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Synthesis of Phosphoenolpyruvate Carboxykinase (Guanosine Triphosphate) by Isolated Liver Polyribosomes

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1. Phosphoenolpyruvate carboxykinase (GTP) (EC 4.1.1.32) was synthesized by post-mitochondrial supernatants of rat liver in the presence of appropriate salts, an energy supply and [^{3}H]leucine. Synthesis of enzyme released from polyribosomes was detected by immunoprecipitation with specific antibody followed by electrophoresis of the dissolved antibody–antigen precipitates on sodium dodecyl sulphate–polyacrylamide gels in the presence of a ^{14}C-labelled enzyme marker. 2. Enzyme synthesis *in vitro* occurs predominantly on free rather than bound polyribosomes. 3. Starved animals in which de-induction of phosphoenolpyruvate carboxykinase (GTP) had been initiated by re-feeding for 2h had a markedly decreased rate of enzyme synthesis, whether the measurements were made after injection of radioactive leucine into the intact animal or if synthesis was determined *in vitro*. 4. The low rate of enzyme synthesis by liver polyribosomes from re-fed animals was not due to the absence of soluble factors, nor could it be increased by the addition of cyclic AMP to the protein synthesis system. 5. Phosphoenolpyruvate carboxykinase (GTP) synthesis *in vitro* is diminished relative to total protein synthesis when the postmitochondrial supernatant is kept at 0°C for several hours before measurement of protein synthesis. Since this effect is blocked by heparin, it is probably caused by selective ribonuclease attack on enzyme mRNA. 6. De-induction of phosphoenolpyruvate carboxykinase (GTP) is tentatively explained as being due to a transcriptional block in specific mRNA synthesis, followed by rapid degradation of existing message.

The cytosol form of rat liver phosphoenolpyruvate carboxykinase (GTP) may be induced *in vivo* by treatment of the animals with cyclic AMP (Wicks *et al.*, 1972), cortisol, glucagon, alloxan (Shrago *et al.*, 1963), thyroxine (Nagai & Nakagawa, 1972) or tryptophan (Ballard & Hopgood, 1973), and is also increased by starvation (Shrago *et al.*, 1963; Hopgood *et al.*, 1973). In those cases where the mechanism of the activity increase has been studied in detail there is evidence for the synthesis of new enzyme protein associated with a higher rate of enzyme activity (Ballard & Hopgood, 1973; Hopgood *et al.*, 1973; Philippidis *et al.*, 1972). The specific events in protein synthesis that are stimulated have not been elucidated, although actinomycin D or cordycepin usually prevents the synthesis of phosphoenolpyruvate carboxykinase (GTP), implying a requirement for mRNA synthesis (Ballard & Hopgood, 1973; Tilghman *et al.*, 1974).

Enzyme activity falls rapidly *in vivo* if starved animals are re-fed with a high-carbohydrate diet or if alloxan-treated animals are injected with insulin. This de-induction occurs as a result of a fall in the rate of enzyme synthesis and has a half-time of approx. 30min (Tilghman *et al.*, 1974). It remains to be answered whether this de-induction is a result of the rapid degradation of specific mRNA or whether some translational effect is involved. It is likely that the availability of a system in which phosphoenolpyruvate carboxykinase (GTP) can be synthesized *in vitro* will help to resolve some of these questions. In this report we describe a postmitochondrial preparation from liver in which the enzyme is synthesized at a relative rate similar to that occurring in the intact animal.

Experimental

Animals

Male rats (7 weeks old) were fed *ad libitum* on a high-carbohydrate diet containing 20% protein and 4% fat. Starvation was for an 18h period, whereas re-fed animals, which had first been starved for 18h, were re-fed for 2h or 3h in the dark before commencing an experiment.

Preparation of liver postmitochondrial supernatant

Livers from either starved or re-fed rats were minced and homogenized at 0°C in 4vol. of a solution containing 0.2M-sucrose, 0.1M-KCl, 0.005M-$MgCl_2$, 0.02M-Tris and 0.001M-dithiothreitol, adjusted to pH7.5 with HCl (solution A). In some experiments heparin was added to this solution to give a concentration of 0.5mg/ml (solution B). Homogenates in either solution A or B were centrifuged at 0°C for 10min at 15000 *g* and the resulting postmitochondrial supernatant was used for the protein-synthesis experiments.

Preparation of cytosol and total polyribosomes

Postmitochondrial supernatants in solution B were centrifuged at 0°C for 60min at 100000*g*. The cytosol thus obtained was used directly in protein-synthesis experiments, and the pellet was suspended in solution B, in 2vol. with respect to the original liver weight.

Isolation of free and bound polyribosomes

The method is essentially that described by Wettstein *et al.* (1963). Postmitochondrial supernatant (3ml) in solution B was layered over a discontinuous gradient consisting of 3ml of 0.3M-sucrose in solution B above 3ml of 1.8M-sucrose in solution B in a 10ml ultracentrifuge tube, and centrifuged at 2°C and 300000 *g* for 2h in a Christ Omega II ultracentrifuge. The pellet of free polyribosomes was suspended in solution B, in 2vol. with respect to the original liver weight. The cloudy layer of bound polyribosomes at the lower gradient interphase was either diluted to 10ml with solution B and centrifuged at 0°C and 100000 *g* for 1h, or mixed with 0.1vol. of 10% sodium deoxycholate (adjusted to pH7.5 with HCl), layered over 3ml of 1.8M-sucrose in solution B and centrifuged at 2°C and 300000 *g* for 2h. In each case the pellets were suspended in solution B, in 2vol. with respect to the original liver weight.

Protein synthesis in vitro

Postmitochondrial supernatant (1ml) was incubated for 30min at 30°C in the presence of 80mM-KCl, 4mM-$MgCl_2$, 20mM-Tris–HCl, 1mM-dithiothreitol, 1mM-ATP, 0.5mM-GTP, 30μg of pyruvate kinase (Sigma Chemical Co., St. Louis, Mo., U.S.A.), 10mM-phosphoenolpyruvate, 50μCi of [4,5-^{3}H]-leucine (specific radioactivity 50–60Ci/mmol; The Radiochemical Centre, Amersham, Bucks., U.K.) and 20 amino acids each at 0.04mM, but excluding leucine. The final volume was 5ml and the pH7.5. In those experiments where pelleted polyribosomes replaced the postmitochondrial supernatant, 1ml of the cytosol fraction and 0.5ml of polyribosomes were used. At the completion of the 30min incubation period, the solutions were cooled to 0°C and centrifuged at 100000 *g* for 60min to obtain a clear supernatant containing the released radioactive protein. This supernatant was concentrated approximately fourfold by vacuum dialysis and centrifuged at 15000 *g* for 2min to remove any insoluble material before the measurement of radioactivity in total protein and phosphoenolpyruvate carboxykinase (GTP).

Measurement of radioactivity in total protein

Protein was precipitated from 50μl of the concentrated extract derived from the protein-synthesizing system by the addition of 2vol. of 10% (w/v) trichloroacetic acid. After these samples had been mixed and centrifuged at 3000 *g* for 5min, the supernatants were discarded and the pellets incubated in 10% trichloroacetic acid at 90°C for 30min. The tubes were cooled, centrifuged, the supernatants discarded and the pellets washed a second time with 10% trichloroacetic acid. These pellets were dissolved in 0.2ml of NCS Solubilizer (Amersham/Searle Corp., Arlington Heights, Ill., U.S.A.) and the radioactivity was measured in a toluene-based scintillation fluid (Hopgood *et al.*, 1973).

Preparation and use of ^{14}C-labelled phosphoenolpyruvate carboxykinase (GTP)

Enzyme labelled with ^{14}C for use as an internal marker on sodium dodecyl sulphate–polyacrylamide gels was obtained as follows. At 1h after the intraperitoneal injection of 100μCi of [U-^{14}C]leucine (340mCi/mmol; The Radiochemical Centre) into a 2-week-old rat, the animal was killed and a liver cytosol fraction obtained (Philippidis *et al.*, 1972). Phosphoenolpyruvate carboxykinase (GTP) activity in this fraction was determined by a modification (Philippidis *et al.*, 1972) of the assay method of Chang & Lane (1966), where 1 unit of enzyme activity represents the fixation of 1μmol of $NaH^{14}CO_3$ into malate in 1min at 37°C. Antibody–antigen precipitates were prepared by mixing 180munits of enzyme, a 50% excess of specific antiserum and 0.6% Triton X-405 (Technicon Equipment, North Ryde, N.S.W., Australia) and incubating these mixtures at 37°C for 15min followed by 0°C for 18h (Philippidis *et al.*, 1972). The precipitates were washed three times with 0.154M-NaCl at 0°C and stored at −20°C. A comparison of the radioactivity in the antibody–antigen precipitates with purified ^{3}H-labelled enzyme showed identical movement of the labelled material when analysed by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis (Ballard *et al.*, 1974). For use as internal electrophoresis standards, the antibody–antigen precipitates were dissolved

and dissociated at 90°C for 10min in 50μl of a mixture containing 2% sodium dodecyl sulphate, 2% dithiothreitol, 25% glycerol and 0.005% Bromophenol Blue (all w/v) in 0.01M-sodium phosphate and adjusted to pH7.0 with NaOH. A portion (20μl) of this solution was mixed with the protein samples for each gel.

Determination of radioactivity in phosphoenolpyruvate carboxykinase (GTP)

After measuring the activity of the enzyme (Chang & Lane, 1966) in the concentrated extract from the protein-synthesis incubations, phosphoenolpyruvate carboxykinase (GTP) was precipitated by the addition of a 50% excess of specific antibody and 30μl of 10% (w/v) Triton X-405 to 360munits of enzyme in a total volume of 0.5ml. These mixtures were incubated for 15min at 37°C and 18h at 0°C, centrifuged and the antibody–antigen precipitates washed four times each with 0.5ml of 0.154M-NaCl at 0°C. The washed precipitates were dissolved in 150μl of a solution containing 2% sodium dodecyl sulphate, 2% dithiothreitol, 25% glycerol and 0.005% Bromophenol Blue in 0.01M-sodium phosphate, pH7.0, and heated for 10min at 90°C. Portions (usually 100μl) of these dissociated proteins were mixed with ^{14}C-labelled phosphoenolpyruvate carboxykinase, separated by electrophoresis on sodium dodecyl sulphate–polyacrylamide-gel columns (55mm× 5.5mm diameter) and the gels fractionated into 1.5mm portions as described previously for the determination of radioactivity (Hopgood *et al.*, 1973). Appropriate corrections were made for ^{14}C counting in the ^{3}H channel and the phosphoenolpyruvate carboxykinase (GTP) radioactivity was taken as the portion of the ^{3}H detected that was associated with the ^{14}C-labelled enzyme peak.

In those experiments where total radioactive proteins were separated by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis, similar procedures were used.

Double-label experiments

Synthesis of phosphoenolpyruvate carboxykinase (GTP) and total proteins were measured with [^{3}H]-leucine in postmitochondrial supernatants prepared from starved or re-fed rats that had been injected with 25μCi of [^{14}C]leucine 1h previously. In these experiments ^{14}C-labelled enzyme was not added to the dissociated samples before electrophoresis.

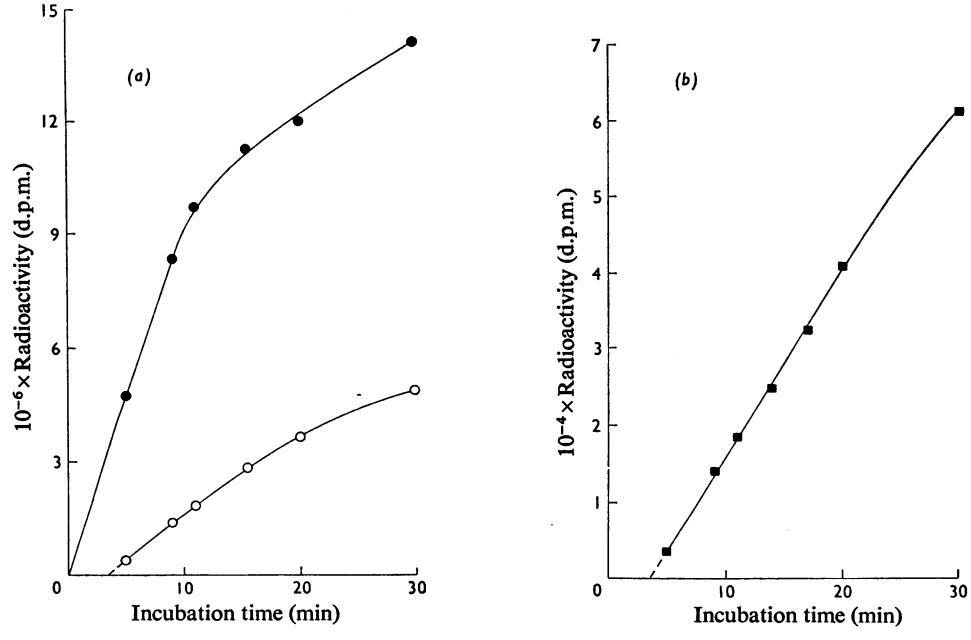

Fig. 1. *Time-course of incorporation of [^{3}H]leucine into proteins synthesized by liver postmitochondrial supernatants*

Radioactivity is expressed as the total incorporation for the 5ml reaction mixture described under 'Protein synthesis *in vitro*' in the Experimental section. Values are the average of two separate experiments. (*a*) Total protein synthesized (●); protein soluble after centrifugation at 100000*g* for 60min (○). (*b*) Phosphoenolpyruvate carboxykinase (GTP) synthesized (■).

Results and Discussion

Synthesis of phosphoenolpyruvate carboxykinase (GTP) and proteins by liver postmitochondrial supernatants

The synthesis of total proteins by postmitochondrial supernatants continues at a linear rate at 30°C for about 12min and then at a diminishing rate between 12 and 30min (Fig. 1*a*). Analysis of polyribosome profiles by sucrose-density-gradient centrifugation (Beck *et al.*, 1972) indicated a change during the incubation period from mostly large polyribosomes to monoribosomes and ribosomal subunits. Protein synthesized and then released from the polyribosomes showed a linear rate of formation for 20min after an initial lag. The proportion of protein synthesized after 30min incubation period and then released varied between 35 and 45% of total protein labelled, giving values that are close to those reported in other studies (Beck *et al.*, 1972).

Phosphoenolpyruvate carboxykinase (GTP) synthesis occurred for at least 20min at a linear rate after an initial lag period. The detection of enzyme synthesis in these and subsequent experiments required a specific separation technique that was not subject to interference by the roughly 100-fold dilution by radioactive protein and 1000-fold excess of precursor [^{3}H]leucine. Although the methods previously used for the isolation of phosphoenolpyruvate carboxykinase (GTP) as an antibody–antigen precipitate were satisfactory (Philippidis *et al.*, 1972), the routine use of the sodium dodecyl sulphate–polyacrylamide-gel electrophoresis for the separation of radioactivity in the antibody–antigen precipitates gave increased specificity, especially when the enzyme represented less than 0.2% of the total protein labelled. The addition of Triton X-405 in the antibody-precipitation reaction markedly decreased the non-specific radioactivity trapped in the precipitate. Detergents that have been used for this purpose with other antibody–antigen reactions (Palmiter *et al.*, 1971) were either less effective in the present system (Triton X-100, Brij 35, Tween 20) or interfered with the precipitation reaction (sodium deoxycholate).

Extrapolation back from the maximum rate of released protein or enzyme synthesis to zero labelling (Fig. 1) gives a measure of the half-transit time for the synthesis of these proteins on a ribosome (Fan & Penman, 1970; Palmiter & Schimke, 1973). The value obtained for phosphoenolpyruvate carboxykinase (GTP) (3.5min) is slightly longer than that found for average proteins (3min), in accordance with the larger-than-average molecular weight of the enzyme peptide chain (74000; Ballard & Hanson, 1969). This measurement of half-transit time is only valid if peptide-chain labelling involves initiation of protein synthesis or if chains initiated before labelling have an average size of one-half the completed length. Although we have no direct evidence on initiation *in vitro*, the time-course of [^{3}H]leucine incorporation into phosphoenolpyruvate carboxykinase (GTP) suggests that initiation is occurring (Fig. 1).

Polyribosomal site of phosphoenolpyruvate carboxykinase (GTP) synthesis

Of the two classes of polyribosomes present in mammalian tissues, free polyribosomes are thought to participate in the synthesis of intracellular proteins, whereas membrane-bound polyribosomes synthesize proteins that are then passed out of the cell (Siekevitz & Palade, 1960; Campbell *et al.*, 1960). Strong evidence for this concept comes from studies of serum albumin synthesis in rats, where the exported protein made in liver is synthesized on membrane-bound polyribosomes (Campbell *et al.*, 1960), but in the Morris 5123 hepatoma albumin is produced on free polyribosomes and is not released from the cell (Uenoyama & Ono, 1972*a*). At least one cytosol protein, serine dehydratase, has been reported to be produced preferentially on bound polyribosomes rather than on free polyribosomes (Pitot & Jost, 1968; Ikehara & Pitot, 1973). This would not be expected if bound polyribosomes made only exported proteins. Since serine dehydratase and phosphoenolpyruvate carboxykinase (GTP) are induced and de-induced under many similar hormonal and dietary conditions (Kenney, 1970), we have determined the polyribosomal site of phosphoenolpyruvate carboxykinase (GTP) synthesis. These experiments (Table 1) show that about 20 times as much enzyme is formed on free polyribosomes as on the membrane-bound polyribosomes derived from an equivalent volume of postmitochondrial supernatant. When the data are expressed as the ratio of the relative rates of enzyme synthesis with the two classes of polyribosomes, the free/bound ratio was approximately 6. No marked differences in either total protein or enzyme synthesis was noted between bound polyribosomes (Table 1, Expt. 1) and bound polyribosomes that had been treated with deoxycholate to release the polyribosomes (Table 1, Expt. 2). These experiments were performed with polyribosomes from livers of starved rats. The sensitivity of the methods used to measure radioactivity in phosphoenolpyruvate carboxykinase (GTP) is not adequate to permit comparable experiments with free and membrane-bound polyribosomes from livers of re-fed animals.

Comparison between phosphoenolpyruvate carboxykinase (GTP) synthesis in starved and re-fed rats

Hopgood *et al.* (1973) and Tilghman *et al.* (1974) have shown that the high rate of phosphoenolpyru-

Table 1. *Synthesis of phosphoenolpyruvate carboxykinase (GTP) by free and membrane-bound polyribosomes*

Free and bound polyribosomes were prepared from the livers of starved animals as described in the Experimental section and incubated with cytosol fraction (1 ml) for the measurement of protein synthesis. Radioactivity is expressed in thousands of d.p.m. incorporated by the polyribosomes present in 1 ml of postmitochondrial supernatant.

	Radioactivity incorporated into:		Percentage of radioactivity in	
	Released protein	Phosphoenolpyruvate carboxykinase (GTP)	phosphoenolpyruvate carboxykinase (GTP)	Free/ bound
Expt. 1				
Free polyribosomes	739	7.91	1.07	6.5
Bound polyribosomes	218	0.36	0.17	
Expt. 2				
Free polyribosomes	772	7.37	0.96	5.7
Bound polyribosomes, deoxycholate-treated	205	0.34	0.17	

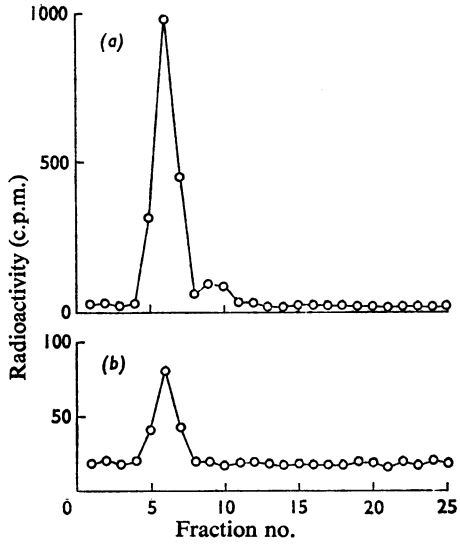

Fig. 2. *Comparison of the phosphoenolpyruvate carboxykinase (GTP) synthesized in vivo and by liver postmitochondrial supernatants*

At 1h after a starved rat had been injected with [^{14}C]leucine, the liver postmitochondrial supernatant was prepared and incubated to measure protein synthesis *in vitro* by using [^{3}H]leucine. Antibody–antigen precipitates were prepared, dissociated and analysed by electrophoresis on sodium dodecyl sulphate–polyacrylamide-gel electrophoresis as described in the Experimental section. Fractions of gel numbered from the sample origin were swollen in NCS solubilizer and the ^{3}H (*a*) or ^{14}C (*b*) radioactivities were determined.

vate carboxykinase (GTP) synthesis that occurs in livers of starved rats is markedly decreased in animals re-fed on a high-carbohydrate diet for 1h or more. Studies with inhibitors of RNA synthesis suggested that this de-induction occurred because the enzyme template had a very short life *in vivo*, so that a block in transcription would result in a rapid cessation of enzyme synthesis. Since it was not possible to exclude other types of post-transcriptional regulation, we have attempted to demonstrate the de-induction process with enzyme synthesized *in vitro*. In these experiments enzyme is synthesized *in vivo* in the presence of [^{14}C]leucine before the incubation of postmitochondrial supernatants with [^{3}H]leucine. We found that the tritiated enzyme formed *in vitro* had an identical mobility on gel electrophoresis with that formed in the intact animal (Fig. 2). The small second peak noted in Fig. 2(*a*) is more pronounced in preparations from re-fed animals, where phosphoenolpyruvate carboxykinase (GTP) represents a smaller fraction of the total protein formed, and probably represents non-specific trapping of radioactivity in the antibody–antigen precipitate. A summary of four double-labelling experiments in which starved and re-fed animals are compared is shown in Table 2. Synthesis of the enzyme *in vivo* accounted for 2.58% of the total protein in starved rats, whereas synthesis *in vitro* represented 1.42% of total protein. With re-fed animals the corresponding relative rates of phosphoenolpyruvate carboxykinase (GTP) synthesis were 0.59 and 0.32%. Thus the same ratio of enzyme synthesis between starved and re-fed animals that is found *in vivo*, 4.5, occurs when protein synthesis is measured in the postmitochondrial supernatant.

The lower relative rate of enzyme synthesis *in vitro* compared with the intact animals (Table 2) could be due to several factors. Enzyme synthesis *in vivo* is expressed as a percentage of cytosol proteins, but *in vitro* as a percentage of total proteins synthesized. An analysis of the molecular-weight spectrum of the peptides labelled in starved animals (Fig. 3) shows that larger peptide chains are formed *in vivo* than *in vitro*. This could also account for the lower relative rate of phosphoenolpyruvate carboxykinase (GTP) synthesis *in vitro*, since this enzyme has a subunit molecular weight of 74000, and is thus

Table 2. *Synthesis of phosphoenolpyruvate carboxykinase (GTP) in vivo and in vitro*

Either starved or 2h-re-fed animals were injected with [^{14}C]leucine to label the proteins synthesized *in vivo* and 1h later liver postmitochondrial supernatants were prepared and incubated with [^{3}H]leucine to measure protein synthesized *in vitro*. Samples of centrifuged incubation mixtures were taken for the measurement of radioactivity in released proteins and in phosphoenolpyruvate carboxykinase (GTP). Values are the means ±S.E.M. for four experiments and radioactivity is expressed as thousands of d.p.m. incorporated/ml of postmitochondrial supernatant.

		Radioactivity incorporated into:		Percentage of radioactivity in phosphoenolpyruvate carboxykinase (GTP)
Dietary state	Label	Released protein	Phosphoenolpyruvate carboxykinase (GTP)	
Starved	[^{3}H]Leucine	1890±370	25.8±6.3	1.42±0.26
	[^{14}C]Leucine	45.2±3.9	1.17±0.26	2.58±0.42
Re-fed	[^{3}H]Leucine	2910±400	9.8 ±3.9	0.32±0.13
	[^{14}C]Leucine	32.2±3.2	0.19±0.04	0.59±0.11

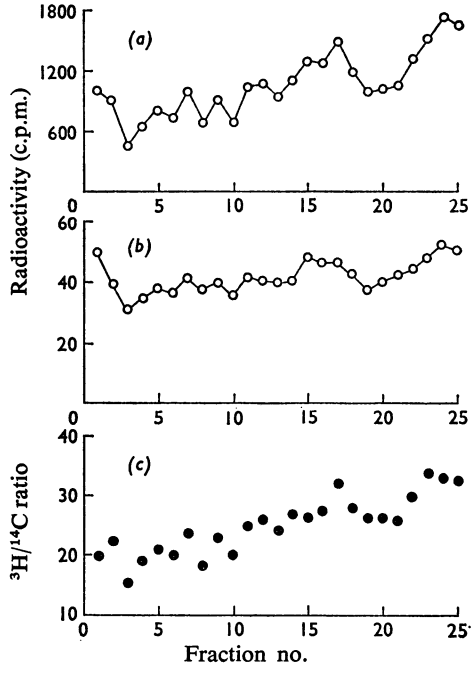

Fig. 3. *Comparison between proteins synthesized in vivo and in vitro*

As described in Table 1 and in the Experimental section, proteins labelled *in vivo* are labelled with ^{14}C, and those formed *in vitro* are labelled with ^{3}H. Protein samples are dissociated and analysed by electrophoresis on sodium dodecyl sulphate–polyacrylamide gels. Fraction 1 represents the sample origin. (*a*) ^{3}H-labelled protein; (*b*) ^{14}C-labelled protein; (*c*) ^{3}H/^{14}C ratio.

larger than the average peptide chain formed. Any process that favours the formation of small chains over large chains will therefore contribute to a lower relative rate of synthesis of the enzyme.

Possible translational control of phosphoenolpyruvate carboxykinase (GTP) synthesis

One translational control that may be pertinent to the rapid changes in phosphoenolpyruvate carboxykinase (GTP) synthesis has been demonstrated with catalase synthesis in rat liver and hepatoma. Uenoyama & Ono (1972*b*, 1973) have shown the presence of both inhibiting and activating factors in the cell cytosol that when added to polyribosomes can alter the rate of catalase synthesis. Analogous experiments with phosphoenolpyruvate carboxykinase (GTP) in which the cytosol fraction from livers of starved rats was added to polyribosomes from livers of re-fed rats did not indicate the presence of any soluble translational activator (Table 3). Likewise the converse experiment gives no suggestion of an inhibitor, since the relative rate of enzyme synthesis is dependent only on the source of polyribosomes (Table 3).

An activator or inhibitor that acts translationally may be tightly bound to the polyribosome and thus not detected by the cytosol exchange experiment. Should such a hypothetical compound act selectively on the initiation of phosphoenolpyruvate carboxykinase (GTP) synthesis, its presence may be detected by the use of inhibitors of initiation. One inhibitor of this type, poly(I), competes with mRNA in binding to the 40S ribosomal subunit–methionyl-tRNA$_F$ complex (Legon *et al.*, 1973). We found that poly(I) inhibited protein synthesis markedly with liver postmitochondrial supernatants from either starved or re-fed animals. However, the synthesis of phosphoenolpyruvate carboxykinase (GTP) by postmitochondrial supernatants from the livers of starved rats was selectively inhibited by poly(I) (Table 4). This selective inhibition may reflect the presence of an activator of initiation in the postmitochondrial supernatant from livers of starved rats, although the finding of a lower proportion of released labelled protein to total protein in the presence of

Table 3. *Polyribosomal location of specificity for the different relative rates of phosphoenolpyruvate carboxykinase (GTP) synthesis*

Cytosol and total polyribosomes were prepared from postmitochondrial supernatants of livers from either starved or 2h-re-fed rats and were mixed in different combinations before the measurement of synthesis of protein and phosphoenolpyruvate carboxykinase (GTP). Incorporated radioactivity is expressed as thousands of d.p.m./ml of postmitochondrial supernatant or the polyribosomes derived from that amount. An equivalent amount of cytosol was added to that present in the postmitochondrial supernatant.

Source		Radioactivity incorporated into:		Percentage of radioactivity in
Starved rat	Re-fed rat	Released protein	Phosphoenolpyruvate carboxykinase (GTP)	phosphoenolpyruvate carboxykinase (GTP)
Postmitochondrial supernatant	—	2320	40.3	1.74
—	Postmitochondrial supernatant	5050	13.7	0.27
Cytosol, polyribosomes	—	2710	45.0	1.66
—	Cytosol, polyribosomes	4740	10.8	0.23
Cytosol	Polyribosomes	2400	7.3	0.30
Polyribosomes	Cytosol	3730	61.9	1.66

Table 4. *Effect of poly(I) on the synthesis of protein and phosphoenolpyruvate carboxykinase (GTP)*

Liver postmitochondrial supernatants from starved or re-fed rats were incubated in the protein-synthesis system with either 0, 250 or 750 μg of poly(I) (Sigma Chemical Co.)/ml of reaction mixture. Radioactivity in total protein, released protein and phosphoenolpyruvate carboxykinase (GTP) is expressed as thousands of d.p.m. incorporated/ml of postmitochondrial supernatant. Values are the means of three experiments.

Liver source of postmitochondrial supernatant	Poly(I) (μg/ml)	Radioactivity incorporated into: Total protein	Released protein	Phosphoenolpyruvate carboxykinase (GTP)	Percentage of radioactivity in phosphoenolpyruvate carboxykinase (GTP)
Starved rat	—	8420	3405	63.3	1.86
	250	4560	969	5.96	0.62
	750	1700	238	1.36	0.57
3h-re-fed rat	—	11000	4825	3.60	0.07
	250	3610	705	0.57	0.08
	750	2200	285	0.25	0.09

poly(I) (Table 4) was unexpected and may mean that poly(I) has effects on protein synthesis other than on initiation.

Phosphoenolpyruvate carboxykinase (GTP) synthesis is stimulated by cyclic AMP in cultured hepatoma cells by a mechanism that is apparently insensitive to actinomycin D (Wicks & McKibbin, 1972) and thus may be a post-transcriptional effect. Dibutyryl cyclic AMP is effective in restoring enzyme synthesis rates when injected into re-fed rats (Table 5), but when either cyclic AMP or dibutyryl cyclic AMP was incubated with postmitochondrial supernatants from livers of re-fed animals in the protein-synthesizing system there was no measurable increase in the relative rate of enzyme synthesis. Should cyclic AMP act post-transcriptionally to increase phosphoenolpyruvate carboxykinase (GTP) synthesis, we are unable to show such an effect. It is unlikely that cyclic AMP acts to stimulate the release of completed enzyme chains from the ribosome, an action that can be demonstrated *in vitro* for tyrosine aminotransferase (Chuah & Oliver, 1971).

The findings illustrated in Fig. 3 could be due to cleavage of inter-ribosomal mRNA by ribonuclease if long mRNA molecules that code for large peptide chains were more likely to be split by ribonuclease. To test this proposal and also measure the relative stability of phosphoenolpyruvate carboxykinase (GTP) template compared with total mRNA, portions of postmitochondrial supernatant were maintained for various times at 0°C before measurement of protein synthesis. We noted a rapid fall in protein labelling owing to storage of the postmitochondrial supernatant (Table 6, Expt. 1) and an even faster decline in phosphoenolpyruvate carboxykinase (GTP) synthesis, so that the relative rate of synthesis of the enzyme fell from 1.11 to 0.32% after 8.5h at 0°C. The decline in protein synthesis and the relatively larger effect on phosphoenolpyruvate carboxykinase (GTP) was prevented by the inclusion

of 0.5mg of heparin/ml of postmitochondrial supernatant (Table 6, Expt. 2). Analysis of the proteins labelled in the four conditions in Expt. 2 (Table 6) shows that heparin is indeed maintaining the spectrum of proteins synthesized (Fig. 4), presumably by inhibiting ribonuclease activity (Rhoads *et al.*, 1973).

Table 5. *Effect of cyclic AMP on phosphoenolpyruvate carboxykinase (GTP) synthesis*

The relative incorporation of [^{3}H]leucine into the enzyme was measured in intact rats after injection of 100μCi of [^{3}H]leucine (Hopgood *et al.*, 1973) or in the protein-synthesizing system *in vitro*. In the intact animals treated with dibutyryl cyclic AMP (Sigma Chemical Co.), 2mg was injected intraperitoneally each hour after the animals had been re-fed for 2h. *In vitro* the cyclic nucleotides were incubated in the protein-synthesis system. Values are the means of three determinations.

Measurements made	Treatment	Percentage of radioactivity in phosphoenolpyruvate carboxykinase (GTP)
In vivo		
	Control, starved	2.29
	Re-fed 2h	0.51
	Re-fed 6h	0.09
	Re-fed 6h, + dibutyryl cyclic AMP	2.16
In vitro		
	Control, starved	1.50
	Re-fed 3h	0.15
	Re-fed 3h + 0.5mM-cyclic AMP	0.18
	Re-fed 3h + 2.5mM-cyclic AMP	0.10
	Re-fed 3h + 0.5mM-dibutyryl cyclic AMP	0.18
	Re-fed 3h + 2.5mM-dibutyryl cyclic AMP	0.14

The relative instability of phosphoenolpyruvate carboxykinase (GTP) mRNA *in vitro* may reflect a comparable instability in the intact tissue. Although

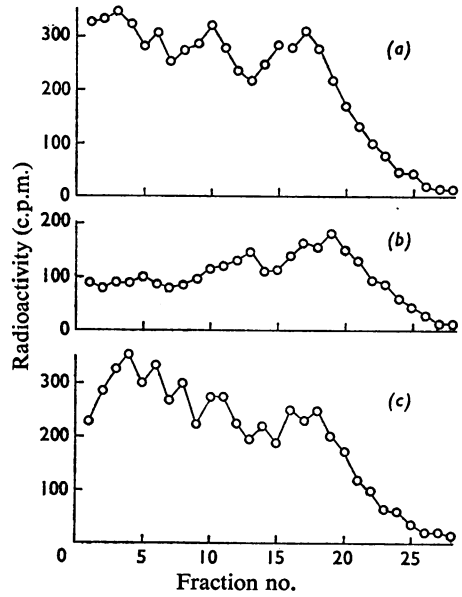

Fig. 4. *Effect of storage of postmitochondrial supernatants on the spectrum of proteins synthesized in a subsequent incubation*

Liver postmitochondrial supernatants from a starved rat were prepared in either the absence or the presence of 0.5mg of heparin/ml and kept at 0°C for 0 or 6h before measurement of protein synthesis. Labelled proteins were separated by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis and the radioactivity was measured. Fraction 1 is the sample origin. (*a*) Time 0h, no heparin; (*b*) 6h, no heparin; (*c*) 6h, plus heparin.

Table 6. *Effect of keeping the liver postmitochondrial supernatant at 0°C on the synthesis of protein and phosphoenolpyruvate carboxykinase (GTP) in vitro*

Liver postmitochondrial supernatants from starved rats were prepared either in the absence or presence of 0.5mg of heparin/ml and kept at 0°C for various times before the measurement of protein synthesis. Radioactivity is expressed as thousands of d.p.m. incorporated/ml of postmitochondrial supernatant.

	Time (h)	Heparin	Radioactivity incorporated into: Released protein	Phosphoenolpyruvate carboxykinase (GTP)	Percentage of radioactivity in phosphoenolpyruvate carboxykinase (GTP)
Expt. 1	0	−	5085	56.3	1.11
	1.5	−	3440	30.4	0.88
	2.8	−	2225	12.6	0.55
	6	−	1545	6.87	0.44
	8.5	−	1240	3.96	0.32
Expt. 2	0	−	2490	36.8	1.48
	0	+	2840	43.5	1.53
	6	−	857	6.11	0.71
	6	+	2080	28.1	1.39

the overall pool of mRNA in mammalian cells has an average half-life of 1 day or more (Greenberg, 1972; Singer & Penman, 1972; Murphy & Attardi, 1973), the template activity for phosphoenolpyruvate carboxykinase (GTP) has a short half-life of 30min *in vivo* both during de-induction produced by insulin or glucose feeding, and after mRNA synthesis is inhibited by cordycepin (Tilghman *et al.*, 1974). If the instability of functional template is caused by an unstable mRNA molecule it would be possible to account for the de-induction response as a transcriptional event rather than any post-transcriptional control. Although this interpretation is tentative and confirmation requires direct measurement of the mRNA present under the various physiological conditions, it is supported by the inability to demonstrate any marked stimulation of phosphoenolpyruvate carboxykinase (GTP) synthesis when liver polyribosomes from re-fed animals are incubated *in vitro* with potential activators.

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