Synthesis and Degradation of Phosphoenolpyruvate Carboxylase in Rat Liver and Adipose Tissue

CHANGES DURING A STARVATION-RE-FEEDING CYCLE

By M. F. HOPGOOD and F. J. BALLARD

*Commonwealth Scientific and Industrial Research Organization,
Division of Nutritional Biochemistry, Adelaide, S. Austral. 5000, Australia*

and LEA RESHEF

Department of Biochemistry, Hadassah Medical School, Jerusalem, Israel

and R. W. HANSON

*Fels Research Institute and Department of Biochemistry,
Temple University School of Medicine, Philadelphia, Pa. 19140, U.S.A.*

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A specific antibody against liver cytosol phosphoenolpyruvate carboxylase (EC 4.1.1.32) was used to isolate the enzyme from liver and adipose tissue. With this technique we have shown that phosphoenolpyruvate carboxylase synthesis in starved rats accounts for 3% of the total synthesis of cytosol protein in each tissue. Re-feeding starved animals decreases this relative rate of phosphoenolpyruvate carboxylase synthesis to 0.2% and 1% respectively in liver and adipose tissue, and the activity of the enzyme in each tissue is decreased to 25% of the starvation value. An additional starvation period is accompanied by an increased rate of enzyme synthesis, but the response to starvation is considerably slower than that caused by re-feeding. The degradation rate of phosphoenolpyruvate carboxylase is also subject to regulation. Thus re-feeding starved animals decreases the half-life of the enzyme in liver from 13 h to 5.2 h, but the rapid rate of degradation is maintained at least during the first 20 h of subsequent starvation. Only slight changes in the degradation rate of phosphoenolpyruvate carboxylase are found in adipose tissue. We conclude that the large alterations in the rate of enzyme synthesis during a starvation-re-feeding cycle are the major cause of fluctuations in activity.

Phosphoenolpyruvate carboxylase (EC 4.1.1.32) activity in livers of adult rats is increased by starving the animals, by injection of cortisol, glucagon, alloxan, mannoheptulose (Shrago *et al.*, 1963), tryptophan (Snook *et al.*, 1971), cyclic AMP (Wicks *et al.*, 1972) and thyroxine (Nagai & Nakagawa, 1972), and high activities are lowered by re-feeding starved rats, by insulin injections to diabetic animals, by adrenalectomy and by thyroidectomy. Some of these effects have also been shown for adipose tissue, where the enzyme is indistinguishable from that present in liver (Ballard & Hanson, 1967, 1969; Reshef *et al.*, 1969a,b, 1972). In many of these studies immunochemical techniques or inhibitors of protein synthesis have been used to show that the changes in enzyme activity do not represent activation of enzyme precursors but the synthesis of new enzyme protein.

In the present work we have taken starvation and re-feeding as examples of conditions when phosphoenolpyruvate carboxylase activities are high and low respectively, and have investigated the mechanisms responsible for enzyme adaptation. In particular, the

rates of synthesis and degradation of enzyme have been measured at various stages in a starvation-re-feeding cycle. The results in both liver and adipose tissue indicate that the rate of phosphoenolpyruvate carboxylase synthesis changes markedly and it is this alteration that is the major factor in the increase or decrease of enzyme activity.

Materials and Methods

Preparation of antiserum against phosphoenolpyruvate carboxylase

Phosphoenolpyruvate carboxylase was purified as described previously (Ballard & Hanson, 1969; Philippidis *et al.*, 1972) to a specific activity of 14–16 μmol of bicarbonate fixed/min at 37°C (units) per mg of protein. Enzyme (5 mg in 2 ml of 0.9% NaCl) was thoroughly mixed with an equal volume of complete Freund's adjuvant (Difco Laboratories, Detroit, Mich., U.S.A.) and injected into a goat in about ten subcutaneous sites. This procedure was repeated at

2-weekly intervals until a titre of greater than 2 units of antibody/ml of serum was obtained. A unit of antibody is defined as that amount necessary to inhibit completely 1 unit of phosphoenolpyruvate carboxylase. Approx. 400ml of blood was obtained from the jugular vein at each bleeding and the serum treated as described for rabbit serum, including the adsorption against a cytosol fraction of foetal rat liver (Philippidis *et al.*, 1972). Each preparation of antibody was checked for specificity by Ouchterlony double-diffusion analysis (Ouchterlony, 1966), freeze-dried and stored *in vacuo* at -20°C . These γ -globulin preparations were reconstituted with water and diluted in 0.15M-NaCl to give an activity of 0.25 unit/100 μl .

Animals

A starvation-re-feeding cycle was set up to accentuate the lower activity of phosphoenolpyruvate carboxylase in re-fed as compared with normally fed rats (Reshef *et al.*, 1969b). All animals were 7-week-old males and weighed 190–210g before starvation. They were starved for 20h, re-fed for periods between 1 and 20h and some animals which had been re-fed for 20h had food withdrawn for an additional period of up to 20h. Animals were fed in darkness. The length of the starvation-re-feeding cycle was determined as the minimum time necessary for hepatic phosphoenolpyruvate carboxylase activities to attain approximate steady states.

At various times after re-feeding or starvation, rats were injected intraperitoneally with 100 μCi of [4,5- ^3H]leucine (specific radioactivity 50–60Ci/mmol; The Radiochemical Centre, Amersham, Bucks., U.K.). Then 60min later the animals were either killed or injected intraperitoneally with a chase of 25 μmol of non-radioactive leucine; animals thus injected were killed at a specified time later. After the animals were killed the liver and epididymal adipose tissue were removed, weighed, and portions homogenized in 3 or 1 vol. respectively of 0.25M-sucrose. Cytosol fractions prepared by centrifuging the homogenates at 100000g for 30min were used for enzyme assay (Chang & Lane, 1966) and measurements of radioactivity in phosphoenolpyruvate carboxylase, as well as for the determination of protein and protein radioactivity.

Incorporation of radioactivity into phosphoenolpyruvate carboxylase

Radioactive phosphoenolpyruvate carboxylase was precipitated from cytosol fractions of liver and adipose tissue by the addition of 0.25 unit of antibody to 0.18 unit of enzyme, followed by incubation at 37°C for 15min and overnight at 0°C (Philippidis *et al.*, 1972). With adipose-tissue extracts, additional non-radioactive enzyme of specific activity 2–4 units/mg

was added to obtain 0.18 unit of phosphoenolpyruvate carboxylase activity. The supernatant from the first precipitation was used for a second antibody-antigen precipitate and the radioactivity in this sample used to correct for non-specific trapping of radioactive material. Antibody-antigen precipitates were washed three times with 0.15M-NaCl at 0°C , dissolved in 0.2ml of NCS solubilizer (Amersham/Searle Corp., Arlington Heights, Ill., U.S.A.) and transferred to scintillation vials containing 10ml of a solution of 4g of 2,5-diphenyloxazole and 100mg of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl) benzene/litre of toluene. After cooling the vial to 1°C radioactivity was measured in a Packard liquid-scintillation spectrometer model 3375. The counting efficiency varied between 33% and 40%.

Protein was precipitated with 10% (w/v) trichloroacetic acid from cytosol fractions, washed as described by Mans & Novelli (1961), dissolved in NCS solubilizer and the radioactivity measured as described above for phosphoenolpyruvate carboxylase.

Sodium dodecyl sulphate electrophoresis

Washed antibody-antigen precipitates were dissolved and the peptides dissociated by heating each sample at 45°C for 1h in 0.1ml of a solution containing 2% sodium dodecyl sulphate, 2% dithiothreitol and 0.01M-sodium phosphate buffer, pH7.0. Pure phosphoenolpyruvate carboxylase (Ballard & Hanson, 1969) and also lactate dehydrogenase, aldolase, bovine serum albumin and pyruvate kinase (Sigma Chemical Co., St. Louis, Mo., U.S.A.) as molecular-weight standards were treated in the same way. After addition of glycerol, 2-mercaptoethanol and Bromothymol Blue, up to 150 μl of sample containing between 10 and 200 μg of protein was subjected to electrophoresis on 10% polyacrylamide gels containing 0.3% bisacrylamide (Weber & Osborn, 1969). Gels and buffer contained 0.1% sodium dodecyl sulphate and 0.1M-sodium phosphate buffer, pH7.0. For gels (5mm diam. $\times$ 55mm length) a current of 3mA was applied for 16h. After this time the light antibody chain had moved close to the bottom of the gel. Gels were removed from the running tubes, stained with 0.25% Coomassie Blue in 50% (v/v) methanol and 10% (v/v) acetic acid for 4h and destained electrophoretically in 7% (v/v) acetic acid (Weber & Osborn, 1969).

In those experiments where radioactive samples were separated by electrophoresis, the gels were removed from the running tubes, extruded into 35 fractions by using an apparatus similar to that described by Ward *et al.* (1970), and the gel fractions swollen in capped tubes containing 2.5ml of a solution of 0.3ml of NCS solubilizer, 0.025ml of 8M- NH_3 and 2.2ml of toluene scintillation fluid. After 6h or more at

room temperature the small tubes ($80\text{mm}^2 \times 50\text{mm}$) were placed inside standard scintillation vials and the radioactivity was determined at 1°C .

Protein measurements

Protein in cytosol fractions of liver and adipose tissue was determined as described by Lowry *et al.* (1951) with crystalline bovine serum albumin as standard.

Results

Enzyme activity changes

The high activities of phosphoenolpyruvate carboxylase in liver and adipose tissue of starved rats are not decreased during the first 4 h of re-feeding (Figs. 1 and 2). Subsequently, activities in liver (Fig. 1) fall to

25% of the starvation value after 20 h of re-feeding, whether activities are expressed as units/total liver or units/g of cytosol protein. Because liver and adipose-tissue weights change markedly during a starvation-re-feeding cycle, we have not expressed activities as units of enzyme/g of tissue. Such a method would unrealistically accentuate the activity changes. In adipose tissue (Fig. 2) from re-fed rats the activity of phosphoenolpyruvate carboxylase was decreased from the high values found in starved animals, although the change was less pronounced and slower than that found in liver.

When re-fed rats are starved, hepatic phosphoenolpyruvate carboxylase activities increase to a maximum of 45 units/total liver over a period of 12 h, although the increase in activity was slow during the first 4 h of starvation. This lag period was more pronounced in adipose tissue, with the activities in animals re-fed for 20 h and in animals re-fed and then starved for 4 h being similar at 50 munits/total epididymal fat or 7–8 units/g of cytosol protein. Phosphoenolpyruvate carboxylase activities in adipose tissue

Fig. 1. Changes in phosphoenolpyruvate carboxylase activity in liver

All animals were starved for 20 h before re-feeding. Values are expressed as means $\pm$ S.E.M. for between six and ten determinations. (a) Activity is expressed as units/g of cytosol protein. (b) Activity is expressed as total units/liver. ○, Starvation; ●, re-feeding.

Fig. 2. Changes in phosphoenolpyruvate carboxylase activity in adipose tissue

All animals were starved for 20 h before re-feeding. Values are means $\pm$ S.E.M. for between six and ten determinations. (a) Activity is expressed as units/g of cytosol protein. (b) Activity is expressed as munits/total epididymal fat. ○, Starvation; ●, re-feeding.

increase between 4h and 20h of starvation, but as found with the re-feeding experiments, the rate of activity change was slower than in liver.

Nature of antibody-antigen precipitates

For our precipitation procedure to be used with reliability, it is crucial that no radioactive material other than phosphoenolpyruvate carboxylase is present in such precipitates. Although we have tested for the specificity of the technique in previous studies (Ballard & Hanson, 1969; Philippidis *et al.*, 1972) by Ouchterlony double-diffusion analysis (Ouchterlony, 1966), considerable radioactivity is always found in a second antibody-antigen precipitate prepared by mixing and incubating the supernatant from the first precipitate with non-radioactive phosphoenolpyruvate carboxylase and additional antibody. Presumably, the finding that the equivalent of between 10 and 25% of the radioactivity in the first precipitate is also found in the second precipitate indicates a non-specific association of radioactive cytosol proteins, peptides or amino acids with the specific antibody-antigen precipitate. This problem is widespread in immunoprecipitation studies, so that Palmiter *et al.* (1971) and Crumpton & Parkhouse (1972) have added 1% deoxycholate to the antibody-antigen mixtures to counteract adsorption phenomena. Sodium deoxycholate is not suitable for use with phosphoenolpyruvate carboxylase, since the enzyme is inactivated, a change that might interfere with the antibody-precipitation reaction. However, we found that other detergents such as Triton X-100, Triton X-405 and Brij 35 neither inhibited the enzyme nor decreased the non-specific radioactivity in the second antibody-antigen precipitate.

We have further characterized the antibody-antigen reaction between phosphoenolpyruvate carboxylase and antibodies prepared against the enzyme, by measuring the radioactivity in polypeptides separated by sodium dodecyl sulphate electrophoresis. The dissolved antigen-antibody precipitate after electrophoresis on sodium dodecyl sulphate gels showed three peptide bands which corresponded to the light and heavy chains of goat γ -globulin, together with phosphoenolpyruvate carboxylase (Fig. 3). No additional peptides were detected, even though a crude cytosol fraction was used as the source of enzyme. Confirmation that the third peptide band was indeed phosphoenolpyruvate carboxylase was obtained from an experiment in which pure enzyme was mixed with a dissociated antigen-antibody precipitate before electrophoresis. As shown in Fig. 3, the addition of pure enzyme increased the intensity of staining in the third band. This peptide stained in the third band has an approximate molecular weight of 65000 based on mobility in the sodium dodecyl sulphate gels, a value not markedly different from that

Fig. 3. Sodium dodecyl sulphate electrophoresis of phosphoenolpyruvate carboxylase and dissociated enzyme-antibody precipitates

Details of the procedure are given in the Materials and Methods section, and of the experiment in the Results section. Samples were placed at the top of the gels. (a) Dissociated enzyme-antibody precipitate with enzyme from the cytosol fraction of rat liver. (b) Phosphoenolpyruvate carboxylase purified from rat liver (approx. 50 μ g, at specific activity 15 units/mg of protein). (c) Mixture of dissociated enzyme-antibody precipitate and purified phosphoenolpyruvate carboxylase.

determined for native phosphoenolpyruvate carboxylase by chromatography on Sephadex G-100 (74000; Ballard & Hanson, 1969). Since dithiothreitol with sodium dodecyl sulphate normally dissociates proteins into peptide subunits, we conclude that native phosphoenolpyruvate carboxylase is a single peptide chain.

Cytosol fractions of liver and adipose tissue prepared from animals injected with 500 μ Ci of [3 H]-leucine 1 h before killing were mixed with antibody, and the antibody-antigen precipitates collected and washed as described in the Materials and Methods section. The supernatant obtained by this procedure was used for a second antibody-antigen precipitate.

Fig. 4. Separation of a phosphoenolpyruvate carboxylase-antibody precipitate by sodium dodecyl sulphate electrophoresis

Enzyme was isolated from liver cytosol of a starved rat injected 1 h previously with $500\mu\text{Ci}$ of $[^3\text{H}]$ leucine. Details of the experiment are given in the Results section. Fraction 1 is the top of the gel. Radioactivity from the first enzyme-antibody precipitate (●) and from the second precipitate (○) is shown.

Each precipitate, derived from 0.36 unit of phosphoenolpyruvate carboxylase, was dissociated and subjected to sodium dodecyl sulphate electrophoresis in a manner similar to that illustrated in Fig. 3. An experiment of this type with a liver cytosol fraction from a starved rat is shown in Fig. 4. All the radioactivity is present in a single peak, which has a mobility identical with that of the phosphoenolpyruvate carboxylase that was detected by staining a duplicate gel with Coomassie Blue. Much less radioactivity was noted in fractions obtained from the second antibody-antigen precipitate, and the recovery of radioisotope through the procedure was greater than 90%. This experiment proves that the antibody-antigen reaction is specific, and the absence of radioactivity with the mobility of phosphoenolpyruvate carboxylase in the gel from the second antibody-antigen precipitate shows it to be a satisfactory control for non-specific adsorption.

A difference was noted when antibody prepared in rabbits was used for the experiments in Figs. 3 and 4. Additional peptides of molecular weights larger than that of phosphoenolpyruvate carboxylase were present, together with peptides between the light and heavy antibody bands. However, these were not radioactive.

Enzyme synthesis and degradation

We have measured the rates of synthesis and degradation of phosphoenolpyruvate carboxylase by isolating the enzyme as antibody-antigen com-

plexes at various times after animals were injected with $[^3\text{H}]$ leucine. Synthesis rates for liver phosphoenolpyruvate carboxylase are expressed both as the total radioactivity incorporated into the enzyme in the whole liver and as the percentage incorporation relative to cytosol proteins (Table 1). The latter method allows for variability in the amount of label injected and also for any changes in the precursor leucine pool. Phosphoenolpyruvate carboxylase accounts for 3% of the total rate of cytosol protein synthesis in livers of starved rats, but re-feeding the animals produces a rapid decline in the enzyme synthesis, so that only 0.2–0.4% of the protein synthesized is phosphoenolpyruvate carboxylase. This lower relative rate of synthesis is maintained for most of the re-feeding period. Starvation of the re-fed rats induces an increase in the relative rate of enzyme synthesis from 0.51% to 1.13% after 2 h and to 2.3% after 12 h of starvation.

Although the relative rate of phosphoenolpyruvate carboxylase synthesis in adipose tissue of starved rats is similar to that found in liver, the changes caused by re-feeding or by starvation are very much slower (Table 2). Further, the lowest rate of enzyme synthesis in adipose tissue, 0.95% of the total protein synthesis, is greater than the lowest found for liver. There was extreme variability in the total incorporation of $[^3\text{H}]$ leucine into adipose-tissue protein, a situation possibly caused by direct absorption of radioactive leucine into the tissue. For this reason, the changes in leucine incorporation into total tissue phosphoenolpyruvate carboxylase do not readily show the trends that become apparent when the results are expressed relative to cytosol protein synthesis.

Rates of phosphoenolpyruvate carboxylase degradation relative to total protein are shown in Figs. 5 and 6. The method used (Philippidis *et al.*, 1972) will underestimate the half-times of degradation, but the error is slight for proteins of short half-life. Further, this procedure does not require exposure of the tissue to the same amount and concentration of radioactive leucine, an assumption that is not valid for adipose tissue.

In livers from starved rats the half-time of phosphoenolpyruvate carboxylase degradation has been reported as 13 h (Ballard *et al.*, 1973) and is decreased to 6 h for the period between 4 and 16 h of re-feeding (Fig. 5). This rapid rate of degradation is continued when re-fed rats are starved for a second time. Although the activity increased as a result of starvation, the half-time of degradation was only 5.2 h.

Degradation of phosphoenolpyruvate carboxylase relative to total cytosol proteins was much slower in adipose tissue than in liver. Between 4 and 20 h of re-feeding and between 4 and 20 h of starvation the half-times of degradation were 12 and 20 h respectively (Fig. 6). The precision of the value for the starvation period is poor, but it is probable that the relative rate

Table 1. *Synthesis of phosphoenolpyruvate carboxylase and cytosol proteins in liver*

Values are expressed as means $\pm$ S.E.M. for between five and seven animals. Details of the starvation-re-feeding cycle and the radioactivity procedures are given in the Materials and Methods section.

Dietary treatment	Radioactivity incorporated (d.p.m./h per mg of liver)		Incorporation into phosphoenolpyruvate carboxylase (%)
	Cytosol protein	Phosphoenolpyruvate carboxylase	
Starved 20h	1052 $\pm$ 102	27.6 $\pm$ 3.0	3.01 $\pm$ 0.22
Starved 20h and re-fed			
1h	670 $\pm$ 34	4.0 $\pm$ 0.6	0.59 $\pm$ 0.09
2h	689 $\pm$ 82	2.0 $\pm$ 0.6	0.36 $\pm$ 0.14
4h	818 $\pm$ 110	3.8 $\pm$ 0.8	0.44 $\pm$ 0.08
8h	760 $\pm$ 100	1.7 $\pm$ 0.4	0.25 $\pm$ 0.06
12h	751 $\pm$ 52	1.3 $\pm$ 0.4	0.17 $\pm$ 0.05
16h	688 $\pm$ 50	4.3 $\pm$ 0.3	0.62 $\pm$ 0.08
20h	502 $\pm$ 61	2.5 $\pm$ 0.5	0.51 $\pm$ 0.08
Starved 20h, re-fed 20h and starved			
2h	510 $\pm$ 71	5.8 $\pm$ 0.8	1.13 $\pm$ 0.16
4h	586 $\pm$ 80	10.2 $\pm$ 2.1	1.68 $\pm$ 0.20
8h	690 $\pm$ 132	12.3 $\pm$ 2.6	1.82 $\pm$ 0.19
12h	662 $\pm$ 190	14.0 $\pm$ 5.1	2.34 $\pm$ 0.23
16h	741 $\pm$ 112	13.8 $\pm$ 2.9	2.02 $\pm$ 0.14
20h	940 $\pm$ 82	20.0 $\pm$ 1.4	2.21 $\pm$ 0.24

Table 2. *Synthesis of phosphoenolpyruvate carboxylase and cytosol proteins in adipose tissue*

Values are expressed as means $\pm$ S.E.M. for results obtained from between five and seven animals in each group. Other details are given in the Materials and Methods section.

Dietary treatment	Radioactivity incorporated (d.p.m./h per mg of adipose tissue)		Incorporation into phosphoenolpyruvate carboxylase (%)
	Cytosol protein	Phosphoenolpyruvate carboxylase	
Starved 20h	792 $\pm$ 170	22.4 $\pm$ 5.0	3.12 $\pm$ 0.26
Starved 20h and re-fed			
1h	550 $\pm$ 142	13.9 $\pm$ 4.0	2.42 $\pm$ 0.18
2h	162 $\pm$ 23	3.0 $\pm$ 0.3	2.00 $\pm$ 0.08
4h	442 $\pm$ 121	7.8 $\pm$ 1.9	1.87 $\pm$ 0.23
8h	498 $\pm$ 100	6.7 $\pm$ 1.5	1.44 $\pm$ 0.37
12h	1322 $\pm$ 281	14.1 $\pm$ 2.8	1.09 $\pm$ 0.05
16h	764 $\pm$ 238	9.9 $\pm$ 3.2	1.34 $\pm$ 0.09
20h	70 $\pm$ 172	7.5 $\pm$ 2.3	0.95 $\pm$ 0.21
Starved 20h, re-fed 20h and starved			
4h	712 $\pm$ 210	8.2 $\pm$ 1.9	1.30 $\pm$ 0.18
8h	947 $\pm$ 282	16.7 $\pm$ 5.9	1.59 $\pm$ 0.16
12h	369 $\pm$ 121	7.2 $\pm$ 2.9	1.80 $\pm$ 0.22
16h	402 $\pm$ 102	8.3 $\pm$ 2.4	2.04 $\pm$ 0.20
20h	640 $\pm$ 122	16.4 $\pm$ 3.3	2.61 $\pm$ 0.13

Fig. 5. Loss of radioactivity from the pool of phosphoenolpyruvate carboxylase in liver

Radioactivity in phosphoenolpyruvate carboxylase is expressed as a percentage of the total radioactivity in cytosol proteins and is plotted on a logarithmic scale. Values are means $\pm$ S.E.M. for duplicate determinations on between six and eight animals. Animals starved for 20h were injected with 100 μ Ci of [3 H]-leucine and were killed 1 h later or were injected with 25 μ mol of unlabelled L-leucine and re-fed for 4, 8, 12, 16 or 20h (●). Animals starved for 20h and then re-fed for 20h were starved for 3h, injected with 100 μ Ci of [3 H]leucine and were killed 1 h later or were injected with 25 μ mol of unlabelled L-leucine and starved for a total period of 8, 12, 16 or 20h (○). Details of the isolation of radioactive enzyme are given in the text.

of degradation was indeed slower than during re-feeding.

Discussion

Changes in the synthesis rate of phosphoenolpyruvate carboxylase

In liver there is a 15-fold difference between the rates of phosphoenolpyruvate carboxylase synthesis in 8–12h-re-fed rats compared with starved rats, whereas in adipose tissue the greatest change was only threefold. In both tissues the fall in synthesis rate caused by re-feeding preceded any measurable lowering in enzyme activity. In liver the rate of enzyme synthesis appears to fall with a half-time of about 20min. This time would be a maximum because no

Fig. 6. Loss of radioactivity from the pool of phosphoenolpyruvate carboxylase in adipose tissue

Details of the animals and injections are given in Fig. 5.

allowance can be made for the time taken for the starved rat to eat, digest and absorb food. Since the half-life of phosphoenolpyruvate carboxylase in starved or re-fed animals is never shorter than 5h it is unlikely that the fall in the rate of synthesis could be related to a major change in enzyme degradation. The most reasonable explanation for the change in synthesis rate would be a fall in some factor limiting phosphoenolpyruvate carboxylase formation. Unless specific initiation, termination or other components are required for the synthesis of this particular enzyme, it is likely that the concentration of available mRNA specific for phosphoenolpyruvate carboxylase is the regulatory factor. If this hypothesis is valid, then either the specific mRNA has a half-life very much shorter than that shown for most other mammalian mRNA species (Revel & Hiatt, 1964; Pitot *et al.*, 1965) or, alternatively, the re-feeding stimulus induces the degradation of phosphoenolpyruvate carboxylase mRNA. A choice between these two possibilities requires a measurement of mRNA concentration, although indirect evidence suggests that normally the half-life of phosphoenolpyruvate carboxylase mRNA is very much longer than 20min. Thus actinomycin D causes a loss of phosphoenolpyruvate carboxylase activity in liver with a half-life of about 15h (Foster *et al.*, 1966; Shrago *et al.*, 1967), a time that is considerably greater than the 6h found during the re-feeding experiments. If the mRNA for phosphoenolpyruvate carboxylase had a very short half-life, one would expect that the inhibition of mRNA synthesis by actinomycin D would result in loss of enzyme activity at the same rate as caused by re-feeding. The hypothesis that re-feeding results in an increased rate of degradation of phosphoenolpyruvate carboxylase

mRNA is consistent with the proposals of Tomkins (Tomkins *et al.*, 1969; Tomkins & Martin, 1970) that there are specific, hormone-sensitive, labile repressors of mRNA translation. The re-feeding stimulus which lowers the rate of phosphoenolpyruvate carboxylase synthesis could well be mediated by the associated rapid changes in insulin and/or glucagon concentrations.

The increase in the rate of phosphoenolpyruvate carboxylase synthesis initiated by starvation is a slower response than the decrease in synthesis caused by re-feeding starved animals. This may be related to the time taken for residual food digestion or to other factors responsible for initiating rapid enzyme synthesis. If these alterations in phosphoenolpyruvate carboxylase synthesis are associated with fluctuations in blood glucose or insulin concentrations, the rapid re-feeding and slower starvation responses seem reasonable. Further, a dietary change would alter the concentration of glucose in portal blood, so that the liver would release or take up glucose. The greater blood-glucose concentration changes in portal blood as compared with the peripheral circulation may account for the slower and less-pronounced alterations in the rate of phosphoenolpyruvate carboxylase synthesis in adipose tissue.

Changes in phosphoenolpyruvate carboxylase degradation

The half-life of phosphoenolpyruvate carboxylase under conditions when the activity is falling is approx. 6h. This value is found in liver (Treadow & Khairallah, 1972) and adipose tissue (Reshef *et al.*, 1972) of animals injected with cycloheximide, in liver (Shrago *et al.*, 1963) and adipose tissue (Reshef *et al.*, 1972) of diabetics injected with insulin, and in foetal liver after treatment with glucagon or cyclic AMP (Ballard *et al.*, 1973). In starved animals the measured activity of hepatic phosphoenolpyruvate carboxylase decreases between 4 and 16h of re-feeding with a half-life of 5.2h, a value very close to that determined in the same animals by immunochemical methods (compare Figs. 1 and 5). This constancy under conditions when the rate of enzyme synthesis is very low shows that reutilization of radioactive amino acids is not occurring to any extent. Such a relationship between declining activity and declining radioactivity in phosphoenolpyruvate carboxylase cannot be established in adipose tissue, because enzyme synthesis continues in this tissue in the re-fed animal. We found the half-life of radioactive enzyme in adipose tissue to be considerably slower than in liver, a result that may reflect either reutilization of radioactive leucine or perhaps the lower activity of a degrading system for phosphoenolpyruvate carboxylase. If degradation is controlled by the activities of non-specific proteases we would not expect to find

the tissue differences, because the method of calculating degradation that we have used is based on the rate of radioactivity loss relative to all cytosol protein. Another alternative would be the existence of more-stable conformations of the enzyme in adipose tissue than in liver. Although this explanation is difficult to test it does not hold generally, because the same degradation rate has been shown in liver and adipose tissue in other conditions, namely insulin-treated diabetics and animals injected with cycloheximide (Reshef *et al.*, 1972; Shrago *et al.*, 1967; Treadow & Khairallah, 1972).

It is perhaps surprising that phosphoenolpyruvate carboxylase degradation in liver is rapid when re-fed animals are starved, a condition where the enzyme activity increases four- to five-fold over a 20h period. In the neonatal rat the initial appearance of phosphoenolpyruvate carboxylase is accompanied by a 20-fold increase in enzyme synthesis, although there is no degradation of enzyme (Philippidis *et al.*, 1972). The lack of enzyme degradation is not a result of a generalized protein stability, and is not caused by the absence of proteolytic enzymes capable of degrading phosphoenolpyruvate carboxylase (Ballard *et al.*, 1973). Apparently the controls that lower the rate of enzyme degradation in the newborn do not operate in adult liver under conditions of increasing enzyme activity. Experiments with other hepatic enzymes have shown a decrease in degradation when changes in diet produce increases in activity. Thus arginase degradation is decreased to zero when rats maintained on a low-protein diet are starved (Schimke, 1964), whereas the degradation rate of acetyl-CoA carboxylase is decreased when starved rats are fed on a fat-free diet (Majerus & Kilburn, 1969).

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