The Effect of Insulin and Glucocorticoids on the Synthesis and Degradation of Phosphoenolpyruvate Carboxykinase (GTP) in Rat Adipose Tissue Cultured *in vitro*

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1. Epididymal adipose tissue from the rat was maintained in culture for periods of up to 96 h. 2. After an initial decrease in protein synthesis during the first 24 h of culture, the adipose tissue recovered its capacity to synthesize and accumulate proteins of a relatively large size. 3. The activity of phosphoenolpyruvate carboxykinase decreased in a parallel manner, but increased again after 24 h of incubation of the tissue in culture, to a value twice that noted in the tissue *in vivo*. This increase in enzyme activity was due to an increase in its rate of synthesis. 4. Both insulin and dexamethasone (9 α -fluoro-16 α -methyl-11 β ,17,21-trihydroxypregna-1,4-diene-3,20-dione) inhibited phosphoenolpyruvate carboxykinase synthesis, but dexamethasone also decreased total protein synthesis. 5. The half-life of phosphoenolpyruvate carboxykinase in adipose tissue cultured *in vitro* was 5–7 h and was not altered by insulin or dexamethasone. 6. It is concluded that both insulin and glucocorticoids lower the activity of phosphoenolpyruvate carboxykinase in rat adipose tissue by decreasing its rate of synthesis.

Studies *in vivo* have clearly indicated that the activity and amount of adipose-tissue phosphoenolpyruvate carboxykinase (GTP) (EC 4.1.1.32) are decreased by corticosteroids and insulin (Reshef *et al.*, 1969b, 1970, 1972; Reshef & Hanson, 1972) and that these hormones affect the enzyme independently and additively (Reshef *et al.*, 1970). It was also found that the rate of synthesis of phosphoenolpyruvate carboxykinase was repressed either by a starvation–re-feeding cycle (Hopgood *et al.*, 1973) or by corticosteroids (Meyuhas *et al.*, 1976), and induced by diabetes and adrenalectomy (Meyuhas *et al.*, 1976). Thus it was difficult to envisage that the additive effect of insulin and corticosteroids is caused in both cases by a repressed synthesis of the enzyme. Experiments with the liver enzyme (Gunn *et al.*, 1975a) indicated that the repressed synthesis and activity of cytosolic phosphoenolpyruvate carboxykinase by corticosteroids were, in fact, mediated by insulin being released under the influence of corticosteroids. Consequently, it was possible that the corticosteroid effect in adipose tissue was also indirect. This possibility was further raised by findings in adipose tissue *in vivo*, since: (a) the activity of the enzyme was

decreased to a greater extent by these hormones than was its rate of synthesis; (b) corticosteroids not only repressed the synthesis of this enzyme, but that of other cytosolic proteins as well (Meyuhas *et al.*, 1976). With this background, the following questions were put forward. (a) Did corticosteroids repress the synthesis of adipose-tissue phosphoenolpyruvate carboxykinase by a direct effect on the tissue? (b) To what extent was this an independent effect or a part of a non-specific decrease in the synthesis rate of cytosol proteins? (c) Did corticosteroids enhance the degradation of the enzyme in adipose tissue and thus affect its content?

These questions could not be answered by experiments *in vivo* and thus an effort was made to develop conditions suitable for a maintenance of adipose tissue *in vitro*. Previous attempts to maintain adipose tissue *in vitro* for longer periods (21 and 6 days respectively) were reported by Smith (1971, 1972) and by Fröhlich *et al.* (1972). The viability of these preparations was judged by histological observations or by metabolic functions, such as the conversion of [¹⁴C]glucose to fat. No indications were given, whether the tissue still maintains the capacity of protein synthesis and susceptibility to hormones. The present paper provides evidence that adipose tissue

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may be kept *in vitro* for up to 96h with preservation of hormonal action and a reasonable capacity to synthesize and degrade specific proteins.

Experimental

Materials

Medium 199 with Earle's salts was from GIBCO, Santa Clara, CA, U.S.A. Tryptose phosphate broth was from Oxoid, London E.C.4, U.K. Heat-inactivated calf serum was from Flow Laboratories, Irvine, Ayrshire, Scotland, U.K. Dexamethasone (9 α -fluoro-16 α -methyl-11 β ,17,21-trihydroxypregna-1,4-diene-3,20-dione) was from Ikapharm, Ramat Gan, Israel. Insulin was a gift from Dr. W. W. Bromer, Eli Lilly and Co., Indianapolis, IN, U.S.A. The L-[4,5-³H]leucine (36.4Ci/mmol) was from the Nuclear Research Center, Negev, Beer-Sheva, Israel, and NaH¹⁴CO₃ (2-10mCi/mmol) was from The Radiochemical Centre, Amersham, Bucks., U.K. NADH, IDP, phosphoenolpyruvate, malate dehydrogenase (EC 1.1.1.37) and trypsin (EC 3.4.21.4) were from Sigma Chemical Co., St. Louis, MO, U.S.A. NCS scintillant was from Amersham/Searle Corp., Arlington Heights, IL, U.S.A.

Tissue preparation

Epididymal fat-pads were obtained from 5-week-old male Sabra albino rats (Wistar origin). The tissues were quickly removed under sterile conditions, followed by three washes, with 5ml portions of sterile 0.15M-NaCl. Tissues from 40-60 rats were thus pooled and minced to small pieces of about 1-2mm³. The minced preparation was then treated with trypsin in a small volume (about 1ml/g) of Medium 199 (Morgan *et al.*, 1950) in 0.01M-NaHCO₃ buffer, containing 0.25mg of trypsin/ml, and carefully shaken at 37°C for 3min. This procedure was found to remove tissue debris (M. Kapeler, unpublished work). The medium was diluted by addition of Medium 199 containing 5% (v/v) calf serum (about 5ml of medium/g of tissue), then immediately aspirated, and the tissue preparation re-washed three additional times with 20ml portions of Medium 199 containing 5% calf serum. About 100mg wet wt. of tissue was incubated in a plastic Petri dish (aerated 35mm-diameter dish from A/S Nunc, Roskilde, Denmark), containing 3ml of Medium 199 in 0.01M-NaHCO₃ buffer supplemented with 5% (v/v) calf serum, 10% (v/v) tryptose phosphate broth, 2mM-glutamine, 10⁵ i.u. of penicillin G/litre and 200mg of streptomycin/litre. The dishes were then incubated in an incubator aerated with CO₂ + air (5:95) at 37°C; humidity was kept at 60-70%. After 2h the medium was replaced, followed by a regular partial exchange (replacing 2ml out of the 3ml of medium) every 12h.

During incubation *in vitro* the tissue floated in the medium for the first 12h, but later tended to stick to the incubation dish.

For maintenance of the tissue in the presence of insulin, this hormone was added to the medium at a final concentration of 0.1 munit/ml and renewed every 12h. In experiments where dexamethasone, insulin or cyclic AMP and theophylline were used, the medium was renewed completely, and replaced by a medium containing these compounds at the following concentrations: dexamethasone, 0.1 μ M; insulin, 1 munit/ml; cyclic AMP, 2mM; theophylline, 1mM.

Measurement of enzyme activity

Phosphoenolpyruvate carboxykinase was measured in a 100000g supernatant fraction prepared from adipose tissue as previously described (Chang & Lane, 1966). One unit of enzyme activity catalyses the reaction of 1 μ mol of NaH¹⁴CO₃/min at 37°C.

Measurement of relative rate of phosphoenolpyruvate carboxykinase synthesis

At various times as indicated in the text, the cultured adipose tissue was pulsed for 60min with [³H]leucine. The culture was thoroughly washed with 0.25M-sucrose, blotted on filter paper, weighed, homogenized in 0.15M-KCl containing 0.02M-sodium phosphate buffer, pH7.0, and a 100000g supernatant fraction obtained. Unlabelled kidney cytosol enzyme (150munits) was added to 0.1-0.2ml of supernatant fractions, and immunoprecipitated by using 70% excess of a specific goat antibody prepared against rat liver cytosol phosphoenolpyruvate carboxykinase (Hopgood *et al.*, 1973). Triton X-405 (final conc. 0.6%) was added to minimize non-specific co-precipitation as previously described (Ballard *et al.*, 1974). Trichloroacetic acid-insoluble radioactivity (Mans & Novelli, 1961) and protein concentration (Lowry *et al.*, 1951) in the supernatants were measured. The specificity of the antibody for rat liver cytosol, kidney and adipose-tissue enzymes *in vivo* has been demonstrated by using sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. This specificity was tested in cultured adipose tissue by dissociating the immunoprecipitate with 1.5% (w/v) sodium dodecyl sulphate, 2% (w/v) dithiothreitol and 0.1M-Tris/HCl buffer, pH8.8. Electrophoresis was carried out for 16h at 3mA per gel as previously described (Ballard *et al.*, 1974). After electrophoresis, the gels were extruded in 1.5mm sections, swollen in NCS and the radioactivity was determined as previously described (Ballard *et al.*, 1974). A second immunoprecipitate was also obtained by the addition of unlabelled antigen and antibody to the supernatants remaining after the precipitation

of the first immunoprecipitate. The immunoprecipitates were dissolved in NCS as previously described (Ballard *et al.*, 1974) and their radioactivity was determined. In some of the experiments the radioactivity in the enzyme was derived by subtracting the radioactivity in the second from those in the first immunoprecipitate (Table 4). In some of the experiments (Fig. 3 and Table 5) the radioactivity in the enzyme was derived from the counts located in the enzyme peak after separation of the immunoprecipitate by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis.

Cytosol proteins in cultured adipose tissue

Trichloroacetic acid precipitates, from adipose tissue pulse-labelled *in vivo* or from cultured adipose tissue pulse-labelled *in vitro*, were dissociated by 3% (w/v) sodium dodecyl sulphate, 3% (w/v) dithiothreitol and 0.1 M-Tris/HCl buffer, pH 8.8, and separated by electrophoresis on sodium dodecyl sulphate/polyacrylamide gels carried out as previously described (Ballard *et al.*, 1974). After 16 h at 3 mA, either gels were stained by Coomassie Blue or 1.5 mm fractions were extruded (30 fractions), swollen in NCS as previously described (Ballard *et al.*, 1974) and the radioactivity was determined.

Results

Activity of phosphoenolpyruvate carboxykinase in cultured adipose tissue

Incubation of rat adipose tissue *in vitro* for 72 h resulted in an increased activity of phosphoenolpyruvate carboxykinase. The activity at 72 h increased beyond that measured in non-incubated tissues, or in tissue samples taken before mincing and trypsin treatment. From the time-course described in Fig. 1, it is evident that the activity started to increase by 36 h of incubation, whereas at 24 h it was similar to or lower than the activity in a non-incubated tissue (0 h). The spontaneous rise in phosphoenolpyruvate carboxykinase activity was prevented by the addition of either corticosteroids (0.1 μ M-dexamethasone) or insulin (1 munit/ml) to the culture medium for the last 24 h of incubation (Table 1). Supplementing the medium with a low concentration of insulin (0.1 munit/ml) from the beginning of incubation resulted in a constant low enzyme activity throughout the experimental period (note that insulin in the medium was renewed every 12 h during the regular exchange of the medium). Under these conditions it was possible to induce phosphoenolpyruvate carboxykinase activity in tissues preincubated for at least 48 h by the addition of cyclic AMP in combination with theophylline for 12 h (Table 2). No effect of cyclic AMP and theophylline was observed in the absence

of insulin (O. Meyuhas & L. Reshef, unpublished work). An alternative way to control the increase in phosphoenolpyruvate carboxykinase activity was to keep insulin (0.1 munit/ml) present in the medium for the initial 48 h. Removal of insulin at this time resulted in an increase in enzyme activity, which was maximal at 24 h (Table 2). This ability to control the time of appearance of increased enzyme activity was advantageous for later experiments designed to measure the synthesis and degradation of the enzyme.

Fig. 1. Time-course of increased phosphoenolpyruvate carboxykinase activity in cultured adipose tissue

Enzyme activities were measured in 100000g supernatant fractions obtained from tissues at various times of incubation (●). Activity of tissue samples taken from pool, before mincing and trypsin treatment, was measured at 0 h (▲). The bars indicate S.E.M. for at least four independent experiments. Each experiment is the mean for three separate Petri dishes.

Table 1. Effect of insulin and dexamethasone on the activity of phosphoenolpyruvate carboxykinase

Cultured adipose tissue was incubated for 72 h in a culture medium as described in the Experimental section. Insulin (1 munit/ml) or dexamethasone (0.1 μ M) was added for the last 24 h and 12 h respectively. Incubation was terminated by washing the tissue with 3 $\times$ 5 ml of 0.9% NaCl, and assaying phosphoenolpyruvate carboxykinase in the 100000g supernatants. Results are the means $\pm$ S.E.M. of the numbers of independent experiments indicated in parentheses. Each experiment is the mean for three separate Petri dishes.

Treatment	Phosphoenolpyruvate carboxykinase activity (munits/g of tissue)
None (6)	115 $\pm$ 15
Dexamethasone (4)	52 $\pm$ 11
Insulin (4)	48 $\pm$ 11

Table 2. Effect of insulin maintenance and removal from the tissue-culture medium on the activity of phosphoenolpyruvate carboxykinase

Cultured adipose tissue was incubated for 72 h. Insulin, as indicated in the Table, was added to the medium at the beginning of incubation with the final insulin concentrations as specified. The medium was replaced every 12 h. In some of the dishes (column *b*), insulin was not added for the last 24 h of incubation. In others (column *a*), where indicated, insulin was present throughout 72 h of incubation. Cyclic AMP (2 mM) and theophylline (1 mM) were added as indicated, for the last 12 h. Each value is the mean for triplicate dishes $\pm$ s.d.

Expt. no.	Treatment	Phosphoenolpyruvate carboxykinase activity (munits/g of tissue)	
		<i>a</i>	<i>b</i>
1	None	163 $\pm$ 10	
	Insulin (0.1 munit/ml)	67 $\pm$ 4	158 $\pm$ 9
	Insulin (0.5 munit/ml)	38 $\pm$ 3	150 $\pm$ 9
2	None	81 $\pm$ 11	
	Insulin (0.1 munit/ml)	37 $\pm$ 3	97 $\pm$ 3
	Insulin (0.5 munit/ml)	28 $\pm$ 4	92 $\pm$ 7
3	Insulin (0.1 munit/ml)	15 $\pm$ 2	56 $\pm$ 3
	Insulin (0.1 munit/ml) + cyclic AMP + theophylline	86 $\pm$ 2	

Immunochemical techniques for measuring the synthesis and degradation of phosphoenolpyruvate carboxykinase

The response of phosphoenolpyruvate carboxykinase activity to either insulin or corticosteroids in cultured adipose tissue *in vitro* was similar to the response of the tissue *in vivo* (Reshef *et al.*, 1969*b*, 1970). It was therefore reasonable to use the system to study the mechanism by which these two hormones decreased the activity of the enzyme by measuring the synthesis and degradation of the enzyme immunochemically (Hopgood *et al.*, 1973). Accordingly the cultured adipose tissue was pulse labelled with [3 H]leucine, and the enzyme was immunoprecipitated from the tissue cytosol by a highly specific antibody prepared against rat liver cytosol phosphoenolpyruvate carboxykinase (Hopgood *et al.*, 1973). The very small amount of phosphoenolpyruvate carboxykinase in the cultured tissue extract required the addition of carrier antigen to ensure the complete precipitation of the labelled antigen. Since the labelled antigen contributed only slightly to the total amount of antigen it was necessary to determine the specificity of the precipitated material in the antibody-antigen complex. To accomplish this the immunoprecipitate was dissociated and separated by electrophoresis on sodium dodecyl sulphate/polyacrylamide gels. As shown in Fig. 2, a major radioactive peak coincided

Fig. 2. Separation of phosphoenolpyruvate carboxykinase from cultured adipose tissue after antibody precipitation

Cultured adipose tissue was incubated for 72 h with insulin (0.1 munit/ml) present for the initial 48 h. The tissue was pulse-labelled with 100 μ Ci of [3 H]leucine for 60 min, then extensively washed in Krebs-Ringer phosphate buffer containing 2 mM-leucine. The enzyme in the cytosol was immunoprecipitated as described in the Experimental section, and the washed immunoprecipitate dissociated and subjected to electrophoresis on sodium dodecyl sulphate polyacrylamide gels. ●, Immunoprecipitate from cultured adipose tissue; ■, phosphoenolpyruvate carboxykinase marker from kidney cytosol.

with marker phosphoenolpyruvate carboxykinase from kidney cytosol. The specificity of the reaction indicated that the amount of radioactivity precipitated by the antibody was a good estimate of the radioactivity in the enzyme. Using this procedure (Fig. 3), we noted that the synthesis of phosphoenolpyruvate carboxykinase increased with time in the cultured adipose tissue, and paralleled the time-course of increased enzyme activity (Fig. 1). The synthesis of total cytosol proteins also increased in the cultured tissue, so that it was not specific for phosphoenolpyruvate carboxykinase alone. However, although the rise in the synthesis of general proteins was already evident at 24 h of incubation (Fig. 3), that of phosphoenolpyruvate carboxykinase increased only after 48 h. The increased capacity of adipose tissue in culture, to synthesize cytosol in general, and phosphoenolpyruvate carboxykinase specifically, indicate the viability of this preparation.

Synthesis of cytosolic proteins by cultured adipose tissue

An electrophoretic analysis, on sodium dodecyl sulphate/polyacrylamide gels, of the molecular weights of cytosolic proteins synthesized by adipose tissue both *in vivo* and *in vitro* was undertaken to determine if culturing the tissue changed the size patterns of cytosol protein. Phosphoenolpyruvate

carboxykinase was used as standard (mol.wt. 80000; S. H. Tilghman, unpublished work). We noted that tissue labelled *in vivo* had a higher percentage of newly synthesized proteins with molecular weights greater than 80000 than did tissue labelled *in vitro* (Table 3). However, the capacity to synthesize large proteins was partially recovered if the time of incubation of the adipose tissue was extended to 48 or 72 h. This is indicated by the increased relative distribution of radioactivity in proteins of a molecular weight greater than 80000 found in these tissues compared with tissues incubated for 24 h (Table 3).

Effect of dexamethasone and insulin on the degradation of phosphoenolpyruvate carboxykinase

Degradation of phosphoenolpyruvate carboxykinase was measured in cultured adipose tissue pre-incubated for 60h *in vitro* (Table 4). This involved pulse-labelling the tissue proteins with [³H]leucine for 60min, incubation for 2h in a medium containing 2mM-leucine, followed by an additional incubation in a regular medium for 5–9h. Degradation was determined by following the rate of decline of radioactivity in cytosol proteins and in the immunoprecipitated enzyme. Results in Table 4 demonstrate that although the radioactivity in cytosolic proteins did not change significantly during each experiment, that in the immunoprecipitate was considerably decreased, with a half-time of about 5–7h (Expt. A, Table 4). This indicated that phosphoenolpyruvate carboxykinase was degraded in the cultured adipose tissue. However, it was clear that neither dexamethasone nor insulin affected the degradation of the enzyme to any extent. On the other hand, the synthesis of the enzyme was inhibited by both insulin (results not shown) and dexamethasone.

Effect of insulin and dexamethasone on the synthesis of phosphoenolpyruvate carboxykinase

Two methods for the maintenance of adipose tissue *in vitro* were used for these experiments (Table 5). In group A, insulin (0.1 munit/ml) was present in the

Fig. 3. Time-course of the synthesis of phosphoenolpyruvate carboxykinase and cytosol proteins in cultured adipose tissue

At various times of incubation cultured adipose tissues were pulse-labelled with [³H]leucine and 100000g supernatants prepared. ■, Radioactivity in cytosol proteins was determined in the trichloroacetic acid precipitates. ●, Radioactivity in phosphoenolpyruvate carboxykinase was determined by dissociating and separating the immunoprecipitates by electrophoresis on sodium dodecyl sulphate/polyacrylamide gels as outlined in Fig. 2, and represent only the counts in the enzyme peak. The bars indicate s.e.m. of four independent experiments.

Table 3. Size of cytosol proteins synthesized in cultured adipose tissue with respect to incubation time

Cultured adipose tissue was pulse-labelled with 100μCi of [³H]leucine for 60min. After extensive washings 100000g supernatants were prepared and precipitated with 10% (w/v) trichloroacetic acid. The washed precipitate was dissolved in sodium dodecyl sulphate and dithiothreitol and analysed by electrophoresis on sodium dodecyl sulphate/polyacrylamide gels as described in the Experimental section. Labelled phosphoenolpyruvate carboxykinase was used as a marker for a protein of 80000mol.wt. The radioactivity in the gels was divided into three groups according to the mobility relative to phosphoenolpyruvate carboxykinase (*a* > 80000mol.wt.; *b* = 80000mol.wt.; *c* < 80000mol.wt.) and the relative distribution of radioactivity in these three groups was calculated. For comparison, adipose tissue was pulse labelled *in vivo* for 30min and the 100000g supernatant obtained was then treated similarly to the cultured tissue. The results are for a representative experiment.

Incubation time (h)	Cytosol proteins (mg/g of tissue)	Mol.wt. ...	Percentage of radioactivity in cytosol proteins		
			>80000	80000	<80000
0	5.0		33	13.5	53.5
24	3.6		25	18.3	56.7
48	4.9		36.4	20.6	43.0
72	5.6		36.4	20.5	43.0
<i>In vivo</i>	7.2		46.7	15.0	38.2

Table 4. *Effect of dexamethasone and insulin on the synthesis and degradation of phosphoenolpyruvate carboxykinase*

In Expt. D, insulin (0.1 munit/ml) was present during the initial 48 h, then removed and the tissues were incubated for an additional 24 h. Dexamethasone (0.1 μ M) and insulin (1 munit/ml) were added where indicated for the last 9 h. In Expts. A–C, insulin was not added during the preincubation. In all experiments the tissues were pulse-labelled for 60 min with [3 H]leucine (50 μ Ci/incubation dish), then chased by replacing the medium by one containing non-radioactive leucine (2 mM) for 2 h. Then, where indicated, the hormones were added for additional incubation as specified for each experiment and the radioactivity in the immunoprecipitated enzyme and in the cytosol proteins was determined.

Experiment	Treatment	10 ⁻³ × Radioactivity (d.p.m./g of tissue) in		Percentage of radioactivity in enzyme
		Cytosol proteins	Enzyme	
A	Pulse	720	14.40	2.0
	2 h chase	600	8.70	1.45
	5 h decay	639	4.74	0.74
	5 h decay + dexamethasone	690	7.95	1.15
B	Pulse	1701	11.10	0.65
	Pulse + dexamethasone	1983	7.62	0.37
	7 h decay	1503	5.97	0.39
	7 h decay + dexamethasone	1380	8.40	0.64
C	Pulse	471	11.76	2.47
	Pulse + dexamethasone	441	5.76	1.31
	9 h decay	429	3.51	0.82
	9 h decay + dexamethasone	387	3.42	0.89
	9 h decay + insulin	432	4.20	0.97
D	Pulse	1071	26.40	2.50
	Pulse + dexamethasone	861	12.90	1.50
	9 h decay	1041	12.10	1.20
	9 h decay + dexamethasone	882	10.95	1.67
	9 h decay + insulin	735	9.18	1.27

incubation medium during the initial 48 h of incubation, then removed for the last 24 h. In group B, no insulin was added throughout the entire incubation period (72 h). For studying the hormonal effects, dexamethasone or insulin were added for the last 12 h of incubation. In these experiments, the immunoprecipitated phosphoenolpyruvate carboxykinase was dissociated and separated by electrophoresis on sodium dodecyl sulphate/polyacrylamide gels, and only the radioactivities located in the peak of phosphoenolpyruvate carboxykinase were taken for the calculation of the rate of enzyme synthesis. By using this procedure (Table 5), it became evident that the specific synthesis of phosphoenolpyruvate carboxykinase was almost twofold higher in tissues from group A than from group B. On the other hand, the rate of synthesis of cytosol proteins was constant under both conditions. Dexamethasone caused a considerable inhibition of the synthesis of cytosol proteins in tissues from group A, but only a slight inhibitory effect was noted in tissues from group B. Irrespective of its effect on the synthesis of cytosol proteins, dexamethasone markedly repressed the synthesis of phosphoenolpyruvate carboxykinase in tissues from both groups A and B. This was evident both from the absolute radioactivity found in the

enzyme and its synthesis relative to that of total cytosol proteins.

Insulin added for the last 12 h caused an increased synthesis of cytosol proteins while at the same time markedly decreasing the specific synthesis of phosphoenolpyruvate carboxykinase.

Discussion

The results of this study clearly indicate that cultured adipose-tissue segments can be used to determine the mechanisms underlying the hormonal regulation of phosphoenolpyruvate carboxykinase. The activity of this enzyme is maintained for up to 96 h *in vitro*; although decreased at 24 h of incubation, the activity gradually recovered and increased later on (Fig. 1). The enzyme is responsive to added hormones such as dexamethasone and insulin. Changes in the synthesis rate of phosphoenolpyruvate carboxykinase are also in close agreement with changes noted *in vivo* (Meyuhas *et al.*, 1976), which indicates that alteration in the specific synthesis of the enzyme accounts for fluctuations in activity noted after treatment with various hormones *in vivo*.

Using these adipose-tissue segments we were able to show that the decrease in phosphoenolpyruvate

Table 5. *Effect of insulin and dexamethasone on the synthesis of phosphoenolpyruvate carboxykinase in cultured adipose tissue*

Cultured adipose tissue was incubated for 72 h. In experiments of group A, insulin (0.1 munit/ml) was present in the medium during the initial 48 h and then removed; 12 h before the termination of the experiments dexamethasone (0.1 μ M) or insulin (1 munit/ml) was added where indicated. In experiments of group B insulin was absent from the medium throughout the incubation period. Dexamethasone (0.1 μ M) was added for the last 12 h. In all cases the tissues were pulse-labelled for 60 min with 100 μ Ci of [3 H]leucine per incubation dish. The 100000g supernatants were used for immunoprecipitation and for determinations of radioactivity in the cytosol proteins. Each immunoprecipitate was analysed by electrophoresis on a sodium dodecyl sulphate/polyacrylamide gel as detailed in Fig. 2 and only the counts in the peak of phosphoenolpyruvate carboxykinase were taken for the specific radioactivity in the enzyme. Results are the means $\pm$ s.e.m. of the number of independent experiments indicated in parentheses. Each experiment is the mean for two or three separate Petri dishes.

Group	Addition to medium	$10^{-3} \times$ Radioactivity (d.p.m./g) in		Percentage of radioactivity in enzyme
		Cytosol proteins	Enzyme	
A	None (3)	636 $\pm$ 41	4.00 $\pm$ 0.69	0.63 $\pm$ 0.13
	Dexamethasone (3)	370 $\pm$ 60	0.90 $\pm$ 0.24	0.24 $\pm$ 0.05
	Insulin (2)	1251 $\pm$ 154	0.92 $\pm$ 0.38	0.08 $\pm$ 0.05
B	None (3)	608 $\pm$ 60	2.09 $\pm$ 0.62	0.34 $\pm$ 0.09
	Dexamethasone (3)	412 $\pm$ 24	0.42 $\pm$ 0.57	0.10 $\pm$ 0.03

carboxykinase activity caused by insulin or dexamethasone was not due to a change in the rate of enzyme degradation. Since both dexamethasone and insulin cause an additive decrease in the activity of the enzyme we infer that this is due to effects of the hormones on the synthesis of adipose-tissue phosphoenolpyruvate carboxykinase. Instances are known of additive effects causing enzyme accumulation by stimulating synthesis alone. Tyrosine transaminase in Reuber H-35 cells, for example, is independently induced by dexamethasone, insulin and cyclic AMP owing to a well-documented increase in enzyme synthesis (Lee *et al.*, 1970; Barnett & Wicks, 1971; Wicks & McKibbin, 1972; Miller *et al.*, 1975). In the chick oviduct, both oestrogen and testosterone induce additively the synthesis of ovomucoid (Palmiter & Haines, 1973). The accumulation and synthesis of serum α_2 u globulins is regulated independently by glucocorticoids and testosterone (Irwin *et al.*, 1971; Sippel *et al.*, 1975). It was found (Gunn *et al.*, 1975b; Tilghman *et al.*, 1975) that the additive increase in the activity of phosphoenolpyruvate carboxykinase in Reuber H-35 cells, caused by corticosteroids and cyclic AMP (Wicks *et al.*, 1973), was again a consequence of an increased enzyme synthesis. Thus two inducers, both affecting the synthesis only, may lead to an additive accumulation of the enzyme. Likewise it is conceivable that an additive decreased enzyme activity is caused by independent effects of the two compounds on enzyme synthesis. Results with cultured adipose tissue support different mechanisms of action for insulin and corticosteroids. First, insulin repressed only the synthesis of phosphoenolpyruvate carboxykinase, whereas it actually prompted an enhanced synthesis of total cytosol proteins. Secondly, the insulin effect was counteracted by cyclic AMP and

theophylline, implying that it repressed the synthesis of the enzyme by decreasing tissue cyclic AMP (Butcher *et al.*, 1968a,b). The reciprocal relationship of insulin and cyclic AMP in regulating phosphoenolpyruvate carboxykinase synthesis was shown both in the rat liver (Tilghman *et al.*, 1974) and in the Reuber H-35 cells (Gunn *et al.*, 1975b), implying a similar mechanism of action for these effectors in liver and adipose tissue. Unlike insulin, dexamethasone not only repressed the synthesis of phosphoenolpyruvate carboxykinase in cultured adipose tissue, but that of total cytosol proteins as well. It should be noted that the concentration of dexamethasone (0.1 μ M) used *in vitro* is considered to be physiological (Allen & Kendall, 1967). Further, it was found that the removal of the adrenals prompted an increased synthesis of total cytosol proteins, whereas injecting adrenalectomized rats with cortisol phosphate considerably repressed their synthesis (Meyuhas *et al.*, 1976). Corticosteroids are known to repress the synthesis of cytosol proteins in tissues other than adipose tissue (Makman *et al.*, 1966, 1968; Chen & Mealy, 1973), an effect that is associated with an inhibited synthesis of nucleic acids and with cell proliferation (Kemper *et al.*, 1969; Drews, 1969; Henderson *et al.*, 1971; Henderson & Loeb, 1974). It is possible that in adipose tissue, corticosteroids affect protein synthesis by inhibiting the synthesis of RNA. Alternatively, a report by Kim & Kim (1975) indicated that corticosteroids elicited a transient inhibition of protein synthesis in the liver. They were able to reproduce this inhibition in a cell-free liver system, derived from rats that were pretreated with the hormone. This implied that the hormone-mediated inhibitory effect took place beyond the point of initiation of protein synthesis. Whether

corticosteroids affect protein synthesis in adipose tissue directly or via an inhibition of RNA synthesis requires further experimentation.

Is the repressed synthesis of phosphoenolpyruvate carboxykinase by corticosteroids associated with the non-specific inhibited synthesis of total cytosol proteins? It is clear that the synthesis of the enzyme is more sensitive to repression by the hormone than is that of total cytosol proteins. However, the inhibited synthesis of cytosol proteins represents an average result, and conceivably there may be proteins in the cytosol that are not at all affected. In that respect, the effect of corticosteroids may be considered specific. It is clear from these studies, however, that corticosteroids affect adipose-tissue phosphoenolpyruvate carboxykinase activity directly by repressing the synthesis of the enzyme.

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