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Acute Regulation of Pyruvate Kinase Activity in Rat Epididymal Adipose Tissue by Insulin

By RICHARD M. DENTON, NIGEL J. EDGELL, BARBARA J. BRIDGES and GRAEME P. POOLE*
Department of Biochemistry, University of Bristol Medical School, University Walk, Bristol BS8 1TD, U.K.

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1. Evidence is presented that exposure of epididymal fat-pads from fed rats to insulin leads to a marked diminution in the K_m for phosphoenolpyruvate of pyruvate kinase. Effects of insulin may be readily demonstrated in experiments both *in vivo* and *in vitro* and are not secondary to the activation by the hormone of glucose transport. No effect of insulin is apparent in tissues from 48h-starved animals. 2. The mechanism of the effect of insulin on pyruvate kinase was not established. The observed changes in K_m do not appear to be the result of alterations in the amounts of bound effectors such as fructose 1,6-bisphosphate and alanine. Rather, as the effect persists in incubated extracts, it appears that a change in the degree of phosphorylation or some other covalent modification of the enzyme may be involved.

At least three different isoenzymes of pyruvate kinase (EC 2.7.1.40) exist in animal tissues (Imamura & Tanaka, 1972; Harada *et al.*, 1978; Engström, 1978). The M_1-type isoenzyme is found in muscle and brain, whereas the L-type isoenzyme is the major type present in liver and is also found as a minor component in kidney. The pyruvate kinase present in most tissues, including adipose tissue, appears to be the third isoenzyme or group of isoenzymes, which have been designated variously by different laboratories as M_2, K or A types (M_2 is used in the present paper).

The activity of the L-type isoenzyme is regulated by a number of effectors, including fructose 1,6-bisphosphate (activator) and alanine (inhibitor). These effectors alter the apparent K_m towards phosphoenolpyruvate with little or no effect on the maximum activity (Seubert & Schoner, 1971). The apparent K_m for phosphoenolpyruvate of the L-type isoenzyme is also markedly increased by phosphorylation brought about by cyclic AMP-dependent protein kinase (Ljungström *et al.*, 1974; Engström, 1978). Changes in phosphorylation have been shown to occur in intact liver preparations associated with increased tissue concentrations of cyclic AMP, for example in the presence of glucagon (Feliú *et al.*, 1977; Ljungström & Ekman, 1977; Riou *et al.*, 1978). The apparent K_m for phosphoenolpyruvate of the M_2-type isoenzyme from a number of cell types may also be increased and decreased by fructose 1,6-bisphosphate and alanine respectively under appropriate conditions (Pogson, 1968*a*,*b*; Imamura & Tanaka, 1972). Early studies by Pogson (1968*a*,*b*) indicated that the adipose-tissue enzyme may exist in interconvertible forms. One, present in tissue extracts prepared with buffers containing EDTA, exhibited sigmoid steady-state kinetics with phosphoenolpyruvate and a high K_m; the other form predominated in tissues extracted in the absence of EDTA and showed hyperbolic kinetics with phosphoenolpyruvate and a much lower K_m. The high-K_m form could be converted into the low-K_m form by incubation of tissue extracts with fructose 1,6-bisphosphate. Pogson (1968*a*) presented evidence that the two forms had different overall charge and apparent molecular weight. Subsequently similar properties have been found with M_2-type enzymes from a number of other sources (Ibsen & Trippet, 1972; Walker & Potter, 1973; Van Berkel *et al.*, 1974; Feliú, 1975; Eigenbrodt & Schoner, 1977*a*; Nishimura *et al.*, 1978). It has been suggested that the two forms represent the enzyme with and without bound fructose 1,6-bisphosphate, since dissociation of fructose 1,6-bisphosphate has been reported to be very slow (Irving *et al.*, 1972; Seubert & Schoner, 1971). More recent studies indicate that the low-K_m form is a tetramer (mol.wt. about 200000) and the high-K_m form is a dimer (mol.wt. about 100000) (Ibsen & Trippet, 1972; Sparmann *et al.*, 1973; Eigenbrodt & Schoner, 1977*b*; Nishimura *et al.*, 1978). The M_2-type isoenzymes are not substrates for the cyclic AMP-dependent protein kinase, but phosphorylation by a cyclic AMP-independent protein kinase leading to inhibition of enzymic activity has been demonstrated with the M_2-type isoenzyme from chicken liver (Berglund *et al.*, 1977; Eigenbrodt & Schoner, 1977*b*).

In this paper, we present evidence that exposure

* Present address: Biological Laboratory, University of Kent, Canterbury, Kent CT2 7NJ, U.K.

to insulin *in vitro* or *in vivo* of rat epididymal adipose tissue from fed, but not from starved, animals leads to a substantial decrease in the K_m of pyruvate kinase. The effect of insulin on adipose-tissue pyruvate kinase does not appear to be the result of changes in the amounts of bound effectors such as fructose 1,6-bisphosphate and alanine. Rather, our results are compatible with insulin causing some change in the extent of phosphorylation or other stable covalent modification of the enzyme.

Experimental

Animals

Epididymal fat-pads were obtained from male Wistar albino rats (170–200g). Unless otherwise stated the rats were fed *ad libitum* with stock laboratory diet (modified 41B; Oxoid, Basingstoke, Hants., U.K.). Before injections of glucose or anti-insulin serum the rats were anaesthetized with Sagatal (pentobarbital; 1ml/kg) and the rats remained under anaesthesia until the pads were removed and immediately frozen with liquid N_2.

Materials

All biochemicals and enzymes, including phosphoenolpyruvate (potassium salt) and pig heart lactate dehydrogenase, were from Boehringer Corp. (London), Lewes, East Sussex, U.K. Chemicals were of the purest grade available from British Drug Houses, Poole, Dorset, U.K. Insulin was obtained from Sigma (London) Chemical Co., Poole, Dorset, U.K. Anti-insulin serum prepared by the method of Robinson & Wright (1961) was the kind gift of Dr. A. S. Cole of this Department. The sera bound approx. 5μg of ^{125}I-labelled bovine insulin/ml at 4°C.

Incubation and extraction of epididymal fat-pads

The pads were preincubated with shaking for 30min at 37°C in bicarbonate-buffered medium (Krebs & Henseleit, 1932) containing 1.25mM-$CaCl_2$, and, unless otherwise stated, glucose (11mM), and gassed with O_2/CO_2 (19:1). The pads were transferred to fresh gassed medium containing additions as indicated in the text and Tables and incubated for 30min. The pads were then removed, quickly blotted and immediately frozen by plunging into liquid N_2.

The frozen pads were powdered and extracted unless otherwise stated in Tris/acetate (100mM), pH6.8, containing KF (100mM) and EDTA (5mM) (4ml/g of frozen powder) by using a Polytron PT10 homogenizer. The homogenates were centrifuged for 30s at about 8000*g* in an Eppendorf 3200 Minifuge and the infranatant was removed from below the fat plug by syringe. The extracts were immediately frozen and stored on solid CO_2 until required for assay (up to 6h); the extracts were then thawed at 0°C and assayed either immediately or after incubation with or without fructose 1,6-bisphosphate as explained in the text. Freezing of extracts immediately after preparation made no appreciable difference to the activity or kinetic behaviour of pyruvate kinase.

Assay of pyruvate kinase in tissue extracts

Samples (10–50μl) of extract were added to 1.5ml of Tris/acetate (125mM), pH7.5, containing KCl (100mM), $MgCl_2$ (10mM), NADH (0.125mM), ADP (1.5mM), lactate dehydrogenase (4 units) plus appropriate concentrations of phosphoenolpyruvate at 30°C, and the rate of oxidation of NADH was followed at 340nm. Rates of NADH oxidation in the absence of added phosphoenolpyruvate were very small (less than 1% of maximum rates with phosphoenolpyruvate).

Assays were essentially linear with time and extract volume over the range used. Particular care was taken that the pH of the buffer was adjusted to pH7.5 at 30°C after all additions (except extract and phosphoenolpyruvate) had been made.

Gel filtration of pyruvate kinase

Extracts were centrifuged at 100000*g* for 1h. A sample (2ml) of the supernatant was applied to a column (110cm×18mm) of Sepharose CL-6B which had previously been equilibrated with the extraction buffer with or without fructose 1,6-bisphosphate as appropriate. The column was eluted with the same buffer at about 20ml/h at 4°C. Pig heart lactate dehydrogenase (mol.wt. 160000) was added to the sample applied to the column to act as an internal reference compound.

Expression of results

A unit of enzyme activity is taken as that amount of enzyme which transforms 1μmol of substrate/min at 30°C. Enzyme kinetic constants were determined by fitting data to $v = V/\{1+(K_m/[S])^h\}$ by using a non-linear least-squares regression program written for the Hewlett Packard 9821 computer by Dr. Paul England of this Department [where K_m is the concentration of substrate S which results in a velocity (v) equal to half maximum velocity, i.e. $V/2$).

Results and Discussion

Studies on the kinetic properties of pyruvate kinase in extracts of rat epididymal fat-pads previously incubated in the presence or absence of insulin

Initial experiments showed that the activity of pyruvate kinase measured in fresh tissue extracts at 0.1mM-phosphoenolpyruvate was increased some

twofold if the pads had been incubated previously in the presence of insulin. No change was apparent if the maximum activity of pyruvate kinase was measured by assaying with 4mM-phosphoenolpyruvate. The activity at both 0.1mM- and 4mM-phosphoenolpyruvate was essentially unaltered if the extracts were incubated at 30°C for up to 1h. However, if fructose 1,6-bisphosphate was added to the extracts, incubation at 30°C quickly resulted in a substantial increase in the activity at 0.1mM-phosphoenolpyruvate; under these conditions the effect of insulin disappeared (Fig. 1*a*). On storage of extracts at 0°C the activity at 0.1mM-phosphoenolpyruvate tended to decline slowly. This decline was greatly facilitated if the extracts were first incubated at 30°C for 10min before being stored at 0°C (Fig. 1*b*). Under these conditions activity at 0.1mM-phosphoenolpyruvate decreased over 20–30min to about 40% of the initial value. The effect of insulin was not lost under these conditions and in fact tended to increase in percentage terms. No appreciable changes in maximum activity of pyruvate kinase were observed during these manipulations (see also Tables 1 and 2).

In the following studies, pyruvate kinase activity has been measured in: (i) initial extracts stored at 0°C for up to 20min; (ii) extracts that had been incubated at 30°C for 10min and then stored at 0°C for 40–60min; (iii) extracts that had been incubated at 30°C for 10min after the addition of fructose 1,6-bisphosphate. The extracts were prepared in buffer adjusted to pH6.8 containing EDTA and KF. It was found that in the absence of EDTA and, to a lesser extent, of KF there was a marked and rather variable decrease in the maximum activity of pyruvate kinase on incubation of extracts at 30°C. The pH of the extraction buffer did not appear critical; results very similar to those shown in Fig. 1 were obtained with extracts prepared at pH7.5. In contrast, the pH at which the assay of pyruvate kinase was conducted was found to be of crucial importance. If activity was assayed at pH6.5, no effects of insulin or of incubation in the presence or absence of fructose 1,6-bisphosphate were apparent. At pH7.0, the changes were appreciably less than those at pH7.5.

The changes in sensitivity to phosphoenolpyruvate concentrations of pyruvate kinase activity in the three types of extract used in the present study are illustrated in more detail in Fig. 2. The kinetic constants derived from the data of this typical experiment are given in Table 1. As reported by others for M_2-type isoenzymes of pyruvate kinase from adipose tissue and other sources (Pogson, 1968*b*; Imamura & Tanaka, 1972; Eigenbrodt &

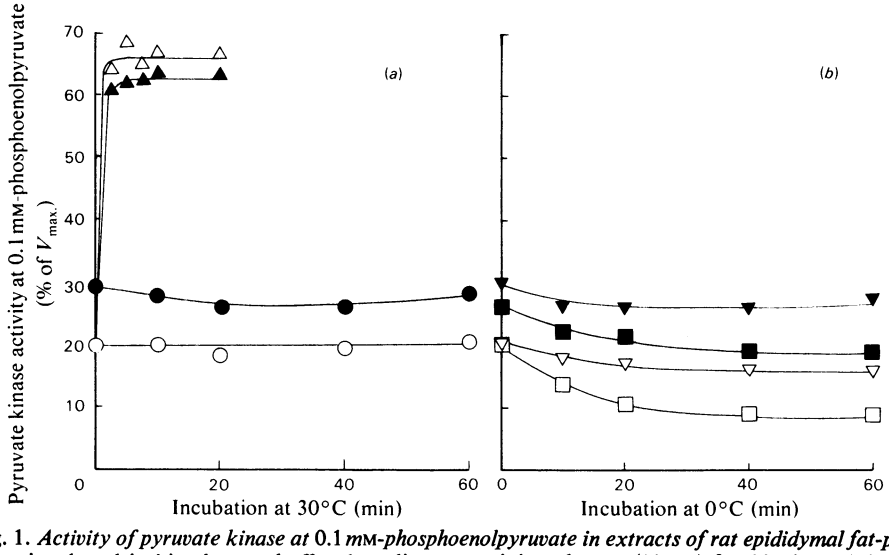

Fig. 1. *Activity of pyruvate kinase at* 0.1 *mM-phosphoenolpyruvate in extracts of rat epididymal fat-pads*
Pads were preincubated in bicarbonate-buffered medium containing glucose (11mM) for 30min and then incubated in fresh medium of the same composition with (solid symbols) or without (open symbols) insulin (0.5μg/ml). The pads were frozen in liquid N_2 and extracts prepared as described in the Experimental section in 100mM-Tris/acetate buffer, pH6.8, containing KF (100mM) and EDTA (5mM). (*a*) Extracts incubated at 30°C without further additions (●, ○) or with added 50μM-fructose 1,6-bisphosphate (▲, △). (*b*) Extracts were stored at 0°C (▼, ▽) or were incubated for 10min at 30°C and then stored at 0°C (■, □). Values of pyruvate kinase activity are expressed as a percentage of the $V_{max.}$ activity of pyruvate kinase (taken as the activity at 4mM-phosphoenolpyruvate). The maximum activity of pyruvate kinase (4.5units/g of tissue) was unaltered by the various treatments. Results shown are from a single experiment and are typical of three separate experiments.

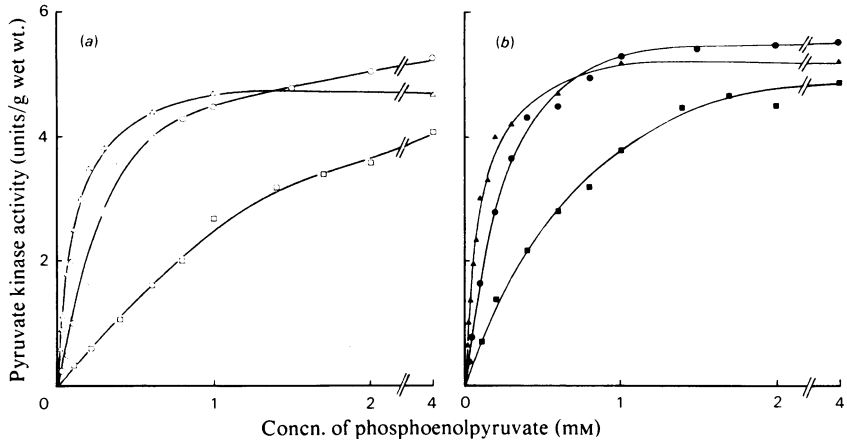

Fig. 2. *Pyruvate kinase activity at various phosphoenolpyruvate concentrations in extracts of rat epididymal fat-pads which had been incubated in (a) the absence or (b) the presence of insulin* (0.5μg/ml)

Incubation conditions were as described in Fig. 1. Pyruvate kinase activity was assayed in: (i) initial extracts stored at 0°C for up to 20min (●, ○); (ii) extracts incubated at 30°C for 10min without addition and then stored at 0°C for 40–60min (■, □); and (iii) extracts incubated at 30°C for 10min with 50μM-fructose 1,6-bisphosphate (▲, △).

Table 1. *Kinetic constants of pyruvate kinase activity in extracts of rat epididymal adipose tissue incubated in the presence and absence of insulin*

Data from Fig. 2 were fitted by non-linear regression analysis. See the legend of Fig. 2 for details of extracts (i), (ii) and (iii). Results are given as calculated value ± S.D. for 9 degrees of freedom in all cases. * $P<0.001$ versus control value, by Student's *t* test. There were 12 observations in all cases.

Tissue	Extract	$V_{max.}$ (units/g wet wt.)	K_m (μM)	*h*
Control	(i)	5.32±0.13	285±16	1.48±0.11
Insulin-treated	(i)	5.60±0.09	191±8*	1.48±0.08
Control	(ii)	5.54±0.21	842±71	1.51±0.14
Insulin-treated	(ii)	5.49±0.28	522±61*	1.49±0.13
Control	(iii)	4.97±0.13	98±8	1.06±0.07
Insulin-treated	(iii)	5.43±0.17	92±8	1.06±0.08

Schoner, 1977*b*), the curves relating activity to phosphoenolpyruvate concentrations were sigmoidal (with values of *h* about 1.5) if the extracts were not treated with fructose 1,6-bisphosphate. After treatment with fructose 1,6-bisphosphate the *h* values became close to 1. There were no significant changes in *V*, but there were marked changes in the K_m for phosphoenolpyruvate with the various treatments. In initial extracts of control tissues the value was about 300μM, which was diminished to about 200μM in initial extracts of insulin-treated tissues. Incubation of extracts with fructose 1,6-bisphosphate lowered the K_m to about 100μM, and this value was the same for extracts from both insulin-treated and control tissues. Incubation in the absence of fructose 1,6-bisphosphate followed by storage at 0°C greatly increased the K_m, but the effect of insulin remained. In a separate but similar experiment to the one reported in Fig. 2 and Table 1, the calculated values for the K_m for phosphoenolpyruvate were as follows: in extract (i), 420±44 and 234±31; in extract (ii), 1186±106 and 636±98; and in extract (iii) 106±10 and 90±11, for extracts prepared from control and insulin-treated tissue respectively (results are means ± S.D. for 5 degrees of freedom in each case). The maximum velocity (overall mean 5.06±0.24units/g) was not significantly different in any of the extracts.

As found previously with the adipose-tissue enzyme (Pogson, 1968*b*) and also with the M_2-type isoenzyme from platelets (Nishimura *et al.*, 1978), we observed little or no activation over 5–10min if fructose 1,6-bisphosphate was added directly to the assay mixture. In contrast, incubation of the undiluted original extracts with fructose 1,6-bisphosphate appeared to result quickly in complete conversion of the enzyme into its tetrameric form (Eigenbrodt & Schoner, 1977*b*; Nishimura *et al.*, 1978). When fat-pad extracts treated with fructose 1,6-bisphosphate

were applied to a Sepharose CL-6B column equilibrated with fructose 1,6-bisphosphate-containing buffer, the pyruvate kinase activity was eluted as a single peak (mol.wt. about 200000) just before lactate dehydrogenase (mol.wt. 160000). Storage of extracts at 0°C in the absence of fructose 1,6-bisphosphate may result in pyruvate kinase of the M_2 type being converted into its dimer form (Schulz *et al.*, 1975). The apparent facilitation of this process by a short preincubation of the extracts at 30°C in the present studies may be because the period at 30°C allows dissociation and loss of bound fructose 1,6-bisphosphate. In any case, when such extracts were applied to the Sepharose CL-6B column equilibrated with fructose 1,6-bisphosphate-free buffer, the activity of pyruvate kinase was eluted as a single peak with a molecular weight (about 100000) which corresponds to the dimer. The same pattern of elution of pyruvate kinase activity was observed with extracts from both control and insulin-treated tissues.

Alanine was found to be a potent and immediate inhibitor of adipose-tissue pyruvate kinase activity when added to the assay mixture, as reported earlier for other M_2-type isoenzymes (Imamura & Tanaka, 1972). Effects of alanine on the activity of pyruvate kinase in extracts from control and insulin-treated tissues are compared in Fig. 3 under two different sets of conditions, where in the absence of alanine there is no effect of insulin. The two situations are: assay of activity in extracts not treated with fructose 1,6-bisphosphate at high saturating phosphoenolpyruvate concentrations (Fig. 3*a*) and assay of activity in fructose 1,6-bisphosphate-treated extracts at low phosphoenolpyruvate concentrations (Fig. 3*b*). It is evident that, in the presence of increasing concentrations of alanine, the effect of insulin becomes clearly manifested again in both cases. Thus it is possible to demonstrate an effect of insulin after additions of substantial amounts of phosphoenolpyruvate, fructose 1,6-bisphosphate and alanine to the extracted enzyme. It is therefore very unlikely that the differences in kinetic behaviour observed after exposure of tissue to insulin are due to varying amounts of effector or substrate remaining bound to the enzyme after extraction. The results of Fig. 3 also suggest that insulin treatment results in the conversion of pyruvate kinase into a form that, particularly at high phosphoenolpyruvate concentrations, is much less sensitive to inhibition by alanine.

Effects of insulin on pyruvate kinase activity in epididymal fat-pads from fed and starved rats

Table 2 shows values of the maximum activity of pyruvate kinase (measured at 4mM-phosphoenolpyruvate) together with values of the activity measured at 0.1mM-phosphoenolpyruvate (expressed as a percentage of the maximum activity).

In the experiments *in vitro* (Table 2*a*), pads were incubated in medium containing glucose in the presence and absence of insulin. Results with tissue

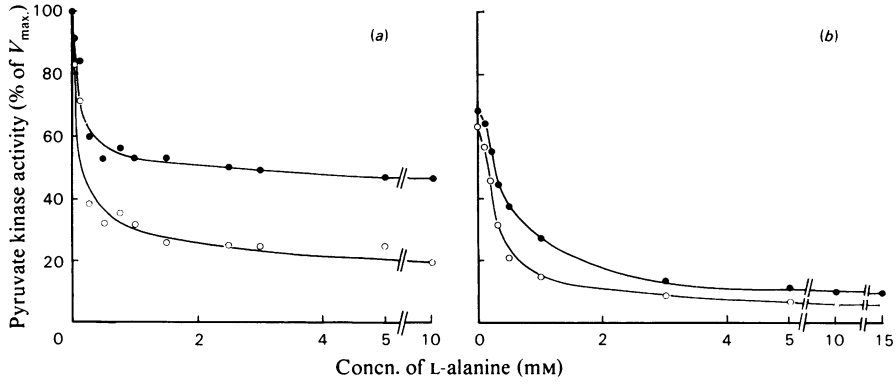

Fig. 3. *Effects of alanine on pyruvate kinase activity in extracts of rat epididymal fat-pads which had been incubated in the absence* (○) *or presence* (●) *of insulin*

Incubation conditions were as described in the legend to Fig. 1. (*a*) Pyruvate kinase activity assayed in extract (ii) as described in Fig. 2 with 4mM-phosphoenolpyruvate and various concentrations of alanine. (*b*) Pyruvate kinase activity assayed in extract (iii) as described in Fig. 2 with 0.2mM-phosphoenolpyruvate and various concentrations of alanine. Results depicted are taken from a single experiment which was typical of three separate experiments. Overall in the presence of 1mM-alanine, activity in extracts of insulin-treated tissue was (*a*) 200±32% and (*b*) 183±18% of control values. The equivalent values in the absence of alanine were 105±8 and 103±9% (mean±S.E.M. of three observations in all cases).

Table 2. *Effects of insulin in vitro and in vivo on the activity of pyruvate kinase extracted from epididymal adipose tissue of fed and starved rats*

In the studies *in vitro* (*a*), epididymal fat-pads from fed or 48h-starved rats were preincubated and incubated in bicarbonate-buffered medium containing glucose (11mM) and where appropriate insulin (0.5μg/ml) as described in the Experimental section. Pads were then frozen in liquid N_2 and extracts (i), (ii) and (iii) prepared and assayed for pyruvate kinase activity as given in the legend to Fig. 2. Activity at 4mM-phosphoenolpyruvate was taken as a measure of $V_{max.}$ activity. In the studies *in vivo* (*b*), fed rats were anaesthetized with Sagatal (1ml/kg) and then injected with anti-insulin serum (400μl given intravenously) and/or glucose (200mg in 0.5ml of 0.9% NaCl given intraperitoneally). After a further 10 or 20min, the fat-pads were removed, frozen in liquid N_2 and analysed for pyruvate kinase activity as in the studies *in vitro*. * $P<0.001$ versus appropriate control.

			$V_{max.}$ of pyruvate kinase activity (units/g) in			Pyruvate kinase activity at 0.1mM-phosphoenolpyruvate as % of $V_{max.}$ in		
		No. of observations	Extract (i)	Extract (ii)	Extract (iii)	Extract (i)	Extract (ii)	Extract (iii)
(*a*) Studies *in vitro*								
Fed rats: pads incubated 30min	Without insulin	6	5.12±0.19	4.73±0.49	5.02±0.31	17.2±0.68	6.6±0.59	54.8±2.4
	With insulin	6	5.14±0.25	5.09±0.40	5.51±0.32	27.5±0.72*	13.0±0.61*	57.9±2.1
Starved rats: pads incubated 30min	Without insulin	4	4.93±0.58	4.25±0.45	4.70±0.20	19.8±0.50	6.2±0.68	54.7±2.0
	With insulin	4	5.46±0.58	4.62±0.49	5.26±0.29	21.2±0.91	7.7±0.12	54.9±2.7
(*b*) Studies *in vivo* with fed rats								
Treatment	Time (min)							
Anti-insulin serum	10	4	6.82±0.32	6.87±0.13	6.55±0.14	12.2±0.17	6.6±0.49	41.9±3.46
Anti-insulin serum plus glucose	10	4	5.90±0.52	5.40±0.65	5.30±0.39	12.2±1.80	7.8±0.8	50.1±2.15
Glucose	10	4	5.10±0.63	5.50±0.47	5.20±0.70	25.0±1.87*	17.1±0.70*	48.3±2.46
Anti-insulin serum	20	6	4.87±0.46	4.67±0.57	4.89±0.41	18.7±0.80	6.1±0.58	50.8±1.6
Glucose	20	6	4.56±0.51	4.02±0.43	4.90±0.50	32.8±0.32*	11.7±0.60*	54.8±1.2

from fed rats were entirely consistent with the studies reported in Figs. 1 and 2, namely, insulin did not affect the maximum activity of pyruvate kinase, but the activity measured at 0.1 mM-phosphoenolpyruvate in extracts (i) and (ii) was significantly increased; the effect of insulin was no longer apparent if extracts were incubated with fructose 1,6-bisphosphate (extract iii). The effects of starvation for 48h on the activity of pyruvate kinase in fat-pads was also investigated. In preliminary experiments it was evident that the kinetic properties of pyruvate kinase in extracts of pads from starved animals incubated for 30min with glucose in the absence of insulin were very similar to those obtained for tissue from fed animals incubated under the same conditions. However, in marked contrast with pads from fed animals, incubation with insulin had little or no effect on pyruvate kinase activity in pads from starved rats (Table 2*a*).

In the experiments *in vivo* (Table 2*b*), the concentration of circulating insulin in anaesthetized rats was manipulated by injection of either anti-insulin serum or glucose 10 or 20min before removal and freeze-clamping of the fat-pads. We have used this technique previously to demonstrate the effects of insulin *in vivo* on fatty acid synthesis and the activities of pyruvate dehydrogenase and acetyl-CoA carboxylase in epididymal fat-pads and interscapular brown adipose tissue (Stansbie *et al.*, 1976; McCormack & Denton, 1977). The technique of injecting glucose has the advantage of ensuring high circulating insulin concentrations in the physiological range without the hypoglycaemia that would follow the injection of insulin itself. Injection of anti-insulin serum causes a marked rise in blood glucose within 15min, from 6–9mM in anaesthetized control animals to 12–20mM; these elevated values are then maintained for at least a further 60min (D. Stansbie, G. L. Evans & R. M. Denton, unpublished work). The activity profiles of pyruvate kinase found in extracts of fat-pads of rats previously treated with anti-insulin serum or glucose for 10 or 20min were very similar to those found *in vitro* in pads incubated with glucose in the absence or presence of insulin respectively (Table 2*b*). When anti-insulin serum and glucose were injected into the same animal, the results were not significantly different from those found after injection of anti-insulin serum alone; this supports the view that the changes in activity of pyruvate kinase observed after injection with either glucose or anti-insulin serum separately are related to changes in the concentration of circulating insulin rather than, for example, changes in the concentration of blood glucose.

Effects of substrate and adrenaline on the activity of pyruvate kinase in rat epididymal fat-pads

Incubation of the pads in the absence of any added substrate appears to have little or no effect on the activation of pyruvate kinase activity by insulin (Table 3). This indicates that the effect of insulin on the enzyme is not secondary to the increased rates of glucose transport and metabolism that occur in the presence of the hormone. This view is supported by the observation that insulin has a similar effect on pyruvate kinase activity when glucose is replaced by fructose (Table 3). Fructose appears to enter fat-cells by a separate carrier system from that involved in glucose transport, and its uptake into fat-cells is increased by insulin to a much smaller extent than the uptake of glucose (Froesch, 1965; Coore *et al.*, 1971).

Table 3. *Effects of incubation of rat epididymal adipose tissue with or without insulin in the presence of no substrate, fructose, glucose and adrenaline on the activity of pyruvate kinase*

Pads from fed rats were preincubated in bicarbonate-buffered medium containing glucose (11mM), fructose (11mM) or no substrate as appropriate for 30min and then transferred to fresh medium of similar composition plus insulin (0.5μg/ml) and/or adrenaline bitartrate (2μg/ml) as indicated and incubated for a further 30min. Pads were then frozen in liquid N_2 and pyruvate kinase activity was assayed in extracts prepared as described for extract (ii) in the legend of Fig. 2. Activity at 4mM-phosphoenolpyruvate was taken as a measure of $V_{max.}$ activity. * $P<0.001$ versus appropriate control (six observations in all cases).

Expt. no.	Additions to incubation media	$V_{max.}$ of pyruvate kinase activity (units/g wet wt. of tissue)	Pyruvate kinase activity at 0.1mM-phosphoenolpyruvate as % of $V_{max.}$
1	None	4.6±0.20	5.4±0.20
	Insulin	4.6±0.33	9.4±0.81*
2	Fructose	5.7±0.46	8.1±0.52
	Fructose plus insulin	5.7±0.22	13.2±0.46*
3	Glucose	5.9±0.41	7.2±0.43
	Glucose plus insulin	6.4±0.44	15.5±0.13*
	Glucose plus adrenaline	5.7±0.26	9.2±0.35
	Glucose plus insulin and adrenaline	6.5±0.84	9.0±0.47

Adrenaline was found to have little or no effect on the basal pyruvate kinase activity when added to the medium of pads incubated in the presence of glucose. However, adrenaline does reverse the activating effects of insulin (Table 3).

General conclusions

The results of this paper appear to represent the first demonstration of the short-term regulation of pyruvate kinase activity in a tissue other than the liver. Changes in the properties of pyruvate kinase of adipose tissue from fed rats have been shown to occur *in vitro* with insulin, and very similar changes are found *in vivo* within a few minutes of altering the concentration of circulating insulin with injections of anti-insulin serum or glucose.

The mechanism of the effect of insulin has not been established. It appears unlikely from the results of the present studies on whole tissue extracts that the changes in activity are related to differing amounts of effectors such as fructose 1,6-bisphosphate remaining bound to the enzyme. The persistence of the effect in extracts incubated at 30°C and/or stored at 0°C suggests that a stable covalent modification is involved, such as a decrease in the ratio of phosphorylated to dephosphorylated forms after exposure of adipose tissue to insulin.

We have not been able to obtain unequivocal evidence that the adipose-tissue enzyme is regulated by a phosphorylation–dephosphorylation cycle to date. This is largely because the enzyme appears to be rather unstable in extracts prepared without EDTA. The changes in apparent K_m for phosphoenolpyruvate observed in the present studies with insulin are rather similar to those which have been shown by others to be associated with dephosphorylation of the L-type isoenzyme (Engström, 1978). The changes in the activity of the M_2-type isoenzyme from chicken liver which have been reported to occur on phosphorylation appear to be largely on the maximum activity of the enzyme rather than the K_m for phosphoenolpyruvate (Eigenbrodt & Schoner, 1977*b*).

The changes in pyruvate kinase activity at low phosphoenolpyruvate concentrations with insulin and adrenaline parallel closely not only the changes in rates of fatty acid synthesis from glucose in epididymal fat-pads but also the changes that we have previously found in the proportion of pyruvate dehydrogenase and acetyl-CoA carboxylase in their respective active forms (for reviews see Denton *et al.*, 1977, 1978). There are thus at least three enzymes in the pathway of fatty acid synthesis from glucose which are activated by insulin. It is not appropriate here to discuss at length the mechanisms that may be involved. However, it should be emphasized that the mechanisms are likely to be closely linked. All three activations are independent of glucose transport and are largely reversed by adrenaline (Denton *et al.*, 1977, 1978). Pyruvate kinase certainly offers a further intracellular target of insulin action which clearly merits further investigation as a possible means of gaining insights into the general problem of the mechanism of insulin action. It would appear to have some advantages for this purpose over other enzymes regulated by insulin: these include the ease of assay and the relatively high activity of the enzyme in most cells. The lack of any insulin effect on the enzyme in adipose tissue from starved animals suggests that the enzyme may prove especially useful as a convenient indicator of insulin 'sensitivity' of fat- and other cells.

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