehydrogenase is the result of an increase in the proportion of the active (dephosphorylated) form of the enzyme. Some further support for this conclusion is provided by the observation (Fig. 3) that insulin treatment of fat-cells increased the $V_{max.}$ of pyruvate dehydrogenase in extracts of mitochondria prepared from them without altering the K_m for pyruvate. (The results in Fig. 3 give K_m±S.E.M. 65±3μM and 79±7μM after non-insulin and insulin treatment respectively.) Likewise almost identical values for the K_m for pyruvate were obtained with extracts of fat-pads incubated in the absence or presence of insulin (78±6μM and 51±6μM respectively). It may be noted that the conditions of

Table 5. *Effect of adrenaline in the presence and absence of insulin on the activity of pyruvate dehydrogenase extracted from epididymal fat-pads and isolated fat-cells*

Procedure was as given in legend to Table 4. Enzyme activities are expressed as μmol/min per g wet wt. for fat-pads and as μmol/min per g dry wt. for fat-cells. The number of observations are given in parentheses.

Preparation	Expt. no.	Additions to incubation medium		Pyruvate dehydrogenase (μmol/min per g wet wt.) (*A*)	Glutamate dehydrogenase (μmol/min per g wet wt.) (*B*)	Activity ratio *A*/*B*
Pads	1	None	(4)	0.067±0.06	0.69±0.10	0.10 ±0.01
		Adrenaline (1 μg/ml)	(4)	0.072±0.12	0.57±0.05	0.12 ±0.01
Pads	2	None	(4)	0.094±0.012	1.21±0.10	0.08 ±0.01
		Insulin (1 mU/ml)	(4)	0.244±0.019*	1.23±0.07	0.19 ±0.01
		Insulin (1 mU/ml)+ adrenaline (1 μg/ml)	(4)	0.141±0.015*	1.23±0.07	0.11 ±0.01*
				(μmol/min per g dry wt.)	(μmol/min per g dry wt.)	
Cells	3	None	(3)	—	—	0.089±0.009
		Adrenaline (1 μg/ml)	(3)	—	—	0.038±0.007*
		Propanolol (10 μg/ml)	(3)	—	—	0.070±0.005
		Propanolol (10 μg/ml)+ adrenaline (1 μg/ml)	(3)	—	—	0.069±0.007
Cells	4	None	(4)	0.162±0.007	0.99±0.03	0.163±0.010
		Adrenaline (1 μg/ml)	(4)	0.048±0.006*	0.89±0.05	0.054±0.008*

* $P<0.05$ versus relevant control.

Table 6. *Effects of insulin, prostaglandin E_1 and adrenocorticotrophic hormone on pyruvate dehydrogenase and glycerol output of epididymal fat-pads*

Procedure was as given in the legend to Table 4, except that anti-insulin serum (1 μl/ml) was added to control and prostaglandin E_1-containing media and normal guinea-pig serum (1 μl/ml) to insulin-containing media. Results are given as means ± S.E.M. of four observations.

Additions to incubation medium	Pyruvate dehydrogenase (μmol/min per g of wet tissue)	Activity ratio pyruvate dehydrogenase / glutamate dehydrogenase	Glycerol output (μmol/h per g of wet tissue)
None (control)	0.193 ± 0.031	0.232 ± 0.026	2.13 ± 0.63
Insulin (1.25 mU/ml)	0.281 ± 0.023*†	0.326 ± 0.027*	0.92 ± 0.17*
Prostaglandin E_1 (1 μg/ml)	0.152 ± 0.024	0.196 ± 0.027	0.83 ± 0.36*
Insulin (1.25 mU/ml) + adrenocorticotrophic hormone (2 μg/ml)	0.178 ± 0.017	0.203 ± 0.014	5.80 ± 0.46**

* $P<0.05$, ** $P<0.01$ versus control value.
† Calculated on the basis of paired differences.

Table 7. *Effects of prostaglandin E_1 and dibutyryl cyclic AMP in the presence of insulin on pyruvate dehydrogenase and glycerol output in epididymal fat-pads*

Procedure was as given in the legend to Table 6.

Additions to incubation medium	Pyruvate dehydrogenase (μmol/min per g of wet tissue)	Activity ratio pyruvate dehydrogenase / glutamate dehydrogenase	Glycerol output (μmol/h per g of wet tissue)
None (control)	0.038 ± 0.007	0.068 ± 0.014	1.43 ± 0.034
Insulin (5 mU/ml)	0.078 ± 0.010**	0.158 ± 0.010**	0.91 ± 0.10*
Insulin (5 mU/ml) + prostaglandin E_1 (2 μg/ml)	0.068 ± 0.010*	0.142 ± 0.017*	0.93 ± 0.11*
Insulin (5 mU/ml) + dibutyryl cyclic AMP (1 mM)	0.046 ± 0.006	0.071 ± 0.003	1.59 ± 0.23

* $P<0.05$, ** $P<0.01$ versus control value.

Table 8. *Effect of ATP on citrate production by and activity of pyruvate dehydrogenase in uncoupled fat-cell mitochondria incubated with pyruvate and malate*

Mitochondria were prepared from non-insulin-treated fat-cells and preincubated at 30°C for 9 min in 130 mM-KCl, 2 mM-$MgCl_2$, 2 mM-ethanedioxybis(ethylamine)tetra-acetate, 0.5 μg of *m*-chlorocarbonyl cyanide phenylhydrazone/ml and 1 μg of oligomycin/ml with or without addition of ATP (5 mM). Pyruvate (5 mM) and malate (5 mM) were then added and the mitochondria incubated for a further 5 min. Some samples of mitochondria and their incubation media were used for determination of citrate production and other samples for the determination of enzyme activities.

Additions to incubation medium	Citrate production (μmol/min per unit of glutamate dehydrogenase)	Activity ratio pyruvate dehydrogenase / glutamate dehydrogenase
None	0.32 ± 0.01 (4)	0.45 ± 0.01 (2)
ATP (5 mM)	0.18 ± 0.02 (4)	0.21 ± 0.03 (2)

assay used to demonstrate effects of insulin and other hormones in earlier sections of this paper were $V_{max.}$ conditions. Moreover the assays were carried out at high dilution and in the presence of 0.5 mM-EDTA so that the possibilities of activation by Mg^{2+} or of inactivation by $MgATP^{2-}$ would appear remote. It may also be noted that fructose was always included in the incubation medium in experiments with adrenaline with the object of preserving tissue concentrations of ATP. Adrenaline (but not insulin) can lower fat-pad concentrations of ATP on incubation without substrate (Hepp, Challoner & Williams, 1968; Bihler & Jeanrenaud, 1970) and this effect might explain the

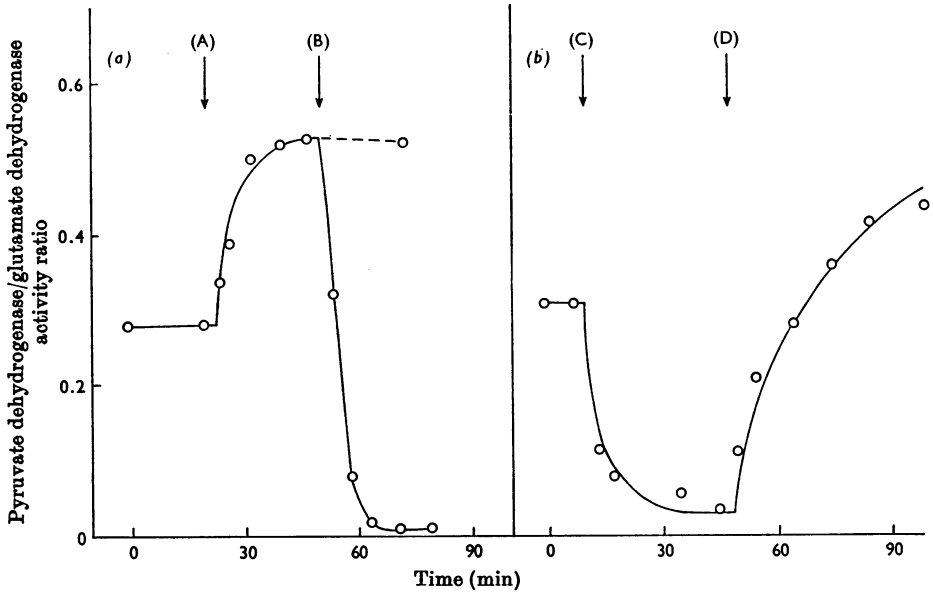

Fig. 2. Time-course of the effect of additions of $MgCl_2$ and ATP on the activity of pyruvate dehydrogenase in extracts of fat-cell mitochondria prepared by freezing and thawing mitochondria in 100 μl of 30 mM-potassium phosphate buffer, pH 7.0, containing $MgCl_2$ (0.5 mM), oligomycin (0.5 μg/ml) and mercaptoethanol (5 mM) and incubated at 30°C. (*a*) At (A) 10 mM-$MgCl_2$ was added and at (B) the remaining extract was diluted tenfold with 30 mM-potassium phosphate buffer, pH 7.0, containing mercaptoethanol (5 mM) and oligomycin (0.5 μg/ml) and incubation continued in the absence (----) or the presence (——) of 0.1 mM-ATP. (*b*) At (C) 0.1 mM-ATP was added and at (D) 10 mM-$MgCl_2$ was added.

Table 9. *Loss of effect of insulin on pyruvate dehydrogenase activity by incubating mitochondrial extracts with magnesium chloride or ATP*

Mitochondria were prepared from insulin-treated or non-insulin-treated cells as described in the legend to Table 2 and extracted by freezing and thawing in 30 mM-potassium phosphate buffer containing oligomycin (0.5 μg/ml) and mercaptoethanol (5 mM). Activities of pyruvate dehydrogenase and glutamate dehydrogenase were assayed immediately and after incubation of the extracts for 30 min at 30°C with the addition of either $MgCl_2$ (10 mM) or $MgATP^{2-}$ (1 mM).

	Activity ratio pyruvate dehydrogenase/glutamate dehydrogenase		
		After incubation	
Source of mitochondria	Initial	With $MgCl_2$ (10 mM)	With ATP (1 mM)
Non-insulin-treated cells	0.30 ± 0.04 (12)	0.58 ± 0.03 (6)	0.06 ± 0.02 (2)
Insulin-treated cells	0.54 ± 0.01 (12)	0.64 ± 0.02 (6)	0.055 ± 0.015 (2)

secondary rise in pyruvate dehydrogenase seen with adrenaline under such conditions (Jungas, 1971).

Effects of cyclic AMP on pyruvate dehydrogenase. These experiments were of two types. In one type cyclic AMP was added to the incubation medium of intact mitochondria and the activity of extracted pyruvate dehydrogenase measured; in the other cyclic AMP was incubated with the enzyme extracted either from whole fat-pads or isolated fat-cell mitochondria and the subsequent time-course of enzyme activity was then followed.

In a series of experiments of the first type mitochondria were prepared from insulin-treated fat-cells and incubated in potassium chloride (130 mM), potassium dihydrogen phosphate (2 mM), pyruvate (1.5 mM), potassium bicarbonate (12.5 mM), pH 7, and either ethanedioxybis(ethylamine)tetra-acetate (2 mM) or ethanedioxybis(ethylamine)tetra-acetate–

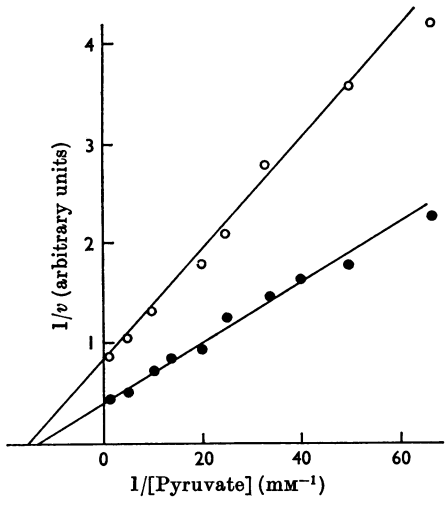

Fig. 3. Dependence of pyruvate dehydrogenase activity on pyruvate concentration in extracts of mitochondria from non-insulin-treated (○) and insulin-treated (●) fat-cells shown as Lineweaver–Burk plots of rate measurements obtained by varying the concentration of pyruvate with 1mM-thiamine pyrophosphate, 0.5mM-NAD and 0.1mM-CoA.

Ca^{2+} buffer such that free Ca^{2+} concentration was 1 or 0.1 μM. Addition of cyclic AMP (250 μM) during 15min incubation at 30°C did not influence activity of extracted pyruvate dehydrogenase.

Because of the possibility that an effect of cyclic AMP might require an extramitochondrial factor another experiment was performed as follows. Insulin-treated fat-cells were broken in sucrose (200mM), magnesium chloride (1.5mM), potassium bicarbonate (20mM), GSH (10mM), tris chloride (20mM), pH 7.3, ethanedioxybis(ethylamine)tetraacetate–Ca^{2+} (2mM) (free Ca^{2+} 1 μM) and 2% defatted bovine serum albumin with and without 0.5mM-ATP, with and without 250 μM-cyclic AMP. After centrifugation the infranatant was removed from the fat plug, gassed with O_2+CO_2 (95:5) and incubated at 30°C with shaking for 10min. The mitochondria were spun down and extracted as usual. A definite inhibition of pyruvate dehydrogenase activity was observed in the presence of ATP but this was not increased by the presence of cyclic AMP at 250 μM.

The experiment described above involved great but unavoidable dilution of the extramitochondrial components of the fat-cell. It could be argued that cyclic AMP required a higher concentration of cytoplasmic protein kinase to exhibit effects on intramitochondrial enzyme activities though the mechanism of such an effect would be obscure. Mitochondria were therefore prepared from insulin-treated fat-cells and incubated in potassium chloride (130mM), magnesium chloride (2mM), tris chloride (20mM) and oligomycin (0.5 μg/ml) with and without ATP (1mM). ATP-containing media were in some cases supplemented with 0.15mg of protein/ml of crude protein kinase from skeletal muscle (prepared as described by Walsh, Perkins & Krebs, 1968, up to the stage of DEAE-cellulose chromatography) and/or cyclic AMP (0.1mM). Extracted pyruvate dehydrogenase activity was unaffected by previous exposure of mitochondria to protein kinase and cyclic AMP alone or combined.

In several experiments extracts of pyruvate dehydrogenase from frozen fat-pads or from isolated mitochondria from non-insulin-treated and insulin-treated fat-cells were incubated in 30mM-phosphate buffer, pH 7, containing oligomycin (0.5 μg/ml), magnesium chloride (0.5mM) and mercaptoethanol (5mM) for up to 45min at 30°C with and without $MgATP^{2-}$ (1 or 0.5mM). Inhibition of enzyme activity by ATP was always shown but this was not increased by the further addition of cyclic AMP (0.1mM). Cyclic AMP by itself was also without effect. However, in an experiment in which skeletal-muscle protein kinase (0.15mg of protein/ml) was also present along with cyclic AMP during 15min incubation, enzyme activity was decreased to 28±3% of control (control: lacking ATP, protein kinase and cyclic AMP) whereas ATP alone only decreased activity to 51±4% of control (mean±S.E.M. for four observations in each case).

From this we conclude that cyclic AMP was unable to activate the intrinsic kinase of pyruvate dehydrogenase under conditions in which it could activate muscle protein kinase. Evidently pyruvate dehydrogenase is susceptible to inhibition, presumably owing to phosphorylation, by extrinsic activated protein kinase, but only when the two enzymes are in free solution not separated by the mitochondrial membrane barrier.

General discussion. These effects of insulin and adrenaline on pyruvate dehydrogenase have clarified some of the earlier observations on the physiological control of fatty acid synthesis in rat epididymal adipose tissue but we wish to emphasize that there is evidence which suggests a further point of control beyond the stage of citrate formation. It is well established that the whole cell concentration of citrate in adipose tissue is unchanged when carbon atom flow from glucose through citrate to fatty acid is maximally stimulated by insulin. Moreover, addition of adrenaline can then greatly increase citrate concentration and decrease the rate of flow of glucose carbon atoms to fatty acid (Denton & Halperin, 1968; Saggerson & Greenbaum, 1970*a*,*b*).

One possibility is that insulin and adrenaline could, by altering the citrate concentration gradient across the mitochondrial membrane, regulate the cytoplasmic citrate concentration and thereby alter the rate of flow through ATP citrate lyase. Another possibility is that insulin and adrenaline could act on a further enzyme in the pathway in the span between citrate and fatty acid.

Our experiments raise the question of the mechanism of action of insulin on pyruvate dehydrogenase but only allow us to suggest plausible alternatives in the light of the vast literature on insulin action. We shall assume for the purposes of discussion that the effect of insulin is to increase the proportion of dephospho-pyruvate dehydrogenase by either inhibition of the kinase or activation of the phosphatase or both. We shall confine our attention almost entirely to the fat-cell and assume not only that insulin acts on the cell but also that it does not enter and act within adipose cells. There appears to be no fully satisfactory demonstration of this though experiments with Sepharose-bound insulin (Cuatrecasas, 1969) have been interpreted in this way. This view implies that the effects of insulin on glycogen synthetase and phosphorylase (Jungas, 1970), on triglyceride lipase (Löffler & Weiss, 1970) and now on pyruvate dehydrogenase are not mediated by direct interaction of insulin with the enzyme systems concerned. In fact, we have not found any effect on pyruvate dehydrogenase of insulin added directly to a mitochondrial suspension but we realise that such an experiment is not conclusive. It would also seem unlikely that insulin added to intact cells is able to penetrate both the plasma membrane and the inner mitochondrial membrane within which pyruvate dehydrogenase is situated.

If then we assume that insulin acts only at the plasma membrane the question arises of how an effect at that site influences the activity of pyruvate dehydrogenase within the mitochondria. Although other possible links could be imagined, some form of 'second messenger' hypothesis seems most likely as a natural extension of Sutherland's concept for the action of adrenaline, glucagon, adrenocorticotrophic hormones and other hormones. Specifically, in view of the reported ability of insulin to lower cyclic AMP in fat-cells (Butcher, Sneyd, Park & Sutherland, 1966) it has been pertinent to consider whether cyclic AMP could mediate the effects of insulin and its hormonal antagonists on pyruvate dehydrogenase.

We have not directly measured fat-cell cyclic AMP in the conditions of our experiments but with fructose as a substrate insulin showed a consistent anti-lipolytic effect, as shown by glycerol release from incubated fat-pads. Butcher (1970) has summarized the evidence correlating the effects on adipose tissue lipolysis with tissue concentrations of cyclic AMP and the extracted triglyceride lipase has been shown to be activated by cyclic AMP (Rizack, 1964; Huttenen *et al.* 1970).

Our results indicate that adrenaline and adrenocorticotrophic hormone both stimulated lipolysis and hence presumably elevated cyclic AMP while antagonizing the stimulation of pyruvate dehydrogenase by insulin. The precise mode of action of dibutyryl cyclic AMP is not known but it is generally accepted as providing a means of reproducing the effects of cyclic AMP in intact cell preparations (Butcher, 1970) and in our experiments its effects were similar to that of adrenocortotrophic hormone and adrenaline.

These results are consistent with the idea of cyclic AMP as a 'second messenger' capable of inhibiting pyruvate dehydrogenase. On this hypothesis insulin would act by lowering tissue cyclic AMP, thus releasing the inhibition. The experiments with prostaglandin E_1, however, cannot be reconciled with this hypothesis. Prostaglandin E_1 diminished lipolysis to an even greater degree than insulin and hence presumably lowered cyclic AMP to at least the same extent, yet it failed to stimulate pyruvate dehydrogenase. Since prostaglandin E_1 did not impede the action of insulin the possibility of some other unrelated action of prostaglandin E_1 on pyruvate dehydrogenase seems remote. Further, we have failed to detect any effects of cyclic AMP on the activity of pyruvate dehydrogenase in intact mitochondria or in mitochondrial extracts incubated under a variety of different conditions. Perhaps the simplest interpretation of these findings is to consider the possibility that insulin action at the fat-cell membrane leads directly or indirectly to the production of a further second messenger which activates pyruvate dehydrogenase, and which is in some way antagonized by cyclic AMP. This antagonism might be the result of an interaction at the level of pyruvate dehydrogenase regulation and/or at sites of formation or destruction of cyclic AMP and this hypothetical second messenger. It may be noted in this connexion that Loten & Sneyd (1970) found that insulin treatment of fat-pads may increase the activity of cyclic AMP phosphodiesterase measured in extracts of the tissue. It is perhaps unlikely that this could result from a fall in the tissue concentration of its substrate (cyclic AMP).

We thank Mrs R. Low for skilled technical assistance. The cost of these investigations was defrayed by grants from the British Diabetic Association, the Medical Research Council and the British Insulin Manufacturers. B.R.M. holds a Medical Research Council Studentship and during part of these investigations H. G. C. held a Wellcome Research Fellowship.

REFERENCES

Bihler, I. & Jeanrenaud, B. (1970). *Biochim. biophys. Acta*, **202**, 496.

Bücher, Th., Czok, R., Lamprecht, W. & Latzko, E. (1963). In *Methods of Enzymatic Analysis*, p. 253. Ed. by Bergmeyer, H.-U. Berlin: Verlag Chemie.

Butcher, R. W. (1970). In *Adipose Tissue: Regulation and Metabolic Functions*, p. 5. Ed. by Jeanrenaud, B. & Hepp, D. London: Academic Press.

Butcher, R. W. & Baird, C. E. (1968). *J. biol. Chem.* **243**, 1713.

Butcher, R. W., Sneyd, J. G. T., Park, C. R. & Sutherland, E. W. (1966). *J. biol. Chem.* **241**, 1652.

Coore, H. G., Denton, R. M., Martin, B. R. & Randle, P. J. (1971). *Biochem. J.* **123**, 38P.

Cuatrecasas, P. (1969). *Proc. natn. Acad. Sci. U.S.A.* **63**, 450.

Denton, R. M., Coore, H. G., Martin, B. R. & Randle, P. J. (1971*a*). *Nature New Biol., Lond.*, **231**, 115.

Denton, R. M. & Halperin, M. L. (1968). *Biochem. J.* **110**, 27.

Denton, R. M., Halperin, M. L. & Randle, P. J. (1968). In *Physiopathology of Adipose Tissue*, p. 31. Ed. by Vague, J. Amsterdam: Excerpta Medica.

Denton, R. M. & Martin, B. R. (1970). In *Adipose Tissue: Regulation and Metabolic Functions*, p. 143. Ed. by Jeanrenaud, B. & Hepp, D. London: Academic Press.

Denton, R. M., Martin, B. R., Coore, H. G. & Randle, P. J. (1971*b*). *Biochem. J.* **123**, 39P.

Denton, R. M. & Randle, P. J. (1967). *Biochem. J.* **104**, 423.

Denton, R. M., Yorke, R. E. & Randle, P. J. (1966). *Biochem. J.* **100**, 407.

Flatt, J. P. & Ball, E. G. (1964). *J. biol. Chem.* **239**, 675.

Halperin, M. L. (1970). *Can. J. Biochem.* **48**, 1228.

Halperin, M. L. & Denton, R. M. (1969). *Biochem. J.* **113**, 207.

Halperin, M. L. & Robinson, B. H. (1970). *Biochem. J.* **116**, 235.

Hepp. D., Challoner, D. R. & Williams, R. H. (1968). *J. biol. Chem.* **243**, 2321.

Huttunen, J. K., Steinberg, D. & Mayer, S. E. (1970). *Biochem. biophys. Res. Commun.* **41**, 1350.

Jacobson, K. B. (1961). *J. biol. Chem.* **236**, 343.

Jungas, R. L. (1966). *Proc. natn. Acad. Sci. U.S.A.* **56**, 757.

Jungas, R. L. (1970). *Endocrinology*, **86**, 1368.

Jungas, R. L. (1971). *Metabolism*, **20**, 43.

Katz, J. & Rognstad, R. (1966). *J. biol. Chem.* **241**, 3600.

Klotzsch, H. & Bergmeyer, H.-U. (1963). In *Methods of Enzymatic Analysis*, p. 156. Ed. by Bergmeyer, H.-U. Berlin: Verlag Chemie.

Krebs, H. A. & Henseleit, K. (1932). *Hoppe-Seyler's Z. physiol. Chem.* **210**, 33.

Linn, T. C., Pettit, F. H. & Reed, L. J. (1969). *Proc. natn. Acad. Sci. U.S.A.* **62**, 234.

Löffler, G. & Weiss, L. (1970). In *Adipose Tissue: Regulation and Metabolic Functions*, p. 32. Ed. by Jeanrenaud, B. & Hepp, D. London: Academic Press.

Loten, E. G. & Sneyd, J. G. T. (1970). *Biochem. J.* **120**, 187.

Martin, B. R. & Denton, R. M. (1970). *Biochem. J.* **117**, 861.

Martin, B. R. & Denton, R. M. (1971). *Biochem. J.* **125**, 105.

Martin, D. B. & Vagelos, P. R. (1962). *J. biol. Chem.* **238**, 1787.

Rizack, M. A. (1964). *J. biol. Chem.* **239**, 392.

Robinson, B. M. B. & Wright, P. H. (1961). *J. Physiol., Lond.*, **155**, 302.

Robison, G. A., Butcher, R. W. & Sutherland, E. W. (1968). *A. Rev. Biochem.* **37**, 149.

Saggerson, E. D. & Greenbaum, A. L. (1970*a*). *Biochem. J.* **119**, 193.

Saggerson, E. D. & Greenbaum, A. L. (1970*b*). *Biochem. J.* **119**, 221.

Siess, E., Wittman, J. & Wieland, O. (1971). *Hoppe-Seyler's Z. physiol. Chem.* **352**, 447.

Simon, E. J. & Shemin, D. (1953). *J. Am. chem. Soc.* **75**, 2520.

Srere, P. A., Barzil, H. & Conen, L. (1963). *Acta chem. scand.* **17**, S129.

Tabor, H., Mehler, A. H. & Stedtman, G. R. (1953). *J. biol. Chem.* **204**, 127.

Walsh, D. A., Perkins, J. P. & Krebs, E. G. (1968). *J. biol. Chem.* **243**, 3763.

Wieland, O. & Siess, E. (1970). *Proc. natn. Acad. Sci. U.S.A.* **65**, 947.