Degradation of Phosphoenolpyruvate Carboxykinase (Guanosine Triphosphate)* *in vivo* and *in vitro*

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1. Phosphoenolpyruvate carboxykinase (GTP) in the cytosol fraction of liver was labelled in young rats by the injection of [^3H]leucine and then isolated with specific antibody. Antibody-antigen precipitates from 'pulse'-labelled animals and from animals in which the content of radioactive enzyme had been decreased by a period of degradation were separated by electrophoresis on sodium dodecyl sulphate-polyacrylamide gels. No radioactive breakdown products were found. 2. ^3H -labelled phosphoenolpyruvate carboxykinase (GTP) was purified from rat liver and used to measure degradation *in vitro*. There was first a loss of catalytic activity, then a disappearance of immunological activity and finally a loss of solubility before any evidence of proteolytic cleavage. Proteolytic-cleavage fragments, when found, were also insoluble. 3. An analysis of the subcellular location of enzyme inactivation showed that phosphoenolpyruvate carboxykinase (GTP) was stable when incubated with liver cytosol fraction and was inactivated most rapidly by the microsomal fraction. 4. We propose that denaturation of the enzyme is the rate-limiting step in degradation *in vivo*, and precedes proteolytic cleavage when the enzyme is incubated with liver preparations *in vitro*.

Although present concepts of protein metabolism include the complete degradation of protein to amino acids before the synthesis of a new protein molecule, there is very little information available on the mechanism of the degradative process. A reason for this lack of knowledge is the inability to either reproduce *in vitro* a sequence of events that may be similar to the reactions occurring *in vivo*, or to detect in intact cells any intermediates between a native protein and the amino acids derived from it.

We have attempted to overcome these restrictions by using ^3H -labelled phosphoenolpyruvate carboxykinase (GTP) (EC 4.1.1.32) purified from the cytosol fraction of rat liver, as a substrate for degradation in broken-cell preparations. The availability of a specific antibody to phosphoenolpyruvate carboxykinase (GTP) (Ballard & Hanson, 1969; Philippidis *et al.*, 1972; Hopgood *et al.*, 1973) permits the isolation of the enzyme together with any degradative products that react with the antibody from liver fractions that are radioactively labelled by injection of labelled precursors into the intact animal. As a

result of these experiments we propose that the initial denaturation process is the rate-limiting step in phosphoenolpyruvate carboxykinase (GTP) degradation.

Materials and Methods

Purification of ^3H -labelled phosphoenolpyruvate carboxykinase (DTP)

Some 20 rats (seven weeks old) were starved overnight to induce the synthesis of phosphoenolpyruvate carboxykinase (GTP) and each animal was injected intraperitoneally with 1 mCi of [4,5- ^3H]leucine (sp. activity 50-60 Ci/mmol; The Radiochemical Centre, Amersham, Bucks., U.K.) and killed 1 h later. Livers were homogenized in 3 vol. of 0.25 M-sucrose and the homogenate was centrifuged at 100000g for 1 h at 2°C to obtain a clear cytosol fraction. Phosphoenolpyruvate carboxykinase (GTP) was purified from this fraction by $(\text{NH}_4)_2\text{SO}_4$ fractionation, Sephadex G-100 chromatography, DEAE-cellulose chromatography, a second $(\text{NH}_4)_2\text{SO}_4$ fractionation (Ballard & Hanson, 1969) and hydroxyapatite chromatography (Philippidis *et al.*, 1972). The enzyme was stored as a suspension in 80% saturated $(\text{NH}_4)_2\text{SO}_4$ containing 1 mM-EDTA

* In accordance with the recommendations (1972) of IUPAC-IUB, this recommended name is used instead of the previous trivial name, phosphopyruvate carboxylase. Further, activities are expressed in katal (mol/s).

and 1 mM-dithiothreitol at pH 7.0. Under these conditions the enzyme lost less than 10% of its activity in 4 months. During the purification and for other studies with the enzyme, activity was measured by a slight modification (Ballard & Hanson, 1969) of the method described by Chang & Lane (1966). Since this assay involves fixation of $\text{NaH}^{14}\text{CO}_3$ (The Radiochemical Centre), care was taken to remove the ^3H -labelled protein by centrifugation after stopping the reaction with 10% (w/v) trichloroacetic acid and before determination of ^{14}C incorporated into oxaloacetate. The purified ^3H -labelled enzyme had a specific enzymic activity of 250 nmol of HCO_3^- fixed/s (nkat) per mg of protein, and a specific radioactivity of $0.24 \mu\text{Ci}/\text{mg}$ of purified enzyme. After allowing for enzyme discarded during the purification procedure, a calculation of the rate of phosphoenolpyruvate carboxykinase (GTP) labelling relative to radioactivity incorporation into total cytosol proteins gives a value of 2.0%, which is comparable with that found by using the specific antibody to precipitate the enzyme from crude liver cytosol fractions (Hopgood *et al.*, 1973). Purity tests on the enzyme indicated no protein-staining bands on sodium dodecyl sulphate-polyacrylamide-gel electrophoresis (see below) other than phosphoenolpyruvate carboxykinase (GTP), and more than 95% of the radioactivity was localized in a single peak with the same mobility as the enzyme (see Fig. 2a).

Immunological procedures

We have reported details of the methods used to prepare antibodies in goats against purified phosphoenolpyruvate carboxykinase (GTP), and of the use of these antibodies for the precipitation of the enzyme from solution (Philippidis *et al.*, 1972; Hopgood *et al.*, 1973). The only modification to these methods is the inclusion of a wetting agent (Triton X-405; Technicon Equipment Pty. Ltd., North Ryde, N.S.W., Australia) at a concentration of 0.6% in all precipitin reactions. This addition decreases the non-specific trapping of radioactivity in the antigen-antibody precipitate.

Preparation of ^{14}C -labelled phosphoenolpyruvate carboxykinase (GTP) in antigen-antibody precipitates

[U- ^{14}C]Leucine ($50 \mu\text{Ci}$; sp. radioactivity $340 \text{ mCi}/\text{mmol}$; The Radiochemical Centre) was injected intraperitoneally into a 3-week-old rat that had been starved for 18 h. At 1 h later the animal was killed, the liver homogenized in 4 vol. of ice-cold 0.25 M-sucrose and the homogenate centrifuged at $100000g$ for 1 h at 2°C to obtain a clear cytosol fraction. Portions of cytosol fraction containing 3 nkat of enzyme were mixed with a 40% excess of specific antibody and Triton X-405 to a final concentration of 0.6%, and

incubated at 37°C for 15 min and at 0°C for 18 h to precipitate the antigen-antibody complex. These precipitates were collected by centrifugation, washed three times with 0.15 M-NaCl, and stored at -20°C . On solubilization in sodium dodecyl sulphate and electrophoresis on polyacrylamide gels, the precipitates showed a single peak of radioactivity (Hopgood *et al.*, 1973).

Preparation and incubation of liver extracts

Liver (1 g) from a rat starved overnight was homogenized in 10 ml of ice-cold Krebs-Ringer phosphate buffer, pH 7.4 (Umbreit *et al.*, 1959). The homogenate was centrifuged at $15000g$ for 10 min at 2°C to pellet the nuclei, mitochondria and lysosomes and then at $100000g$ for 30 min to separate the microsomal fraction from the cytosol fraction. Each pellet was suspended in either homogenization buffer or in cytosol fraction, to produce a concentration equivalent to that of the original homogenate.

In those experiments where radioactive phosphoenolpyruvate carboxykinase (GTP) was incubated *in vitro*, approx. 1×10^5 d.p.m. of ^3H -labelled enzyme in an 80% (w/v) $(\text{NH}_4)_2\text{SO}_4$ suspension was centrifuged and the pellet suspended in 3 ml of the appropriate liver extract prepared as above, but with 5 mM-L-cysteine (pH 7.4) added. The preparation was incubated with shaking for various times at 37°C . After the incubation, samples were cooled in ice and centrifuged at $100000g$ for 30 min at 2°C to obtain the 'supernatant' and 'pellet' fractions referred to in Tables 2 and 4. The supernatant fraction was used for the measurement of total radioactivity, the assay of phosphoenolpyruvate carboxykinase (GTP) (Chang & Lane, 1966), the measurement of radioactivity that could be precipitated by a 40% excess of specific antibody, the determination of radioactivity soluble in 5% (w/v) trichloroacetic acid and for the measurement and analysis on sodium dodecyl sulphate-polyacrylamide gels of radioactivity precipitated by 5% trichloroacetic acid. The pellet was suspended by homogenization in Krebs-Ringer phosphate buffer, pH 7.4, and portions were taken for the measurement of total radioactivity, the determination of radioactive material soluble in 5% trichloroacetic acid and for the measurement and separation on sodium dodecyl sulphate-polyacrylamide gels of radioactivity precipitated by 5% trichloroacetic acid.

Measurement of radioactivity

Portions ($25 \mu\text{l}$) of solutions containing ^3H - or ^{14}C -labelled samples were mixed with 0.2 ml of NCS tissue solubilizer (Amersham Searle Corp., Arlington Heights, Ill., U.S.A.) and 10 ml of a scintillation fluid containing 4 g of 2,5-diphenyloxazole and 100 mg

of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene per litre of toluene. Radioactive material that had been pelleted by centrifugation of Krebs–Ringer phosphate buffer extracts or after the addition of trichloroacetic acid was dissolved in 0.2 ml of NCS tissue solubilizer and transferred to 10 ml of the above scintillation fluid. All samples were counted at 1°C in a Packard liquid-scintillation spectrometer model 3375. Appropriate corrections were made for ^{14}C counting in the ^3H channel.

Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate

Antibody–antigen precipitates or trichloroacetic acid precipitates were dissolved in 0.15 ml of a solution containing 2% (w/v) sodium dodecyl sulphate, 2% (v/v) dithiothreitol, 0.005% Bromophenol Blue and 20% (w/v) glycerol in 0.01 M-sodium phosphate buffer pH 7.0, by heating the mixtures at 90°C for 10 min. Soluble samples were mixed with a more concentrated dissociation solution so that the final concentrations were as above.

Portions (100 μl) of these dissociated protein solutions, containing up to 500 μg of protein, were separated by electrophoresis on 10% (w/v) polyacrylamide gels containing 0.1% sodium dodecyl sulphate (Weber & Osborn, 1969) for 30 min at 3 mA and for 5 h at 5 mA. The gels were either stained with Coomassie Blue and destained in aq. 7% (v/v) acetic acid to detect proteins (Weber & Osborn, 1969), or fractionated by using an apparatus similar to that described by Ward *et al.* (1970). Each 40 mg gel fraction was swollen in capped tubes containing 2.5 ml of a solution of 0.3 ml of NCS tissue solubilizer, 0.025 μl of 8 M- NH_3 and 2.2 ml of toluene scintillation fluid. The tubes were placed inside scintillation vials after being left overnight at room temperature and their radioactivity was determined at 1°C.

Assay of β -glucuronidase

Soluble β -glucuronidase activity and activity released by treating particulate fractions with 0.5%

Triton X-100 was measured as the formation of phenolphthalein from phenolphthalein glucuronide (Bowers *et al.*, 1967).

Results

Degradation of phosphoenolpyruvate carboxykinase (GTP) in vivo

If the first reaction in phosphoenolpyruvate carboxykinase degradation is rate limiting, no products will be discernible until amino acids are formed. Should, however, the initial proteolytic cleavage not be rate limiting, some breakdown products will accumulate and some of these may retain the antigenic determinants that will permit their isolation and characterization with antibody. To test these possibilities we 'pulse'-labelled liver proteins in 3-week-old rats and also measured the radioactivity remaining in liver protein after 17 h of a decay period. Table 1 shows a decrease in phosphoenolpyruvate carboxykinase (GTP) labelling from 1.35 to 0.56% of the radioactivity in total cytosol protein over the decay period. When the radioactive protein that reacts with antibody specific to phosphoenolpyruvate carboxykinase (GTP) was separated on sodium dodecyl sulphate–polyacrylamide gels, a single radioactive peak was found from livers after both 'pulse' and decay treatment. The mobility of this peak corresponded exactly to the ^{14}C -labelled enzyme in an antigen–antibody precipitate that was added as an internal standard (Fig. 1). There was thus no indication of either endoproteolytic cleavage products or a small increase in electrophoretic mobility that would be caused by gradual exopeptidase action.

Degradation of ^3H -labelled phosphoenolpyruvate carboxykinase (GTP) in liver homogenates

Although no cleavage products could be detected *in vivo* by the above procedure, it is possible that the

Table 1. Decay of phosphoenolpyruvate carboxykinase (GTP) radioactivity *in vivo*

Two 3-week-old rats were each injected with 100 μCi of $[4,5\text{-}^3\text{H}]$ leucine. After 1 h one animal was killed and the other was injected with a 'chase' of 12.5 μmol of non-radioactive leucine and killed 17 h later. Livers were removed, homogenized and cytosol fractions prepared (Philippidis *et al.*, 1972) for the measurement of radioactivity in total protein (Mans & Novelli, 1961) and phosphoenolpyruvate carboxykinase (GTP) (Hopgood *et al.*, 1973).

Time after $[^3\text{H}]$ leucine injection (h)	Enzyme activity (nkat/g)	Radioactivity incorporated (d.p.m./10 mg of liver)		Radioactivity in phosphoenolpyruvate carboxykinase (GTP) (%)
		Cytosol protein	Phosphoenolpyruvate carboxykinase (GTP)	
1	120	38 000	511	1.35
18	111	28 400	158	0.56

cell disruption and dilution that is associated with homogenization would dissociate the various steps in protein breakdown so that intermediate products would be found. Further, the availability of a pure

Fig. 1. Sodium dodecyl sulphate-polyacrylamide-gel electrophoresis of phosphoenolpyruvate carboxykinase (GTP)-antibody precipitates

Antigen-antibody precipitates from the 'pulse'- (a) or decay- (b) treated animals described in Table 1 were prepared and the protein subunits separated by electrophoresis in the presence of ^{14}C -labelled phosphoenolpyruvate carboxykinase (GTP). The radioactivity in each fraction was measured as described in the Materials and Methods section. ^3H -labelled enzyme ($\circ$); ^{14}C -labelled internal enzyme standard ($\bullet$). Under the counting conditions used both the ^3H and ^{14}C background counts were approx. 18 c.p.m. The direction of protein migration is from left to right.

radioactive protein that can be added to homogenates avoids the necessity of using the specific antibody to isolate the protein and thus removes the condition that for breakdown products to be detected they must react with and be precipitated by antibody.

When ^3H -labelled phosphoenolpyruvate carboxykinase (GTP) was added to liver homogenates and incubated in the presence of 5 mM-cysteine, enzyme activity was lost rapidly, with only 15% remaining after a 4 h incubation (Table 2). The radioactive material precipitated by antibody is also decreased under these conditions, but the rate of loss of antibody-reacting radioactivity was somewhat slower since 31% remained after the 4 h incubation period. Initially all of the radioactive protein added to the homogenate was precipitated by the antibody, but by 4 h some 28% of the radioactivity remaining in the supernatant was not precipitated. This radioactive material cannot be accounted for by amino acids or small peptides because all the radioactivity was precipitated when trichloroacetic acid was added to a final concentration of 5%. A comparison of antibody-precipitated radioactivity and trichloroacetic acid-precipitated radioactivity by electrophoresis on sodium dodecyl sulphate-polyacrylamide gels showed a single peak of radioactivity at all incubation times (i.e. comparable with Figs. 2a or 2b). This radioactivity had a mobility identical with the ^{14}C -labelled phosphoenolpyruvate carboxykinase (GTP) that was added as an internal marker.

Since not all of the radioactivity added to the liver homogenate could be recovered in the supernatant obtained by centrifuging the incubated homogenate at 100000g for 30 min, an analysis was made of the radioactive material in the pellet. We found that all of this pelleted radioactivity was insoluble in 5% trichloroacetic acid, implying the absence of small peptides and amino acids.

Table 2. Degradation of ^3H -labelled phosphoenolpyruvate carboxykinase (GTP) in liver homogenates

Purified ^3H -labelled enzyme (approx. 1×10^5 d.p.m.) was incubated at 37°C with 3 ml of a 10% (w/v) liver homogenate in Krebs-Ringer phosphate buffer, pH 7.4, containing 5 mM-cysteine. At 0, 2 and 4 h, samples were taken for measurements on a supernatant and pellet obtained by centrifuging the homogenate at 100000g for 30 min. All measurements of radioactivity are $10^{-3} \times$ d.p.m./total incubation mixture.

Fraction	Measurement	Incubation time (h)	...	0	2	4
Supernatant	Enzyme activity (nkat)	63.8		31.0	9.3	
	Total radioactivity	105		81.2	43.7	
	Radioactivity precipitated by antibody	97.5		74.6	29.9	
	Radioactivity not precipitated by antibody	4.7		5.7	11.7	
	Radioactivity precipitated by trichloroacetic acid	104		80.5	42.8	
	Radioactivity soluble in trichloroacetic acid	<2		<2	<2	
Pellet	Total radioactivity	5.5		21.8	61.5	
	Radioactivity precipitated by trichloroacetic acid	5.5		21.9	61.3	
	Radioactivity soluble in trichloroacetic acid	<2		<2	<2	

Table 3. *Loss of phosphoenolpyruvate carboxykinase (GTP) activity after incubation with subcellular fractions of liver*

Livers were homogenized in Krebs–Ringer phosphate buffer, pH 7.4, to give a 10% (w/v) homogenate which was further fractionated by differential centrifugation as described in the Materials and Method section. Various fractions were incubated with 5 mM-cysteine and the activities of phosphoenolpyruvate carboxykinase (GTP) and β -glucuronidase measured in a supernatant obtained by centrifuging the incubation mixture for 30 min at 100000g. Total activities of β -glucuronidase were also measured in a sample of incubation mixture that was treated with 0.5% Triton X-100. For further details see the text.

Cell fraction	Measurement	Incubation time (h)	Enzyme activity (nkat/ml)				
			0	1	2	3	4
Homogenate	Phosphoenolpyruvate carboxykinase (GTP)	...	2.45	1.82	1.57	1.27	0.98
	Released β -glucuronidase		0.075	0.093	0.088	0.205	0.400
	Total β -glucuronidase		—	—	—	—	1.38
Microsomal fraction plus cytosol fraction	Phosphoenolpyruvate carboxykinase (GTP)		2.45	1.93	1.77	1.40	0.75
	Released β -glucuronidase		0.057	0.053	0.042	0.063	0.138
	Total β -glucuronidase		—	—	—	—	0.318
Homogenate minus microsomal fraction	Phosphoenolpyruvate carboxykinase (GTP)		2.45	1.90	1.70	1.65	1.27
	Released β -glucuronidase		0.136	0.126	0.138	0.283	0.407
	Total β -glucuronidase		—	—	—	—	0.95
Cytosol fraction	Phosphoenolpyruvate carboxykinase (GTP)		2.45	2.22	2.28	2.28	2.18
	Released β -glucuronidase		0.057	0.050	0.043	0.057	0.058
	Total β -glucuronidase		—	—	—	—	0.038

Inactivation of phosphoenolpyruvate carboxykinase (GTP) by subcellular fractions of liver

We noted from Table 2 and additional experiments that none of the parameters appeared to follow the kinetics of a first-order reaction. The loss of enzyme activity, the loss of antibody-reacting material and the loss of soluble radioactivity was greater during the second 2 h period of a 4 h incubation. Such a result would be expected if the active factors in degradation were retained within a cell organelle and only became functional with disruption of the organelle during incubation. Since lysosomes contain proteolytic enzymes and have been implicated in protein degradation, we measured the loss of enzyme activity in various cell fractions and related this to the total and soluble activities of a lysosomal marker enzyme, β -glucuronidase. These experiments (Table 3) showed that although lysosomal breakage did occur during incubation, it was not the lysosome fraction that contained the greatest ability to inactivate phosphoenolpyruvate carboxykinase (GTP). Loss of enzyme activity was most rapid in the microsomal fraction, even though this preparation contained less than one-quarter of the total β -glucuronidase content. Phosphoenolpyruvate carboxykinase (GTP) activity was stable in the incubated cytosol fraction. Other experiments (results not given) showed that intact lysosomes prepared by the method of Sawant *et al.* (1964) and incubated with the cytosol fraction did not produce any enzyme inactivation.

Degradation of ^3H -labelled phosphoenolpyruvate carboxykinase (GTP) by an enriched microsomal fraction

A subcellular fraction that was pelleted by centrifuging a post-mitochondrial supernatant at 100000g for 30 min was suspended in Krebs–Ringer phosphate buffer, pH 7.4, to give a fivefold enrichment with respect to the original homogenate. This microsomal fraction was incubated with ^3H -labelled enzyme and 5 mM-cysteine for 2 and 4 h. Under these incubation conditions activity is lost much more rapidly than in homogenates, with less than 5% of the original enzyme activity remaining after 2 h.

An electrophoretic analysis of the total radioactivity remaining in the supernatant showed that 18% of the initial radioactivity was present after 4 h, with essentially all of this radioactivity having a mobility identical with the internal enzyme standard (Fig. 2b). No difference in electrophoretic pattern was seen between 0 h (Fig. 2a) and 4 h (Fig. 2b), other than the decrease in total radioactivity.

After incubation of the microsomal fraction- ^3H -labelled phosphoenolpyruvate carboxykinase (GTP) mixture for 4 h, 83% of the radioactivity was pelleted by a centrifugation at 100000g for 30 min (Table 4). It was considered unlikely that this pelleted radioactivity could be associated with a membrane fraction, since neither treatment with 0.5% Triton X-100 (Table 4) nor sonication solubilized any radioactivity. About 63% of the initial radioactivity was present as

protein in the pellet, and on electrophoresis of the pelleted material approx. 40% of the applied radioactivity had a mobility greater than phosphoenolpyruvate carboxykinase (GTP) and was presumably

some degradation products of the enzyme (Fig. 2c). More extensive degradation was indicated by the finding that 25% of the initial radioactivity could be accounted for by trichloroacetic acid-soluble material in the pellet (Table 4).

Fig. 2. Sodium dodecyl sulphate-polyacrylamide-gel electrophoresis of ^3H -labelled phosphoenolpyruvate carboxykinase and degradation products

Supernatant fractions of 0 (a) and 4 h (b) incubation mixtures and pelleted radioactivity from 4 h incubations (c) of microsomal fraction with ^3H -labelled enzyme (see Table 4) were separated by electrophoresis in the presence of ^{14}C -labelled phosphoenolpyruvate carboxykinase (GTP). The radioactivity in each fraction was determined as described in the Materials and Methods section. ^3H -labelled protein ($\circ$), ^{14}C -labelled enzyme ($\bullet$). The background was approx. 18 c.p.m. The direction of protein migration is from left to right.

Discussion

Pathway of phosphoenolpyruvate carboxykinase (GTP) degradation

Any mechanism put forward to explain the degradation of phosphoenolpyruvate carboxykinase (GTP) must incorporate the findings that the enzyme degradation rate is altered under some conditions existing in the intact liver cell. Thus phosphoenolpyruvate carboxykinase (GTP) is stable during the period when the enzyme first appears in the newborn rat (Philippidis *et al.*, 1972), but if either slices or homogenates are prepared from liver at this age and then incubated *in vitro*, enzyme activity is lost (Ballard *et al.*, 1973). Analogous results are found in adult rats where tryptophan inhibits phosphoenolpyruvate carboxykinase (GTP) degradation *in vivo* (Ballard & Hopgood, 1973), but is ineffective if added to slices or homogenates incubated *in vitro* (M. F. Hopgood & F. J. Ballard, unpublished work). We interpret such results as evidence for the existence of at least two forms of active enzyme, one of which is stable *in vivo*, possibly owing to the binding of a ligand that dissociates on disruption of the tissue, whereas the other form is unstable (see Scheme 1). We assume in this hypothesis that both 'stable' and 'unstable' enzyme have the same catalytic activity and are inhibited equally by the specific antibody prepared against phosphoenolpyruvate carboxykinase (GTP) that had been purified from starved adult rats, i.e. 'unstable' enzyme (Hopgood *et al.*, 1973). Support for this assumption comes from the

Table 4. Degradation of ^3H -labelled phosphoenolpyruvate carboxykinase (GTP) in an enriched microsomal fraction

Microsomal fractions were prepared from liver as described in the Materials and Methods section and incubated at five times the original concentration with ^3H -labelled enzyme (3.3×10^4 d.p.m.) and 5 mM-cysteine for 2 or 4 h at 37°C . Samples were taken and centrifuged at $100000g$ for 30 min to separate supernatant from pellet. All measurements of radioactivity are $10^{-3} \times$ d.p.m./total incubation mixture.

Fraction	Radioactivity measurement	Incubation time (h)	2	4
Supernatant	Total		14.7	5.8
	Precipitated by trichloroacetic acid		13.4	5.5
	Soluble in trichloroacetic acid		<2	<2
	With gel mobility of phosphoenolpyruvate carboxykinase (GTP)		12.5	4.8
	Degradation products		<1	<1
Pellet	Total		16.8	27.4
	Released by 0.5% Triton X-100		<2	<2
	Precipitated by trichloroacetic acid		10.9	20.7
	Soluble in trichloroacetic acid		6.0	8.5
	With gel mobility of phosphoenolpyruvate carboxykinase (GTP)		7.0	14.6
	Degradation products		5.6	9.3

Scheme 1. *Proposed pathway for phosphoenolpyruvate carboxykinase (GTP) degradation*

finding that the enzyme in liver cytosol fractions from 1-day-old rats ('stable' enzyme) shows an equivalence point identical with that of the 'unstable' enzyme in the livers of starved adult rats (Philippidis *et al.*, 1972).

The experiments in which homogenates or microsomal fractions were incubated with ^3H -labelled enzyme indicates that there is a progression in the degradation processes in which catalytic activity is lost before immunological activity, which in turn decays more rapidly than soluble enzyme protein. Since all three forms of the enzyme have the same subunit molecular weight, whereas the native enzyme has only a single subunit (Ballard & Hanson, 1969; Hopgood *et al.*, 1973), we have included both loss of catalytic activity and loss of immunological activity as a single step in Scheme 1: 'unstable' enzyme to denatured enzyme. We recognise, however, that such an analysis is probably an oversimplification and that a set of sequential reactions may be involved. Incubation of the radioactive enzyme in homogenates or microsomal fractions led to an increasing proportion of the radioactive protein being sedimented at 100000g for 30 min. Initially this pelleted radioactivity had the same subunit molecular weight as native enzyme, but after prolonged incubation with microsomal fractions approximately one-third of the radioactive material had a smaller subunit molecular weight. These degradation products were never found in the supernatant fraction after high-speed centrifugation, so presumably they were sufficiently altered to restrict solubility. Further degradation of radioactive enzyme to acid-soluble material occurs (Table 4), but since this radioactivity is also retained in the pellet it probably represents peptides rather than free leucine.

Degradation of other proteins

Somewhat similar studies to those reported here have been carried out by Li & Knox (1972) with tryptophan oxygenase. Under some conditions *in vitro* the loss of catalytic activity preceded loss of immunological activity, but there was essentially no conversion of denatured radioactive tryptophan oxygenase

into acid-soluble products. Li & Knox (1972) did not report whether the denatured enzyme became insoluble, but concluded that the initial step in degradation was related to denaturation, with this reaction prevented by combination of the enzyme with substrate.

It is perhaps generally accepted that most proteins act as substrates for the few endoproteases and exoproteases present in tissues, although specific or group-specific proteases probably occur (Schimke & Doyle, 1970; Kominami *et al.*, 1972). There is also a positive correlation between the subunit size and the rate of degradation of proteins, although no such correlation occurs between the total molecular weight and degradation rate (Dice & Schimke, 1972; Dice *et al.*, 1973). It would appear, therefore, that proteins must be in equilibrium with their subunits and these subunits are the substrates for intracellular proteases.

There are reports that protease activity of the liver cytosol fraction is insufficient to account for the observed rate of degradation of cytosol proteins *in vivo* (Bohley *et al.*, 1971a,b). Similarly, phosphoenolpyruvate carboxykinase (GTP) and tyrosine aminotransferase are rapidly degraded *in vivo* and in homogenates, but are quite stable when cytosol fractions are incubated (Auricchio *et al.*, 1972; M. F. Hopgood & F. J. Ballard, unpublished work). A search for cell constituents that could degrade cytosol proteins led Bohley *et al.* (1971a) to postulate that either microsomal fractions or lysosomes could serve this function, since both have sufficient protease activity. In the present study we find both rapid loss of phosphoenolpyruvate carboxykinase (GTP) activity and degradation of the protein by microsomal fractions, whereas intact lysosomes do not inactivate the enzyme. This negative result with lysosomes agrees with studies on the degradation of total cytosol protein (Bohley *et al.*, 1971a), albumin (Davies *et al.*, 1971; Mego, 1971), alanine aminotransferase and arginase (Haider & Segal, 1972) where lysosomes were only effective if disrupted. However, there is little doubt that liver lysosomes take up labelled, denatured albumin after it has been injected into the portal vein, and continue to degrade the albumin when the lysosomes are isolated and incubated *in vitro* (Davies *et al.*, 1971; Mego, 1971).

If denaturation rather than proteolysis is the limiting step in phosphoenolpyruvate carboxykinase (GTP) degradation *in vivo* but not in homogenates, then why does denaturation not occur in the cytosol fraction? Further, the loss of enzyme activity is greatly accelerated by the addition of cysteine to homogenates (M. F. Hopgood & F. J. Ballard, unpublished work). This effect of cysteine has been noted with other proteins (Bohley *et al.*, 1971b) and has been interpreted as an activation of proteinases. We suggest that cysteine may stimulate the degradation process other than by an activation of proteinases,

perhaps as a substrate of a protein disulphide reductase that is present in the microsomal fraction, but absent from the liver cytosol.

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