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Properties of Phosphoenolpyruvate Carboxykinase (Guanosine Triphosphate) Synthesized in Hepatoma Cells in the Presence of Amino Acid Analogues

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1. Phosphoenolpyruvate carboxykinase (GTP) was induced by a combination of dibutyryl cyclic AMP, theophylline and dexamethasone in Reuber H35 hepatoma cells under conditions where an amino acid in the medium was replaced by an appropriate analogue. 2. With canavanine replacing arginine or with 5-fluorotryptophan or 6-fluorotryptophan replacing tryptophan the induced enzyme had a lower catalytic activity relative to antibody reactivity. 3. These aberrant enzyme molecules were heat-labile *in vitro*. 4. Measurements of enzyme degradation *in vivo* indicated that the canavanine-containing enzyme and the 6-fluorotryptophan-containing enzyme were degraded more rapidly than the enzyme containing all natural amino acids.

Phosphoenolpyruvate carboxykinase (GTP) (EC 4.1.1.32) is normally degraded in rat liver with a half-time of approx. 6h (Hopgood *et al.*, 1973), but during the initial appearance of the enzyme at birth or after adult animals are injected with actinomycin D, cycloheximide or large doses of tryptophan, the turnover-time of the enzyme is much longer (Philippidis *et al.*, 1972; Ballard & Hopgood, 1973). As a result of these experiments and studies on the degradation of the enzyme *in vitro*, we have proposed a model in which phosphoenolpyruvate carboxykinase (GTP) exists in two or more forms, only one of which is susceptible to rapid degradation (Ballard *et al.*, 1974). We have no evidence on the reactions that transform the unstable enzyme into a more stable form, but it may be postulated that ligand binding is involved, thus leading to altered physical properties of the enzyme molecule (see Goldberg & Dice, 1974).

A direct method by which the physical properties of a protein may be altered is to insert unnatural amino acids into the peptide chain (Pine, 1967). In the present study with Reuber H35 hepatoma cells we have accomplished this by inducing phosphoenolpyruvate carboxykinase (GTP) synthesis under conditions whereby an essential amino acid is replaced by an appropriate analogue in the incubation medium. We find that the altered enzyme differs from the normal enzyme in heat-stability and in the reaction with specific antiserum. Studies with intact cells show that the enzyme with canavanine replacing arginine in the molecule is recognized by the cell as aberrant, and is rapidly degraded.

Experimental

Cell culture conditions

Reuber H35 hepatoma cells were grown as monolayers in plastic Petri dishes incubated at 37°C under an atmosphere of CO_2 + air (5:95) (Knowles *et al.*, 1975).

Induction of phosphoenolpyruvate carboxykinase (GTP)

The growth medium was removed from confluent cells and replaced by a similar medium but with the foetal calf serum omitted and in some experiments with either arginine or tryptophan omitted and with L-canavanine, DL-5-fluorotryptophan, DL-6-fluorotryptophan or DL-7-azatryptophan added at the concentrations indicated. Dibutyryl cyclic AMP, theophylline and dexamethasone phosphate were added at final concentrations of 0.5mM, 1mM and 5μM respectively to induce phosphoenolpyruvate carboxykinase (GTP), and the dishes were left for 15h before isolation of cells (Knowles *et al.*, 1975).

Preparation of cell extracts

Extracts prepared by homogenizing washed cells in water and then freezing and thawing the mixture three times (Knowles *et al.*, 1975) were centrifuged at 100000*g* and 0°C for 30min. The supernatant was used for the assay and immunochemical isolation of phosphoenolpyruvate carboxykinase (GTP).

Protein determinations

Protein was measured in uncentrifuged cell homogenates as described by Lowry *et al.* (1951) with crystalline bovine serum albumin as standard.

Assay of phosphoenolpyruvate carboxykinase (GTP)

The enzyme activity was measured in cytosol fractions as described (Ballard & Hanson, 1969). One unit of enzyme activity catalyses the fixation of 1 μmol of $NaH^{14}CO_3$/min at 37°C.

Immunological procedures

Antibodies against rat liver cytosolic phosphoenolpyruvate carboxykinase (GTP) were prepared in goats and treated with a cytosol fraction of foetal rat liver to attain specificity (Ballard & Hanson, 1969; Hopgood *et al.*, 1973). One unit of antibody activity is defined as the amount that completely inactivates 1 unit of phosphoenolpyruvate carboxykinase (GTP) in a cytosol fraction from rat liver.

Two types of quantitative antibody titration were performed. In the first method, different volumes of cell cytosol were incubated with a fixed amount of antibody for 15min at 37°C and then for 3h at 0°C. The solutions were centrifuged at 3000g for 5min and phosphoenolpyruvate carboxykinase (GTP) activity was measured in the supernatant. The second method differed in that increasing volumes of antibody were incubated with a constant amount of cytosol enzyme.

Degradation experiments

Degradation of phosphoenolpyruvate carboxykinase (GTP) was measured in cells grown, labelled and induced as described by Knowles *et al.* (1975). The three types of experiments mentioned below are described in detail by Knowles *et al.* (1975).

Type 1 *experiment.* Normal enzyme was induced in the presence of [^{14}C]leucine, after which analogue-containing enzyme was labelled with [^{3}H]leucine.

Type 2 *experiment.* Enzyme was induced in the presence of the amino acid analogue and [^{14}C]leucine before labelling of normal enzyme with [^{3}H]leucine.

Type 3 *experiment.* Normal enzyme and the analogue-containing enzyme were induced in separate culture dishes in the presence of [^{3}H]leucine.

In each type of experiment the subsequent 'chase' and degradation conditions were exactly as described by Knowles *et al.* (1975).

Measurement of radioactivity in phosphoenolpyruvate carboxykinase (GTP)

Cytosol fractions prepared from cells that had been previously labelled with either [^{3}H]leucine or [^{3}H]leucine plus [^{14}C]leucine were assayed for phosphoenolpyruvate carboxykinase (GTP) activity. To 0.3ml of these extracts was added sufficient unlabelled, purified enzyme (Ballard & Hanson, 1969) to bring the total activity to 150 munits, Triton X-405 at a final concentration of 0.6% and 250 munits of specific antibody. The final volume was 0.5ml. These solutions were mixed, incubated for 15min at 37°C and then overnight at 0°C, and centrifuged at 3000g for 5min. A second control precipitation was performed on the supernatant of this centrifugation step with an additional 150 munits of non-radioactive enzyme and 250 munits of antibody. The incubation conditions were the same as for the first precipitation. Each antibody–antigen precipitate was washed four times by suspension in 0.5ml of 0.15M-NaCl. The washed precipitates were dissolved in 250μl of NCS solubilizer (Amersham/Searle Corp., Arlington Heights, Ill., U.S.A.) added to 5ml of a toluene scintillation fluid (Hopgood *et al.*, 1973), and the radioactivity was measured by liquid-scintillation spectrometry.

Sodium dodecyl sulphate–polyacrylamide-gel electrophoresis

The details have been described (Hopgood *et al.*, 1973). Briefly, washed antibody–antigen precipitates containing phosphoenolpyruvate carboxykinase (GTP) that had been labelled with [^{14}C]leucine in hepatoma cells were dissolved and dissociated, a sample of pure ^{3}H-labelled enzyme (Ballard *et al.*, 1974) was added, and the samples were applied to polyacrylamide gels. After electrophoresis the gels were extruded, 1.5mm gel portions were swollen overnight and the radioactivity was measured.

Results and Discussion

Heat inactivation of phosphoenolpyruvate carboxykinase (GTP)

Enzyme induced in the hepatoma cells in the presence of normal medium, or with one amino acid replaced by an analogue, was extracted and its stability measured in cytosol fractions at 48°C. Results of one experiment are shown in Table 1, where only the activities remaining after 20min incubation are given. Enzyme from cells induced for 15h in the presence of all amino acids or with arginine or tryptophan omitted from the medium retained approx. 80% of the initial activity during the 20min period. With increasing concentrations of 5-fluorotryptophan, 6-fluorotryptophan or canavanine in the induction medium, the enzyme became increasingly heat-labile. However, cells induced with 7-azatryptophan replacing tryptophan in the medium attained high enzyme activities, with the enzyme having a similar heat-stability to the enzyme induced without analogues present.

Table 1. *Heat inactivation of phosphoenolpyruvate carboxykinase (GTP) in cytosol fractions*

Enzyme in hepatoma cells was induced as described in the Experimental section with the omissions from or additions to the medium that are shown in the Table. Cells were homogenized and the activity of cytosolic phosphoenolpyruvate carboxykinase (GTP) was measured after 0 or 20min at 48°C. Values are the means of two experiments on separate culture dishes.

Omissions from medium	Additions to medium (mM)	Enzyme activity (munits/ml) at		Relative inactivation rate
		0min	20min	
Trp	—	208	171	1.0
—	—	164	127	
Arg	—	264	199	
Trp	5-Fluorotryptophan (0.1)	140	60	3.4
Trp	5-Fluorotryptophan (0.25)	123	44	4.1
Trp	5-Fluorotryptophan (1.0)	94	24	5.4
Trp	6-Fluorotryptophan (0.1)	173	58	4.3
Trp	6-Fluorotryptophan (0.25)	121	32	5.3
Trp	6-Fluorotryptophan (1.0)	173	23	8.1
Trp	7-Azatryptophan (0.1)	269	187	1.5
Trp	7-Azatryptophan (0.25)	213	167	1.0
Trp	7-Azatryptophan (1.0)	250	173	1.5
Arg	Canavanine (0.1)	97	59	2.0
Arg	Canavanine (0.5)	87	57	1.7
Arg	Canavanine (1.0)	77	38	2.8

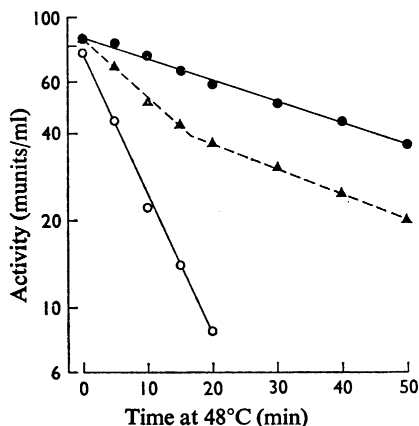

Fig. 1. *Heat inactivation of phosphoenolpyruvate carboxykinase (GTP)*

Semi-logarithmic plots of inactivation of enzyme in cytosol from normal cells or cells induced in the presence of 1mM-6-fluorotryptophan measured at 48°C are shown. ●, Normal cells; ○, 6-fluorotryptophan-treated cells; ▲, 1:1 mixture of cytosol from normal and 6-fluorotryptophan-treated cells.

Inactivation rates were averaged for the three conditions in Table 1 where amino acid analogues were absent, and all other rates were expressed relative to this value (Table 1, column 5). By this technique it can be seen that the greatest rate of inactivation was found for the enzyme from cells induced in the presence of 1mM-6-fluorotryptophan, whereas the lowest concentrations of 5-fluorotryptophan or 6-fluorotryptophan resulted in phosphoenolpyruvate carboxykinase (GTP) being formed that was three to four times as labile as the normal enzyme.

The results in Table 1 may be interpreted as showing the existence of unstable enzyme in cells induced in the presence of an inhibitor of inactivation in the cytosol from normal cells, or the presence of an activator of inactivation in the cells induced with analogues present. A distinction between these possibilities can be made if the cytosol from cells induced in the presence of an analogue is mixed with cytosol from normal cells, and the rate of heat inactivation of the enzyme measured. An experiment of this type, where the cytosol was from cells induced in the presence of 1mM-6-fluorotryptophan, is shown in Fig. 1. The inactivation curve of the mixed cytosol is approximately biphasic, indicating a mixture of two forms of phosphoenolpyruvate carboxykinase (GTP), one more stable than the other. If activators or inhibitors had been responsible for the differences in heat inactivation, the mixed cytosol would be expected to give an intermediate plot with a single first-order component of inactivation.

Other studies have shown that altered enzymes have accelerated rates of heat inactivation. Thus tyrosine aminotransferase in hepatoma cells is heat-labile when the enzyme was induced in the presence of amino acid analogues (Johnson & Kenney, 1973), whereas isocitrate lyase (Gershon & Gershon, 1970), glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (Holliday & Tarrant, 1972) in aged organisms appear to be made up of normal enzyme plus some enzyme that is heat-labile.

Antibody-titration experiments

Since it was necessary to isolate phosphoenolpyruvate carboxykinase (GTP) from hepatoma cells by means of an immunoprecipitation reaction, it was essential to show that the enzyme reacted with antibody prepared against the rat liver enzyme.

The equivalence-point titration (Fig. 2) indicates two phosphoenolpyruvate carboxykinase (GTP) activities, one that does not react with antibody and appears quantitatively in the supernatant, and one that is quantitatively precipitated. The extrapolations in Fig. 2 give the amounts of enzyme activities that are completely precipitated by the 12 munits of antibody used in the experiment. The enzyme present in normally induced cells gives an equivalence point that is close to the predicted value of 12 munits, whereas the equivalence point from cells induced in the presence of canavanine gives a lower value, suggesting the presence of antibody-reacting material that has a lower catalytic activity than the normal enzyme.

In similar experiments, Johnson & Kenney (1973) showed that tyrosine aminotransferase from hepatoma cells treated with 5-fluorotryptophan gave a lower antibody equivalence point than the enzyme from normal cells, suggesting the selective loss of catalytic activity as compared with antibody reactivity. An altered reactivity of aldolase towards specific antiserum has been shown in livers of aging mice, where the aldolase present has been reported to consist in part of a catalytically inactive form that still retains antibody reactivity (Gershon & Gershon, 1973). Further studies on aldolase in aged rabbits support this concept and also provide evidence that the aldolase has an altered amino acid sequence (Anderson, 1974).

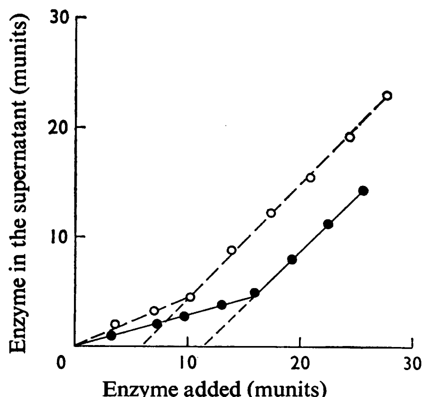

Fig. 2. *Equivalence-point titration of phosphoenolpyruvate carboxykinase (GTP) from hepatoma cells*

Various amounts of enzyme extracted from normal (●) or canavanine-treated (○) cells were mixed with 12 munits of specific antibody, and after incubation at 37°C for 15 min and 0°C for 3 h the tubes were centrifuged and activity was measured in the supernatant.

Quantitative measurement of the differences between catalytic activity and amount of enzyme antigen is most conveniently performed by measuring the precipitation of activity caused by increasing amounts of antibody (Fig. 3), since the intersection or extrapolation of the ordinate gives the amount of antibody required to completely precipitate the enzyme added. If the enzyme from hepatoma cells was identical with the rat liver enzyme, then the number of antibody units required just to precipitate the enzyme should equal the number of units of enzyme added. This relationship holds because an antibody unit was defined in such terms. We have also defined the equivalence ratio (see Table 2) as the ratio between the units of antibody required to precipitate a certain number of catalytic units and the units of catalytic activity added (Fig. 3, ordinate intercept/abscissa intercept). It is apparent that phosphoenolpyruvate carboxykinase (GTP) from hepatoma cells that were induced with arginine and tryptophan present (Fig. 3*e*) gives an equivalence ratio close to unity, whereas the enzyme in cells where induction was carried out in the presence of analogues gives a higher ratio. These equivalence ratios are given in Table 2 together with measurements of protein and enzyme activity for the same experiments as shown in Fig. 3. We have calculated the amount of enzyme antigen in the cells as the product of the equivalence ratio and the catalytic activity, assuming that the different enzyme molecules all react equally with the antibody. Thus we assume that the incorporation of analogue amino acids only alters the catalytic activity of the enzyme. The calculated amount of enzyme antigen in normal cells and those induced in the presence of canavanine, 5-fluorotryptophan or 6-fluorotryptophan are all similar when allowance is made for the protein content of each culture dish (Table 2, bottom line). This result supports the assumption made above.

The antibody titration for liver phosphoenolpyruvate carboxykinase (GTP) shown in Fig. 3(*d*) differs from the various titrations of hepatoma-cell enzyme in that all the liver activity is precipitated or inhibited. With the hepatoma enzyme, between 10% (Fig. 3*a*) and 40% (Fig. 3*c*) of the activity is not precipitated by antibody, even under conditions of considerable antibody excess. This fraction of the enzyme activity represents phosphoenolpyruvate carboxykinase (GTP) that does not react with antibody and is the cause of the titration lines in Fig. 2 that intercept the origin. We believe that this activity represents mitochondrial enzyme that had been shown not to react with the antibody to the cytosol enzyme (Ballard & Hanson, 1969). Mitochondrial

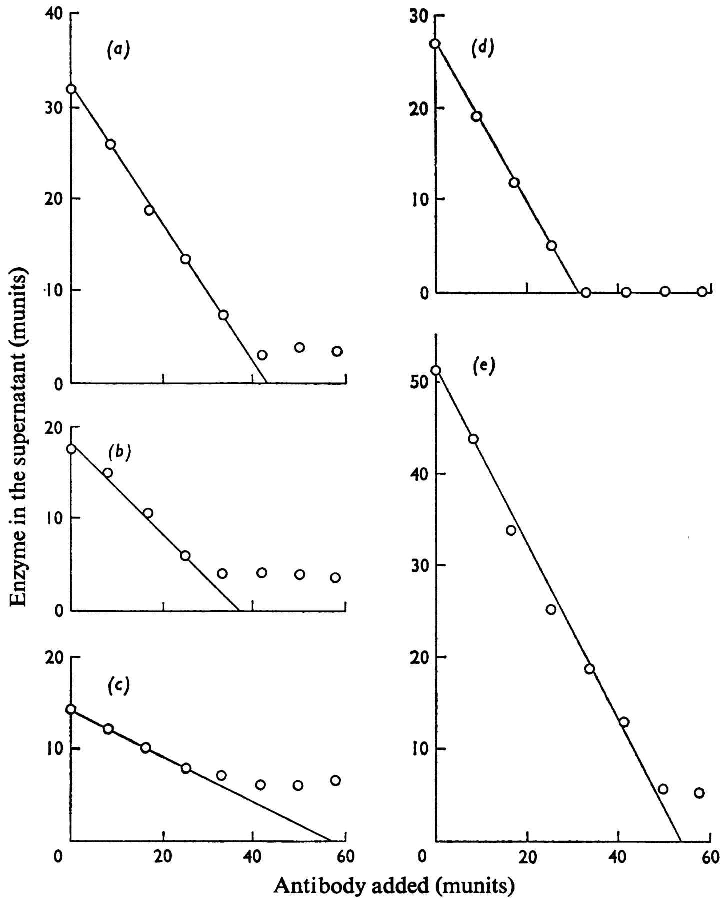

Fig. 3. *Quantitative immunotitration of phosphoenolpyruvate carboxykinase (GTP) extracted from hepatoma cells or liver*

Enzyme was extracted from cells induced in the presence of (*a*) 1mM-6-fluorotryptophan, (*b*) 1mM-5-fluorotryptophan, (*c*) 0.5mM-canavanine, (*e*) no analogue, and (*d*) was obtained with a cytosol fraction from liver of starved rats (Ballard & Hanson, 1969). To each enzyme extract were added different amounts of specific antibody and after incubation at 37°C for 15min and 0°C for 3h the tubes were centrifuged and activity was measured in the supernatant.

Table 2. *Phosphoenolpyruvate carboxykinase (GTP) activity and antigen in hepatoma cells*

Cells were induced in the presence of normal medium or with 0.5mM-canavanine replacing arginine, 1mM-5-fluorotryptophan replacing tryptophan or 1mM-6-fluorotryptophan replacing tryptophan. The cells were homogenized and cytosol extracts prepared. Protein and enzyme activity were measured as described in the Experimental section and the equivalence ratio was determined from Fig. 3. Values are for the total amount in each culture dish.

Analogue present in inducing medium ...	None	Canavanine	5-Fluorotryptophan	6-Fluorotryptophan
Protein (mg)	1.37	1.89	1.21	1.11
Enzyme activity (munits)	237	79	98	163
Enzyme activity (munits/mg)	173	42	81	147
Equivalence ratio	1.03	3.98	2.00	1.35
Enzyme antigen (munits)	244	314	196	220
Enzyme antigen (munits/mg)	178	166	162	198

enzyme would be present in the hepatoma cytosol fraction, since cell breakage is accomplished by repeated freezing and thawing, a treatment that would also break mitochondria. On the other hand, liver cytosol fractions (Fig. 3*d*) were prepared by homogenizing tissue in 0.25M-sucrose, a condition in which most mitochondria remain intact. We have shown that cytosol and mitochondrial activities of phosphoenolpyruvate carboxykinase (GTP) may be separated on columns of hydroxyapatite (Ballard, 1971). When the cytosol fraction from hepatoma cells was chromatographed by this procedure, the proportion of activity occurring in the initial 'mitochondrial' peak closely agreed with the amount of activity that was not inactivated by antibody.

Sodium dodecyl sulphate–polyacrylamide-gel electrophoresis of hepatoma phosphoenolpyruvate carboxykinase (GTP)

To establish that the altered enzyme in hepatoma cells induced in the presence of 0.5mM-canavanine had

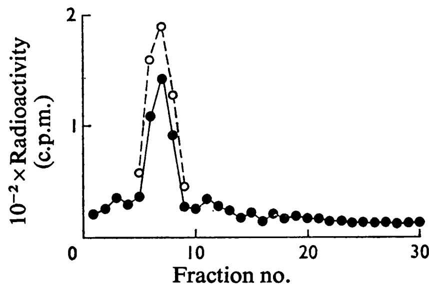

Fig. 4. *Sodium dodecyl sulphate–polyacrylamide-gel electrophoresis of phosphoenolpyruvate carboxykinase (GTP) from canavanine-treated hepatoma cells*

Enzyme was labelled with 5μCi of [^{14}C]leucine in canavanine-treated cells for 3h and isolated as an antibody–antigen precipitate. To this was added pure ^{3}H-labelled enzyme, and after dissociation and electrophoresis the gel was extruded into fractions and the radioactivity measured (Hopgood *et al.*, 1973). Fraction 1 is the origin. ●, Radioactivity in canavanine-containing enzyme; ○, ^{3}H-labelled liver enzyme.

a similar molecular weight to the liver enzyme, antibody–antigen precipitates were prepared from cells labelled with [^{14}C]leucine, dissociated, mixed with pure ^{3}H-labelled enzyme and subjected to electrophoresis on sodium dodecyl sulphate–polyacrylamide gels. The two radioisotopes coincided exactly (Fig. 4), implying that canavanine incorporation did not alter the molecular weight of the enzyme protein.

Degradation of phosphoenolpyruvate carboxykinase

Enzyme degradation in the Reuber hepatoma cells was determined in the same culture dishes as used for measurements of total protein turnover (Knowles *et al.*, 1975). Usually less than 50 munits of enzyme were available in the 0.3ml of hepatoma-cell cytosol used for immunoprecipitation, so it was necessary to add non-radioactive purified liver enzyme to give a total activity of 150 munits. A large excess of antibody was used to precipitate the enzyme from these solutions to allow for the antigen of low catalytic activity present, at least in cells exposed to canavanine.

The results in Table 3 were obtained from an experiment in which phosphoenolpyruvate carboxykinase (GTP) was induced in the presence of [^{14}C]-leucine before [^{3}H]leucine and canavanine were added for a second labelling period (type 1 experiment). The method adopted for this type of experiment is essentially similar to that used by Johnson & Kenney (1973) for studies on the degradation of tyrosine aminotransferase. During the degradation period the inducers were omitted from the medium, a procedure which results in a rapid decrease of the enzyme synthesis rate (S. M. Tilghman, J. M. Gunn, F. J. Ballard, L. Reshef & R. W. Hanson, unpublished work). Leucine (2mM) was also added to the medium during the degradation period. Both these procedures will lessen the possibility of radioisotope re-utilization into the phosphoenolpyruvate carboxykinase (GTP) pool.

We found that the enzyme activity, expressed as munits/mg of protein, fell during the degradation period (Table 3), but at a slower rate than the loss of ^{14}C from the enzyme pool. Since there is considerable

Table 3. *Degradation of phosphoenolpyruvate carboxykinase (GTP)*

Details of this example of a type 1 experiment are given in the Experimental section and by Knowles *et al.* (1975). Values are the means ± S.E.M. for determinations on three culture dishes at each time-period.

Degradation period (h) ...	0	1	2	3	4	5
Protein (mg)	2.91 ± 0.68	3.40 ± 0.66	3.52 ± 0.50	2.97 ± 0.23	3.27 ± 0.58	3.36 ± 0.36
Enzyme (munits)	117 ± 23	131 ± 34	144 ± 26	134 ± 23	100 ± 22	110 ± 19
Enzyme (munits/mg of protein)	41.1 ± 2.3	37.5 ± 3.2	40.5 ± 2.0	44.6 ± 4.0	30.2 ± 2.4	32.5 ± 3.4
$10^{-2}\times$ ^{14}C-labelled enzyme (d.p.m.)	32.9 ± 5.3	42.2 ± 7.1	30.7 ± 5.3	25.6 ± 0.4	19.9 ± 3.4	18.9 ± 3.2
$10^{-2}\times$ ^{3}H-labelled enzyme (d.p.m.)	114.8 ± 23.1	88.4 ± 19.0	74.7 ± 13.3	55.5 ± 3.0	39.0 ± 6.0	31.2 ± 4.5

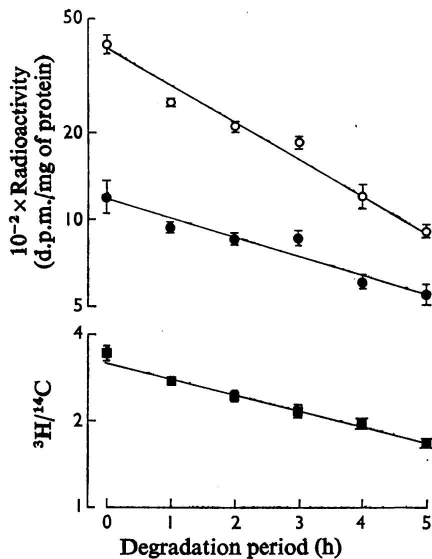

Fig. 5. *Degradation of phosphoenolpyruvate carboxykinase (GTP) in hepatoma cells; type 1 experiment*

The enzyme was induced in the presence of [^{14}C]leucine and absence of added arginine for 15h, after which the medium was replaced with one containing [^{3}H]leucine, inducers and 0.5mM-canavanine. Then 3h later the medium was replaced with one lacking radioisotope and inducers. Three culture dishes were taken each hour and radioactivity in phosphoenolpyruvate carboxykinase (GTP) was measured by antibody precipitation from cell cytosol fractions. Values are calculated from the data in Table 3 and represent the means ± S.E.M. at each time-point. They have been plotted with a logarithmic vertical scale. ●, ^{14}C-labelled normal enzyme; ○, ^{3}H-labelled canavanine-containing enzyme; ■, ^{3}H/^{14}C ratio.

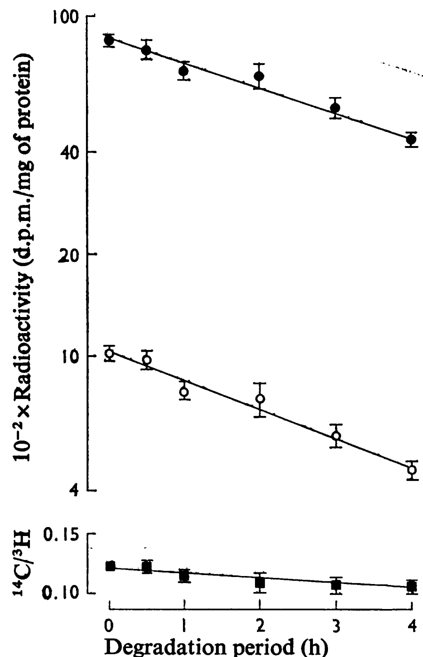

Fig. 6. *Degradation of phosphoenolpyruvate carboxykinase (GTP) in hepatoma cells; type 2 experiment*

The enzyme was induced in the presence of 0.5mM-canavanine and [^{14}C]leucine for 15h, after which the medium was replaced with one containing arginine, [^{3}H]leucine and inducers but with canavanine omitted. Details of the degradation measurements are given in Fig. 5 and by Knowles *et al.* (1975). Values at each point are the means ± S.E.M. for six Petri dishes and are expressed as d.p.m. in enzyme/mg of total cell protein. They have been plotted with a logarithmic vertical scale. ●, ^{3}H-labelled normal enzyme; ○, ^{14}C-labelled canavanine-containing enzyme; ■, ^{14}C/^{3}H ratio.

variability between the different Petri dishes, the radioisotope data have been expressed relative to total protein, and the ratio of ^{3}H-labelled enzyme/^{14}C-labelled enzyme has also been calculated (Fig. 5). The loss of radioactivity from the ^{14}C-labelled enzyme is considerably slower than that found with the ^{3}H-labelled enzyme, a conclusion that is clearly evident from the fall in the ^{3}H/^{14}C ratio. All three plots in Fig. 5 approximate to first-order processes, unlike the loss of radioactivity from total proteins (Knowles *et al.*, 1975). This suggests that there are only two pools of enzyme in the cells, one containing arginine and the other canavanine. The degradation of the canavanine-containing enzyme (^{3}H-labelled) is approximately twice as rapid as that of the arginine-containing enzyme (^{14}C-labelled).

It is possible that the enzyme labelled in the presence of arginine and [^{14}C]leucine in the experiment illustrated in Table 3 and Fig. 5 consisted in part of a rapidly degraded form of phosphoenolpyruvate carboxykinase (GTP). A labile enzyme would have been degraded during the subsequent [^{3}H]leucine-labelling period, and would not be detected. The second type of degradation experiment rules out this possible interpretation, since the arginine-containing enzyme is now labelled last, and it still has a long half-time of degradation (Fig. 6). In this experiment the initial labelling with [^{14}C]leucine was performed with canavanine present and should form aberrant phosphoenolpyruvate carboxykinase (GTP). Most of this form of the enzyme would have been degraded at the rapid rate shown in the previous experiment (Fig. 5), so the residual ^{14}C-labelled enzyme probably represents arginine-containing enzyme. As would be expected, this enzyme has a similar degradation rate to the ^{3}H-labelled enzyme which was synthesized in

the presence of added arginine (Fig. 6). This interpretation is supported by the almost constant $^{14}C/^{3}H$ ratio throughout the degradation period.

Phosphoenolpyruvate carboxykinase (GTP) degradation in culture dishes that were labelled simultaneously with [^{3}H]leucine, but with canavanine replacing arginine in one of the two groups, is shown in Fig. 7. Loss of ^{3}H from the enzyme pool occurs with a half-time of 2h when canavanine was present in the labelling period and with a half-time of 5h when arginine was present during synthesis. Although the variability is greater than in an experiment of type 1 (Fig. 5), additional evidence is provided that the canavanine-containing enzyme is unstable *in vivo*.

Canavanine was used as the analogue amino acid in these degradation experiments because its incorporation into the protein chain can be readily detected by amino acid analysis (Knowles *et al.*, 1975). However, phosphoenolpyruvate carboxykinase (GTP) synthesized in the presence of 5-fluorotryptophan or 6-fluorotryptophan was more heat-labile than the canavanine-containing enzyme (Table 1), so that degradation of these enzyme forms may also be accelerated. To test this hypothesis we carried out a type 1 experiment with 6-fluorotryptophan and tryptophan. The results show a slight increase in the degradation of analogue-containing enzyme over that found with the normal enzyme (Fig. 8).

It is noteworthy that Johnson & Kenney (1973) did not find an accelerated rate of tyrosine aminotransferase degradation in hepatoma cells treated with 6-fluorotryptophan, even though the enzyme was heat-labile. They considered that although this negative finding may argue against the hypothesis that physical properties of a protein are rate-limiting factors in protein degradation, tyrosine aminotransferase was unusual, since the native enzyme was degraded at a very high rate. Our experiments with phosphoenolpyruvate carboxykinase (GTP) show that changes in antibody reactivity, degradation *in vivo* and heat-lability need not correlate with one another. Thus the enzyme from cells induced in the presence of 6-fluorotryptophan was more heat-labile than the enzyme from canavanine-treated cells, whereas the reverse order was found for antibody reactivity and degradation rate. We consider that the insertion of an amino acid analogue at or near the catalytic site of an enzyme may alter the catalytic reactivity or heat-lability, but it need not alter the recognition of the molecule by the protein-degrading

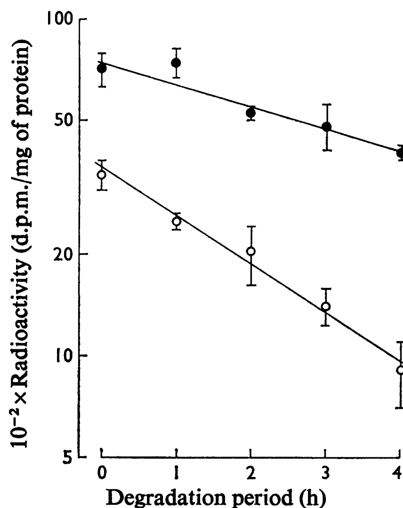

Fig. 7. *Degradation of phosphoenolpyruvate carboxykinase (GTP) in hepatoma cells; type 3 experiment*

Enzyme was induced in cells in two groups of Petri dishes, one of which contained [^{3}H]leucine, 0.5mM-canavanine but no arginine, and the other contained [^{3}H]leucine, arginine but no canavanine. The label and inducers were removed after 3h and degradation was measured as described in the legend to Fig. 5 and by Knowles *et al.* (1975). Values are means±S.E.M. for groups of three Petri dishes at each time-point and are expressed as d.p.m. in enzyme/mg of cell protein. They have been plotted with a logarithmic vertical scale. ●, Arginine-containing enzyme; ○, canavanine-containing enzyme.

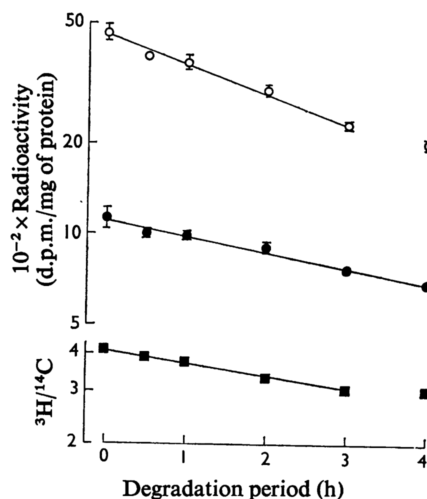

Fig. 8. *Degradation of phosphoenolpyruvate carboxykinase (GTP) in hepatoma cells; type 1 experiment with 6-fluorotryptophan*

This experiment is similar to that reported in Fig. 5 except that 1mM-6-fluorotryptophan replaced canavanine in the appropriate media. Values are means±S.E.M. for data on six Petri dishes at each time-point and are presented as semi-logarithmic plots. ●, ^{14}C-labelled normal enzyme; ○, ^{3}H-labelled 6-fluorotryptophan-containing enzyme; ■, $^{3}H/^{14}C$ ratio.

systems of the cell. Further experiments will be necessary to clarify these differences, and to establish whether degradation of error or aberrant proteins involves a unique protein-removal system that has been evolved to clear the cell of aberrant proteins.

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