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The Relative Stability of Liver Cytosol Enzymes Incubated *in vitro*

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1. Relative rates of enzyme inactivation were measured in liver slices, homogenates and cytosol fractions as well as in the presence of trypsin and at acid pH. The enzymes chosen are all present in the cytosol fraction of rat liver, and have widely different degradation rate constants *in vivo*. 2. The inactivation rates of lactate dehydrogenase, fructose bisphosphate aldolase, glucose 6-phosphate dehydrogenase, glucokinase, phosphoenolpyruvate carboxykinase (GTP), L-serine dehydratase and thymidine kinase in liver preparations at neutral pH are in a similar order to the rate constants of degradation of these enzymes in the intact animal. 3. The two exceptions of this general correlation were tyrosine aminotransferase, which was stable *in vitro* but not *in vivo*, and glyceraldehyde phosphate dehydrogenase, which shows the reverse pattern. 4. These findings generally support the concept that the same factors are responsible for enzyme inactivation *in vitro* as occur in the intact tissue.

The degradation rates of an individual protein can be determined in whole animals by isolating and measuring the radioactivity in the protein at various times after the injection of a radioactive precursor. With this technique it has been shown that many proteins in the cytosol fraction of rat liver are degraded at rates proportional to their tissue content (see Schimke & Doyle, 1970) and thus follow the kinetics of first-order reactions. Since the sequence of reactions that make up intracellular protein degradation cannot readily be investigated *in vivo*, we have attempted to recreate the relevant events *in vitro* (Ballard *et al.*, 1974). These experiments, and studies by other workers (Li & Knox, 1972; Varandani & Shroyer, 1973), suggest that the initial step in protein degradation need not be a proteolytic cleavage, but may be a denaturation reaction, perhaps involving reduction of disulphide bonds.

Half-times of degradation differ markedly from one cytosol protein to another, and range from 12min for ornithine decarboxylase (Russell & Snyder, 1969) to 6 days for lactate dehydrogenase (Kuehl & Sumsion, 1970). In the present report we have examined the rates of inactivation *in vitro* of enzymes with widely different degradation rates in the intact tissue, in an attempt to show whether the regulatory factors that control protein degradation *in vivo* may be demonstrated in less organized tissue preparations.

Materials and Methods

Chemicals

Trypsin (chymotrypsin-inactivated) was obtained from Calbiochem, Los Angeles, Calif., U.S.A.; [2-^{14}C]thymidine and $NaH^{14}CO_3$ were from The Radiochemical Centre, Amersham, Bucks., U.K.; all enzymes and nucleotides required for measurements of enzyme activities were from the Sigma Chemical Co., St. Louis, Mo., U.S.A.

Animals

Fed male rats, aged 7 weeks, were used in all experiments.

Preparation and incubation of liver slices

Livers were removed from animals, cooled to 0°C in ice-cold 0.154M-NaCl, and slices were cut as described by Ballard & Oliver (1964). Portions weighing approx. 200mg were added to flasks containing 3ml of Eagle's Minimal Essential Medium (Eagle, 1959), and incubated with shaking under an atmosphere of O_2+CO_2 (95:5) for various times at 37°C. The pH of the medium was maintained in the range 7.2–7.4 by the addition of 1M-$NaHCO_3$ at various times during the incubation. At the completion of the incubation period the medium and tissue were homogenized together by using a coaxial homogenizer, and the homogenate was centrifuged at 100000*g* and 0°C for 30min to obtain a clear supernatant.

Incubation of liver homogenates

Portions of liver from the same animals used to prepare liver slices were homogenized in 8vol. of Krebs–Ringer phosphate buffer, pH7.4 (Umbreit *et al.*, 1959), containing 0.25M-sucrose, and incubated at 37°C in the presence of 5mM-L-cysteine (adjusted

to pH7.4 with HCl). Portions were taken and centrifuged at 0°C and 100000*g* for 30min to obtain the supernatant.

Incubation of liver cytosol fraction

Homogenates prepared as described in the preceding section were centrifuged at 0°C and 100000*g* for 30min to obtain the cytosol fraction. L-Cysteine (adjusted to pH7.4 with HCl) was added to give a concentration of 5mM, and the solutions were incubated at 37°C. Samples were cooled in ice at the completion of the incubation period.

Inactivation with trypsin

Liver cytosol fractions, prepared as described in the preceding section, were incubated at 37°C with trypsin at a concentration of 30μg/ml. Samples were removed and cooled in ice for the measurement of enzyme activities.

Enzyme-inactivation measurements at pH6.0

Livers were homogenized in 4vol. of 0.25M-sucrose and centrifuged at 0°C and 100000*g* for 30min. The cytosol thus obtained was mixed with an equal volume of 0.1M-potassium phosphate buffer, pH6.0, and incubated at 37°C. Portions of this solution were taken at various times, cooled in ice, the pH adjusted to 7.5 with 1M-Tris, and then centrifuged at 0°C and 100000*g* for 30min to obtain a clear supernatant.

Enzyme measurements

Enzyme activities were measured in the cytosol or supernatant fractions from liver slices, homogenate or cytosol incubations. The following methods were used for enzyme assays at 25°C: lactate dehydrogenase (EC 1.1.1.27) (Kornberg, 1955); glucose 6-phosphate dehydrogenase (EC 1.1.1.49) (Kornberg & Horecker, 1955); fructose bisphosphate aldolase (EC 4.1.2.13) (Blostein & Rutter, 1963); glyceraldehyde phosphate dehydrogenase (EC 1.2.1.12) (Kuehl & Sumsion, 1970); glucokinase (EC 2.7.1.2) (Ballard *et al.*, 1972); and L-serine dehydratase (EC 4.2.1.13) (Ishikawa *et al.*, 1965). Assay methods described for the following enzymes were performed at 37°C: phosphoenolpyruvate carboxykinase (GTP) (EC 4.1.1.32) (Ballard & Hanson, 1969); tyrosine aminotransferase (EC 2.6.1.5) (Granner & Tomkins, 1970); and thymidine kinase (EC 2.7.1.75) (Klemperer & Haynes, 1968). Units of all enzymes except thymidine kinase are defined as μmol of product formed/min, whereas a unit of thymidine kinase activity is 1nmol of TMP formed/min.

Results and Discussion

To demonstrate a correlation between degradation rate constants of enzymes *in vivo* and inactivation rates *in vitro*, it is important that the degradation rate constants for the chosen enzymes cover a wide range. We have measured inactivation of four enzymes that turn over rapidly in the intact liver [tyrosine aminotransferase, thymidine kinase, L-serine dehydratase and phosphoenolpyruvate carboxykinase (GTP)], three enzymes that are relatively stable *in vivo* lactate dehydrogenase, glyceraldehyde phosphate dehydrogenase and fructose bisphosphate aldolase and two enzymes of intermediate stability (glucose 6-phosphate dehydrogenase and glucokinase) (see Table 2). Another important consideration for this study is that the half-lives of the enzymes as determined by different authors should have been measured by comparable techniques. With the exception of glucokinase, glucose 6-phosphate dehydrogenase and thymidine kinase, the degradation rates quoted in Table 2 were determined from the decay of radioactivity from the enzyme pool after an injection of labelled leucine into the animal. Although the half-lives determined by this method may be overestimated owing to reincorporation into the enzyme pool of radioactive leucine that has come from degraded protein, it is unlikely that this problem causes a serious error when relative rates of degradation are compared.

The half-lives of glucokinase and glucose 6-phosphate dehydrogenase given in Table 2 were calculated from the decay of enzyme activity after the removal of an inducing stimulus. These values would certainly be an overestimate, since some enzyme synthesis would occur during de-induction, albeit at rates sufficiently low to permit a net loss of enzyme protein. The half-life of thymidine kinase given in Table 2 was determined by measuring the decrease in activity after protein synthesis has been stopped by injection of cycloheximide into the animal (Bresnick *et al.*, 1967). Since this drug inhibits general proteolysis (Hershko & Tomkins, 1971) as well as the turnover of some specific proteins (Kenney, 1967; Ballard & Hopgood, 1973), it is likely that the half-life of 0.1 day (Table 2) is also an overestimate of the true stability of thymidine kinase.

Enzyme inactivation in liver slices, homogenates and cytosol fractions incubated in vitro

Rates of enzyme inactivation were measured in liver slices, homogenates and cytosol fractions from the same animals. Slices were incubated in a culture medium containing amino acids, vitamins and a balanced salt mixture to preserve, as much as possible, a situation comparable with the intact tissue. In preliminary experiments, it was shown that protein synthesis in liver slices, measured as the incor-

Table 1. *Enzyme inactivation in liver preparations*

Tissue preparations were obtained as described in the Materials and Methods section, incubated at 37°C for the times indicated, and enzyme activities were determined in the resultant cytosol fractions. Values are means from measurements on tissue from three animals.

	Liver preparation	Enzyme activity (units/g of liver) after incubation at 0h	1h	2h	3h	4h	6h
Lactate dehydrogenase	Slice	281	—	293	—	301	281
	Homogenate	506	508	530	534		
	Cytosol	503	512	499	533		
Glyceraldehyde phosphate dehydrogenase	Slice	111	—	101	—	86	76
	Homogenate	174	27	8.7	8.0		
	Cytosol	163	177	154	135		
Fructose bisphosphate aldolase	Slice	4.47	—	4.77	—	4.33	3.93
	Homogenate	5.47	5.47	6.30	5.73		
	Cytosol	4.97	5.03	4.97	5.00		
Glucose 6-phosphate dehydrogenase	Slice	4.03	—	3.45	—	2.92	2.45
	Homogenate	4.68	4.19	3.94	2.19		
	Cytosol	4.78	4.46	4.60	4.45		
Glucokinase	Slice	2.78	—	1.19	—	1.28	1.04
	Homogenate	3.99	2.20	1.10	0.65		
	Cytosol	3.89	3.11	3.04	2.86		
Phosphoenolpyruvate carboxykinase (GTP)	Slice	1.48	—	1.36	—	1.21	1.04
	Homogenate	1.80	1.21	0.91	0.26		
	Cytosol	1.71	1.68	1.73	1.76		
L-Serine dehydratase	Slice	0.51	—	0.37	—	0.39	0.51
	Homogenate	0.34	0.08	0.12	0.15		
	Cytosol	0.44	0.35	0.35	0.27		
Thymidine kinase	Slice	0.75	—	0.25	—	0.19	0.13
	Homogenate	0.64	0.31	0.28	0.19		
	Cytosol	0.92	0.28	0.20	0.16		
Tyrosine aminotransferase	Slice	1.27	—	1.47	—	1.50	1.27
	Homogenate	2.12	2.15	1.93	1.07		
	Cytosol	2.10	2.09	2.27	2.03		

poration rate of [^{14}C]leucine into total protein (Liberti *et al.*, 1971), was linear for the 6h incubation period. Lactate dehydrogenase and tyrosine aminotransferase were stable in the incubated liver slices (Table 1), although an activation of tyrosine aminotransferase was noted at the early time-periods. In the same experiments, glyceraldehyde phosphate dehydrogenase, fructose bisphosphate aldolase, glucose 6-phosphate dehydrogenase, L-serine dehydratase and phosphoenolpyruvate carboxykinase (GTP) lost less than 50% of their initial activities during the 6h incubation, whereas glucokinase and thymidine kinase were rapidly inactivated (Table 1).

To obtain rapid rates of enzyme inactivation in homogenates and cytosol fractions, L-cysteine, an activator of cathepsin B (Otto, 1971), was included in the incubation mixtures. Under these conditions lactate dehydrogenase and fructose bisphosphate aldolase were stable throughout the 3h incubation of homogenates, whereas tyrosine aminotransferase and glucose 6-phosphate dehydrogenase activities were decreased by half (Table 1). Glucokinase, thymidine kinase, L-serine dehydratase, phosphoenolpyruvate carboxykinase (GTP) and especially glyceraldehyde phosphate dehydrogenase were rapidly inactivated in homogenates.

The cytosol was also used for inactivation studies, since this fraction does not contain lysosomes, and it is reported to have a low proteolytic activity (Bohley *et al.*, 1971). Thus it may be possible to distinguish between enzyme inactivation caused by proteolysis and inactivation produced by some other reaction. All the enzymes except L-serine dehydratase were more stable in the cytosol than in homogenates (Table 1), and substantial inactivation was noted only with glucokinase, thymidine kinase and L-serine dehydratase.

Effects of trypsin on enzyme inactivation

The effects of trypsin on enzyme inactivation at a concentration of 30μg/ml of cytosol is shown in Fig. 1. In control experiments under identical conditions but with trypsin omitted from the

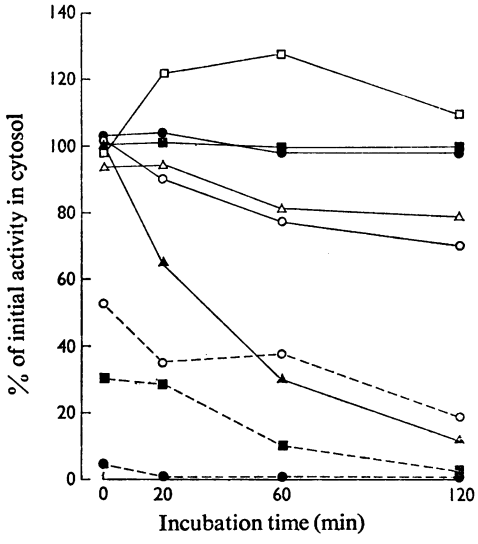

Fig. 1. *Enzyme inactivation by trypsin*

Trypsin was added to a liver cytosol fraction at a concentration of 30μg/ml and the enzyme activities were measured after various times of incubation at 37°C. Values are the means of three separate experiments, and are expressed as the percentage of the enzyme activity in the absence of trypsin. Lactate dehydrogenase (●——●); fructose bisphosphate aldolase (○——○); glyceraldehyde phosphate dehydrogenase (▲——▲); glucose 6-phosphate dehydrogenase (△——△); glucokinase (●----●); phosphoenolpyruvate carboxykinase (GTP) (■——■); L-serine dehydratase (○----○); thymidine kinase (■----■); tyrosine aminotransferase (□——□).

incubation solutions, approximately half of the glucokinase and thymidine kinase activities were lost after 1h. The remaining enzymes were stable under control conditions. With trypsin added, lactate dehydrogenase and phosphoenolpyruvate carboxykinase (GTP) were stable, whereas tyrosine aminotransferase showed some activation, as it did in the control. Glucose 6-phosphate dehydrogenase and fructose bisphosphate aldolase lost between 20 and 30% of their initial activity after incubation for 2h, whereas glyceraldehyde phosphate dehydrogenase, L-serine dehydratase, glucokinase and thymidine kinase were extensively inactivated over the same period. L-Serine dehydratase, thymidine kinase and especially glucokinase were inactivated by trypsin at 0°C during the 2h between addition of trypsin and measurement of the enzyme activities. Further experiments showed that both glucokinase and thymidine kinase were unstable at 0°C at trypsin concentrations as low at 10μg/ml.

Bond (1971) has compared the inactivation rates of lactate dehydrogenase, arginase, catalase, L-serine dehydratase and tyrosine aminotransferase by trypsin and found that the two enzymes with short half-lives *in vivo*, L-serine dehydratase and tyrosine aminotransferase, were the most rapidly inactivated. Although we confirm that L-serine dehydratase is rapidly inactivated by trypsin, the data in Fig. 1 and experiments by Schimke *et al.* (1965) argue that tyrosine aminotransferase is resistant to trypsin. These differences are explained by the high concentration of trypsin used in the experiments reported by Bond (1971), but clearly many enzymes that are relatively more stable *in vivo* than tyrosine aminotransferase are inactivated by trypsin at this concentration.

Effects of incubation at pH 6 on enzyme stability

It has been inferred that degradation of cytosol proteins occurs within lysosomal autophagic vacuoles and that the differential sensitivities of various proteins is a result either of a selective sieving or pumping action at the lysosome membrane, or by different specificities as substrates for lysosomal proteinases (Schimke, 1970; Deter *et al.*, 1967; Coffey & de Duve, 1968; Pontremoli *et al.*, 1973). Since the pH of the lysosome is considered to be acid, it would be expected that stable proteins *in vivo* would be stable at pH6. In Fig. 2, however, it can be seen that neither glyceraldehyde phosphate dehydrogenase nor fructose bisphosphate aldolase are stable at this pH. Further, fructose bisphosphate aldolase is one of the very few enzymes that are inactivated by cathepsin B (Otto, 1971).

Tyrosine aminotransferase, which is rapidly degraded *in vivo* (Kenney, 1967), is quite stable at pH6 (Fig. 1) and is not inactivated by purified lysosomal cathepsin B (Otto, 1971).

General considerations

The rates of degradation of the nine enzymes measured in this study have been rated on the scale – (no inactivation) to +++ (rapid inactivation) for the five incubation conditions (Table 2). With this means of comparison it can be seen that relative degradation rates *in vivo* are not uniformly reflected in the various tissue preparations, since tyrosine aminotransferase, which is the least stable enzyme *in vivo*, is not inactivated to any extent *in vitro*. Tyrosine aminotransferase and L-serine dehydratase, however, are the only enzymes in the group that contain prosthetic groups. It has been argued that the apoenzyme of such enzymes is the form degraded *in vivo* (Kominami *et al.*, 1972), whereas the holoenzyme is relatively stable. If this interpretation is correct, one would predict that tyrosine aminotransferase holoenzyme is stable *in vitro*, a result at variance with measurements of coenzyme dissociability (Litwack & Rosenfield,

1973). Further, the pyridoxal phosphate prosthetic group dissociates more rapidly from tyrosine aminotransferase than from L-serine dehydratase (Litwack & Rosenfield, 1973), although L-serine dehydratase is rapidly inactivated *in vitro* under all conditions we have tested. Coenzyme dissociation *in vitro* may explain the anomalous activity changes for these enzymes *in vitro*. Thus tyrosine aminotransferase activity was found to increase during incubation (Table 1, Fig. 1) and L-serine dehydratase activity often increased after an initial fall (Table 1). Although we can offer no explanation for these changes, comparable effects noted *in vivo* were interpreted as reversible inactivation (Grossman & Boctor, 1972) and did not represent net enzyme synthesis.

The stability of tyrosine aminotransferase in cytosol preparations and at acid pH agrees with the results of Auricchio *et al.* (1972), although we find that the enzyme is somewhat more stable in slices and homogenates than reported in that investigation.

Glyceraldehyde phosphate dehydrogenase is a relatively stable enzyme *in vivo* (Kuehl & Sumsion, 1970), but it is rapidly inactivated in tissue extracts, especially homogenates (Table 1). This instability has been noted previously (Furfine & Velick, 1965), and it was suggested that inactivation was caused by heavy metals (Kuehl & Sumsion, 1970).

With the exception of tyrosine aminotransferase, L-serine dehydratase and glyceraldehyde phosphate dehydrogenase, the enzymes measured do show a correlation between degradation in intact liver and inactivation in slices or homogenates. Thus lactate dehydrogenase and fructose bisphosphate aldolase are relatively stable, glucose 6-phosphate dehydrogenase and phosphoenolpyruvate carboxykinase (GTP) are inactivated slowly, whereas glucokinase and thymidine kinase are rapidly inactivated. Although glucokinase has a longer half-life *in vivo* than phosphoenolpyruvate carboxykinase (GTP) the method used

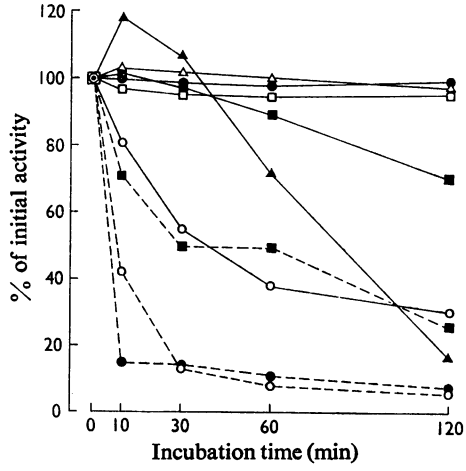

Fig. 2. *Enzyme inactivation at pH6*

Cytosol fractions were mixed with phosphate buffer, pH6.0, as described in the Materials and Methods section and incubated at 37°C for various times. At the completion of the incubation period the mixtures were cooled in ice, the pH was adjusted to 7.5 and the solutions were centrifuged at 100000*g* for 30min. Enzyme activities measured in the clear supernatant are expressed as the percentage of initial activities. Enzymes are indicated by the same symbols as used in Fig. 1. Values are the means of three separate experiments.

Table 2. *Relative rates of enzyme inactivation in vitro as compared with enzyme degradation in vivo*

Enzyme inactivation values from the data in Table 1 and Figs. 1 and 2 were assessed according to the range – (no inactivation) to +++ (rapid inactivation). A symbol in parentheses indicates an initial fall in activity followed by an increase.

	$t_{\frac{1}{2}}$ *in vivo* (days)	Relative inactivation:				
		Slice	Homogenate	Cytosol	Trypsin	pH6
Lactate dehydrogenase	6.0 (Kuehl & Sumsion, 1970)	–	–	–	–	–
Glyceraldehyde phosphate dehydrogenase	5.4 (Kuehl & Sumsion, 1970)	+	+++	+	++	++
Fructose bisphosphate aldolase	4.9 (Kuehl & Sumsion, 1970)	+	–	–	+	++
Glucose 6-phosphate dehydrogenase	Approx. 1 (Freedland, 1968)	+	+	+	+	–
Glucokinase	Approx. 1 (Weber *et al.*, 1966)	++	++	++	+++	+++
Phosphoenolpyruvate carboxykinase (GTP)	0.3 (Hopgood *et al.*, 1973)	+	++	–	–	+
L-Serine dehydratase	0.2 (Pitot & Jost, 1968)	(+)	(+++)	++	+++	+++
Thymidine kinase	Approx. 0.1 (Bresnick *et al.*, 1967)	+++	++	+++	+++	+++
Tyrosine aminotransferase	0.06 (Kenney, 1967)	–	+	–	–	–

to determine glucokinase stability (Weber *et al.*, 1966) depended entirely on activity changes and may substantially overestimate stability. So long as attention is given to the exceptions, the correlation between degradation in the intact animal and inactivation in slices or homogenates should prove useful in resolving the steps and regulatory controls in protein breakdown.

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