Control of cell protein catabolism in rat liver

Effects of starvation and administration of cycloheximide

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1. The loss of liver protein occurring in rats starved for 24 h was largely prevented by the administration of repeated doses of cycloheximide, an inhibitor of protein synthesis. Similar effects were produced on tubulin, a 'fixed' liver protein. 2. Starvation accelerated, whereas cycloheximide markedly lowered, the rate of protein radioactivity decay after labelling with [³H]valine or [¹⁴C]bicarbonate, indicating that changes in catabolic rates played an important role in the above regulations of liver protein mass. 3. The total activity of several lysosomal hydrolases showed little change in livers of starved rats, but a marked progressive decline developed after the administration of cycloheximide, particularly in the activities of cathepsins B, D and L as well as acid ribonuclease. There was no evidence that these changes might be due to endogenous inhibitors (at least for cathepsin B activity, which fell to less than 30% of the control values) or enzyme leakage into the bloodstream; rather, plasma β -galactosidase and β -N-acetylglucosaminidase activities fell progressively during the cycloheximide treatment. 4. Endogenous proteolytic rates, measured *in vitro* by incubating subcellular preparations from livers prelabelled *in vivo* with [³H]valine, were markedly decreased in cycloheximide-treated animals. 5. The osmotic fragility of hepatic lysosomes, appreciably enhanced in starved animals, after cycloheximide treatment was found to be even lower than in fed controls. 6. The present data are consistent with the view that in starved animals the loss of liver protein is mostly accounted for by increased breakdown, due, in part at least, to enhanced autophagocytosis. 7. Cycloheximide largely counteracted these effects of starvation, altering the liver from being 'poised' in a proteolytic direction to a protein-sparing condition. The present data suggest that, besides suppression of the autophagic processes, a decrease in the lysosomal proteolytic enzyme system may also play a role in this regulation, and they seem to provide further circumstantial evidence for the existence of co-ordinating mechanisms between protein synthesis and degradation.

How general cell protein catabolism is controlled is still not comprehensively understood, yet its pivotal role in regulating the protein mass of cells and tissues is widely acknowledged (Garlick *et al.*, 1975; Tanaka & Ichihara, 1977; Hendil, 1977; Scornik & Botbol, 1976; Warburton & Poole, 1977; Amenta *et al.*, 1978b; Otsuka & Moskowitz, 1978). It has been suggested that the regulation of protein synthesis and degradation may be closely co-ordinated (Amenta *et al.*, 1978b; Mayer *et al.*, 1980; Millward *et al.*, 1980). In this regard, a common technique has been to inhibit protein synthesis by the administration of chemical agents (most frequently

cycloheximide) and to monitor the resulting effect on protein catabolism. Apart from the consideration that inhibitors of protein synthesis may also have independent effects on protein degradation (Kovács & Seglen, 1981), results of studies with such inhibitors vary widely, depending on the experimental conditions. There appear to be two main sources of variation: the length of the experimental period and the kind of degradative process involved. In the short-time range (a few hours), the degradation of some individual proteins (see, e.g., Louis-Ferdinand, 1975; Tsurugi & Ogata, 1977; Pegg, 1979) as well as general cell protein catabolism

(Amenta *et al.*, 1977a,b; Hendil, 1977) were little affected, if at all, by inhibitors of protein synthesis; by contrast, these agents promptly suppressed the degradation of some other proteins (Kenney, 1967) or the deprivation-induced acceleration of protein catabolism in cultured cells (Hershko & Tomkins, 1971; Epstein *et al.*, 1975; Amenta *et al.*, 1976a,b, 1977b, 1978a) and in the perfused liver (Woodside, 1976; Khairallah & Mortimore, 1976).

In long-term experiments cycloheximide, although without effect on protein catabolism in growing or resting KB cells (Feldman & Yagil, 1969), markedly slowed down this process in rat embryo fibroblasts (Amenta *et al.*, 1977a, 1978b); a role for lysosomal proteinases in this regulation was suggested by the strict correlation between lowered catabolic rates and decreased enzyme activities. Moreover, cycloheximide was able to inhibit protein degradation in organ-cultured foetal hearts, thereby decreasing their negative nitrogen balance; it has been suggested that a decrease in the lysosomal proteolytic capacity contributed to this effect (Wildenthal & Griffin, 1976).

The present paper examines the effects of cycloheximide on rat liver protein catabolism *in vivo* as well as on lysosomal proteinase activities and on the state of the lysosomal system. The results show that the administration of cycloheximide decreased liver protein catabolism and caused a marked fall in several lysosomal enzyme activities (particularly proteinases and acid ribonuclease), while simultaneously blocking the starvation-induced increase in lysosome fragility. These observations lend support to the view that a co-ordinating mechanism may exist between protein synthesis and degradation (Amenta *et al.*, 1978b) and that alterations in the activities of lysosomal proteinases and/or state of the lysosomal system may be an important factor in this co-ordination.

Experimental

Materials

L-[3,4(n)-³H]Valine (36 Ci/mmol), sodium [¹⁴C]-bicarbonate (58 Ci/mmol) and [*ring-C-methoxy*-³H]-colchicine (5 Ci/mmol) were from The Radiochemical Centre (Amersham, Bucks., U.K.); leupeptin and pepstatin were from Protein Research Foundation (Osaka, Japan); L-leucylglycine was from Fluka (Buchs, Switzerland); GPT Monotest kit was from Boehringer (Mannheim, Federal Republic of Germany); Unisolve 1 was from Koch-Light Laboratories (Colnbrook, Bucks., U.K.); Picofluor 30 was from Packard Instruments International (Zurich, Switzerland); β -glycerophosphate (disodium salt, grade I), *p*-nitrophenyl *N*-acetyl- β -D-glucosaminide, phenolphthalein mono- β -glucuronic acid (disodium salt), RNA (grade VI, from

Torula yeast), bovine haemoglobin (type II), azocasein, α -*N*-benzoyl-DL-arginine *p*-nitroanilide, bovine serum albumin (crystallized), GTP (sodium salt, type I), EGTA and cycloheximide were from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Animals and treatments

Male Wistar rats (Nossan, Milano, Italy), weighing 180–200 g, were maintained on regular laboratory chow (Piccioni, Brescia, Italy) and housed under controlled lighting conditions (dark period from 8:00 p.m. to 8:00 a.m.).

Experiments started at 9:00 a.m. (t_0) and ended 24 h later, with the following design: rats were weighed (initial body weight) and randomly assigned to three groups: (1) fed controls, with free access to food and water; (2) starved animals, with only water *ad libitum*; (3) starved animals treated with cycloheximide, with water *ad libitum* (a fourth experimental group of drug-treated fed animals was precluded since rats soon ceased eating once given cycloheximide). Beginning at t_0 and thereafter at 6 h intervals, rats of group 3 received intraperitoneally four doses of cycloheximide, dissolved in 0.15 M-NaCl, each amounting to 1.1 mg per kg initial body wt., whereas rats of groups 1 and 2 received the saline solution alone (5 ml per kg body wt.). This schedule was adopted to ensure a long-lasting inhibition of protein synthesis by using sublethal doses of cycloheximide. Although extensive inhibition of protein synthesis is rapidly achieved after a single dose of cycloheximide (Yeh & Shils, 1969; Baccino *et al.*, 1977), this effect is limited to a few hours and may be followed by a rebound phenomenon (Ch'ih *et al.*, 1977).

Animals were killed by decapitation, and in most cases the liver was rapidly perfused with 0.15 M-NaCl to wash out the blood. The whole liver was excised, weighed and, unless otherwise specified, homogenized to 10% (w/v) in distilled water with a Polytron 10-ST apparatus (Kinematika, Luzern, Switzerland). To prepare platelet-poor plasma, we collected blood with a syringe from the abdominal aorta of animals under light ether anaesthesia; $\frac{1}{3}$ vol. of 0.135 M-sodium citrate was added as anti-coagulant. Then the blood was centrifuged at 3500 g (r_{av} , 11 cm) for 10 min at room temperature; the resulting supernatant was decanted and further spun at 20000 g (r_{av} , 17.5 cm) for 30 min at 2°C, and the final supernatant was used for enzyme assays.

Analytical procedures

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard. Total and polymerized tubulin were quantified by a [³H]colchicine-binding assay, liver being homogenized in a microtubule-stabilizing buffer (Pipeleers *et al.*, 1977a).

For measurements of protein radioactivity, liver proteins were processed as indicated by Swick & Ip (1974), dissolved in 0.3M-NaOH, and their radioactivities counted in a liquid-scintillation counter with Picofluor 30 as scintillation mixture. For measurements of acid-soluble radioactivity in particle suspensions, we first precipitated protein with 0.27M-HClO₄ for 15 min at 0°C. After centrifugation at 3000g (r_{av} , 18 cm) for 10 min at 2°C, the supernatant was collected, bovine serum albumin was added to it to a final concentration of 0.5 mg/ml, and, after a further 15 min at 0°C, this mixture was again centrifuged as above. The radioactivity of the final supernatant was counted with Unisolve 1 as scintillation mixture.

Enzyme activities were assayed, always in the presence of 0.1% (w/v) Triton X-100, unless otherwise specified, by the following methods: acid phosphatase (EC 3.1.3.2), β -glucuronidase (EC 3.2.1.31) and acid ribonuclease (EC 3.1.27.1) by the methods of de Duve *et al.* (1955), β -N-acetylglucosaminidase (EC 3.2.1.30) by the method of Sellinger *et al.* (1960), β -galactosidase (EC 3.2.1.23) by the method of Baccino *et al.* (1975), cathepsin B (EC 3.4.22.1) by method I of Barrett (1972), cathepsin D (EC 3.4.23.5) by the method of Barrett (1967), cathepsin L (EC 3.4.22.15) by the method of Kirschke *et al.* (1977), leucylglycine dipeptidase (EC 3.4.13.-) by the method of Bouma *et al.* (1976), tyrosine aminotransferase (EC 2.6.1.5) by the method of Diamondstone (1966), and alanine aminotransferase (EC 2.6.1.2) by the method of Wróblewski & LaDue (1956). According to present knowledge (Barrett & McDonald, 1980), the assays for cathepsins B and L cannot be regarded as strictly specific. As to cathepsin L, the assay was based on measurement of the azocasein-splitting activity (Kirschke *et al.*, 1977). We found, however, that most of this activity at pH 6.0 in liver homogenates

is inhibited by pepstatin, which does not affect cathepsin L; moreover, the pepstatin-insensitive activity, amounting to 30–40% of the total, is suppressed by leupeptin, an inhibitor of cysteine proteinases, which include cathepsin L (Kirschke *et al.*, 1977; Riemann *et al.*, 1982). Therefore, to increase its specificity for cathepsin L, the assay was performed in the presence of pepstatin (final concn. 50 μ g/ml), after a 10 min preincubation at 37°C of liver homogenates with this inhibitor.

Presentation of results

Protein and enzyme activity data are expressed as total hepatic content per 100g initial body wt. (recorded at t_0); values for starved and cycloheximide-treated animals are in part presented as percentages of values for fed controls to facilitate comparisons. The rationale for this method of reporting data is discussed elsewhere (Freedland, 1967; Garlick *et al.*, 1975).

Data are presented as means $\pm$ S.D. (or $\pm$ S.E.M. where specified). The significance of the differences was evaluated by analysis of variance.

Results

Total protein and tubulin

Along with a marked decrease in liver weight (Table 1), in 24 h-starved rats there occurred a 28% decrease in the protein mass (ranging from 25 to 30% in different experiments). This value is close to the maximal diurnal excursion observed in rats trained to eat a single 4 h daily meal (Millward *et al.*, 1974). In rats treated with cycloheximide, liver protein decreased by only 12%, demonstrating that cycloheximide by some means inhibited the starvation-induced protein loss.

That these changes in starved and cycloheximide-treated rats primarily reflected modifications

Table 1. *Effects of starvation and the administration of cycloheximide on liver weight, total protein content and tubulin content*

All data are expressed per 100g initial body wt., except for the percentage ratio between polymerized and total tubulin. Values in parentheses are percentages with respect to fed controls. There were ten animals in each group. See the Experimental section for treatments and other details. The significance of the differences was as follows: starved or cycloheximide-treated versus fed: ^a $P < 0.025$, ^b $P < 0.005$; cycloheximide-treated versus starved: ^c $P < 0.025$, ^d $P < 0.005$.

	Fed controls	Starved	Cycloheximide-treated
Wet weight (g)	4.61 $\pm$ 0.41 (100%)	2.73 $\pm$ 0.26 ^b (59.2%)	3.38 $\pm$ 0.24 ^{b,d} (73.3%)
Total protein (mg)	858 $\pm$ 121 (100%)	616 $\pm$ 78 ^b (71.8%)	754 $\pm$ 82 ^d (87.9%)
Total tubulin (μ g)	1402 $\pm$ 199 (100%)	923 $\pm$ 104 ^b (65.8%)	1206 $\pm$ 91 ^{a,d} (86.0%)
Polymerized tubulin (μ g)	359 $\pm$ 70 (100%)	199 $\pm$ 34 ^b (55.4%)	311 $\pm$ 72 ^d (86.6%)
Polymerized tubulin (% of total tubulin)	25.4 $\pm$ 2.05	21.5 $\pm$ 2.74 ^b	25.8 $\pm$ 4.26 ^c

in 'fixed' liver proteins was corroborated by observations on a major slowly-turning-over cell structural protein, namely tubulin (Table 1). The decrease in total tubulin induced by starvation (in agreement with Pipeleers *et al.*, 1977b) and its partial prevention by cycloheximide closely paralleled the findings for total protein, suggesting a common regulation, at least in the present experimental conditions. The ratio of unpolymerized to polymerized tubulin increased in starved animals (cf. Pipeleers *et al.*, 1977b), whereas cycloheximide restored its normal value.

Since cycloheximide at this dosage is known to inhibit protein synthesis extensively (Baccino *et al.*, 1977), it is most probable that a marked restriction in liver protein catabolism is also effected by this agent.

Decline in protein radioactivity

Short-term variations in hepatic protein mass in response to changes in food supply mostly reflect regulation in the rate of protein breakdown (Waterlow *et al.*, 1978, pp. 697–746; Khairallah, 1978, 1981). Our observations are consonant with this view (Table 2). Rats were injected with either [³H]valine or [¹⁴C]bicarbonate 24 h before t_0 ; total liver protein radioactivity was measured at the beginning (t_0) and the end (24 h later) of the experimental period. With this observation window, which takes into account a fairly long-lived component of protein turnover, the apparent degradation rate constant (k_d) measured in fed animals was quite similar with the two labels, 0.272 and 0.317 day⁻¹ respectively. Therefore this component of protein turnover most closely corresponds to the middle (66% of liver protein, $k_d = 0.274$ day⁻¹) of the three arbitrary components obtained by Garlick *et al.* (1976) with [¹⁴C]bicarbonate. In starved rats

the protein degradation rate increased almost 2-fold with both labels; cycloheximide, as inferred previously, inhibited protein degradation in starved animals. A major difference between the two labelling procedures emerged in the cycloheximide-treated animals; with [¹⁴C]bicarbonate the apparent decline in radioactivity decreased to the fed control values, but to well below this value with [³H]valine. The reason for this discrepancy is not clear. Although [¹⁴C]bicarbonate is regarded as a suitable precursor for studying liver protein turnover (Swick & Ip, 1974), results with [³H]valine are probably biased by re-utilization, unless this is prevented by adding an excess of unlabelled amino acid (such as can be done in the perfused liver; Mortimore & Mondon, 1970) or by halting protein synthesis (Khairallah & Mortimore, 1976). In our experiments with [³H]valine, one would expect the k_d in the cycloheximide-treated rats to be approximately equal to the k_d observed in the comparable group that had previously received [¹⁴C]bicarbonate, namely 0.31 day⁻¹. The observed k_d of 0.124 day⁻¹ suggests that even in the presence of cycloheximide significant re-utilization of [³H]valine occurs, a not unreasonable explanation in view of our previous observations that this schedule of cycloheximide treatments effects a less than complete inhibition of protein synthesis (Baccino *et al.*, 1977). The question remains: why should the degree of recycling be so much greater in the cycloheximide-treated animals than in the corresponding fed controls? Although this point was not further investigated, we consider that the observed difference could be accounted for by a greater degree of external recycling of [³H]valine (cf. Felig *et al.*, 1977; Waterlow *et al.*, 1978, pp. 169–170). As an example of a similar phenomenon, a massive transfer of labelled arginine has been suggested to occur from

Table 2. *Effects of starvation and the administration of cycloheximide on decline in liver protein radioactivity*
Either [³H]valine (0.5 mCi/kg body wt.) or sodium [¹⁴C]bicarbonate (0.1 mCi/kg), dissolved in 0.15 M-NaCl (1 ml/kg), was injected into the saphena 24 h before t_0 , under light ether anaesthesia. Total protein radioactivity is expressed as d.p.s. per 100 g initial body wt. Numbers of rats are given in parentheses. The apparent degradation rate constant (k_d) is given in day⁻¹, the apparent half-life ($t_{1/2}$) in days.

	[³ H]Valine			[¹⁴ C]Bicarbonate		
	Radioactivity (d.p.s.)	k_d	$t_{1/2}$	10 × Radioactivity (d.p.s.)	k_d	$t_{1/2}$
At t_0	126 330 ± 10 800 (8)			2776 ± 363 (10)		
At 24 h:						
Fed controls	96 170 ± 7750 (8)	0.272	2.54	2022 ± 421 (10)	0.317	2.19
Starved	74 880 ± 6750 (8)	0.523	1.32	1624 ± 328 (10)	0.536	1.29
Cycloheximide-treated	111 570 ± 7060 (8)	0.124	5.58	2034 ± 331 (6)	0.311	2.23

extrahepatic sources into the regenerating liver in animals given [*guanidino*-¹⁴C]arginine, but not in those receiving [¹⁴C]bicarbonate as precursor (Swick & Ip, 1974).

Lysosomal enzymes

Table 3 shows that in 24h-starved rats there occurred only minor, if any, changes in the total activity per 100g initial body wt. for most of the hydrolases examined and a 22% decrease for cathepsin D. In terms of specific activity, owing to the decrease in total liver protein, there was no change for cathepsin D and a very moderate increase, if any, for the others (cf. Desai, 1969). Thus the acceleration of liver protein catabolism in starved animals did not seem to require any reinforcement of the lysosomal proteolytic enzyme system (at least as determined in whole homogenates).

By contrast, after the administration of cycloheximide (Table 3) all the enzyme activities were markedly decreased, in both absolute (per 100g initial body wt.) and relative terms (per mg of protein; values not presented), except for β -glucuronidase, which showed little change (this is possibly a separate problem, related to the dual location of the enzyme in lysosomes and endoplasmic reticulum; de Duve *et al.*, 1955). The proteolytic activities and acid ribonuclease activity showed the largest decrease; the fall in cathepsin B activity was particularly sharp, reaching 27% of control values. Of note is the observation (Fig. 1) that the decline in hydrolase activities could be detected early (6h was the shortest time interval considered) during the cycloheximide treatment and showed a regular progression until 18–24 h.

Table 3 also includes data on tyrosine amino-transferase activity, which decreased in starved animals (cf. Kato & Saito, 1980), but was considerably increased after the administration of cycloheximide. The paradoxical response to cyclo-

Fig. 1. Time course of liver hydrolase activity changes after the administration of cycloheximide. Cathepsin B (●), cathepsin D (■) and acid phosphatase (○) activities per total liver/100g initial body wt., expressed as percentages of the *t*₀ values, are shown at various experimental time periods. For experimental details see the text. Each point is the mean for six animals; vertical bars denote S.E.M.

Table 3. Effects of starvation and the administration of cycloheximide on liver hydrolase activities

Data are calculated per 100g initial body wt. and are presented as absolute values for fed controls and as percentages of the fed control values for starved and cycloheximide-treated rats. Numbers of animals are given in parentheses. Activity units are nkat, except for cathepsin D (1 unit corresponding to an increase of 1*A*₂₈₀ unit/s in the trichloroacetic acid-soluble phase from a 6ml reaction volume) and cathepsin L (1 unit corresponding to an increase of 1*A*₃₆₆ unit/s in the acid-soluble phase from a 2ml reaction volume). Acid ribonuclease activity is expressed in terms of liberated mononucleotides, assuming an average molar absorption coefficient of 8500 at 260nm (de Duve *et al.*, 1955).

	Activity units (fed controls)	Percentage values	
		24 h-starved	Cycloheximide-treated
Cathepsin B	0.912 ± 0.233 (12)	97.8 ± 9.50 (12)	27.9 ± 8.59 (12)
Cathepsin D (10 ⁴ units)	308 ± 53.8 (14)	78.1 ± 13.1 (14)	51.6 ± 9.71 (14)
Acid ribonuclease	155 ± 31.8 (12)	86.5 ± 12.4 (20)	58.8 ± 7.44 (20)
Cathepsin L (10 ⁵ units)	523 ± 87.2 (14)	84.9 ± 12.0 (12)	60.2 ± 12.1 (12)
Leucylglycine dipeptidase	69.0 ± 6.83 (8)	87.2 ± 28.6 (16)	63.8 ± 15.7 (16)
β-Galactosidase	23.0 ± 2.93 (8)	90.4 ± 20.2 (16)	65.9 ± 9.46 (16)
Acid phosphatase	413 ± 24.8 (10)	89.7 ± 7.20 (14)	66.1 ± 8.07 (16)
β-N-Acetylglucosaminidase	168 ± 47.0 (12)	87.4 ± 18.7 (8)	66.7 ± 11.5 (16)
β-Glucuronidase	33.0 ± 4.68 (8)	87.9 ± 15.2 (16)	88.8 ± 19.2 (16)
Tyrosine aminotransferase	159 ± 47.1 (12)	69.0 ± 13.1 (12)	575 ± 91.9 (12)
Protein (mg)	872 ± 107 (14)	73.4 ± 10.9 (20)	87.4 ± 10.8 (20)

heximide treatment probably reflects, at least in part, an impairment of enzyme degradation (Kenney, 1967; Baccino *et al.*, 1977) and, additionally, a less than total inhibition of translation by our dosage schedule of cycloheximide. In this regard it is worth noting that the degradation of the short-lived tyrosine aminotransferase mRNA is also inhibited by the administration of cycloheximide, as observed on cultured hepatoma cells (Stiles *et al.*, 1976).

Many proteinase inhibitors are known to occur in liver as well as in blood plasma (Laskowski & Kato, 1980); an endogenous thiol-proteinase inhibitor has been recently characterized in rat liver (Kominami *et al.*, 1981). We therefore considered the possibility that such inhibitors may be involved in the decrease in proteinase activities after the administration of cycloheximide, particularly in the case of cathepsin B. As shown in Fig. 2, substrate hydrolysis was a linear function of the homogenate concentration for both control and experimental groups, well beyond

Fig. 2. Measured cathepsin B activity (hydrolysis of α -N-benzoyl-DL-arginine p-nitroanilide) as a function of liver homogenate concentration

The amount of liver homogenate from either control (●) or cycloheximide-treated (○) rats was varied. In combination experiments, a varied amount of homogenate from control (●) or cycloheximide-treated (○) rats was added to a fixed amount of homogenate from cycloheximide-treated or control rats respectively. For experimental details see the text. The final concentration of homogenate in the assay mixture is expressed as equivalents of wet tissue wt./ml. The homogenate concentration used in the routine assay is indicated with an asterisk (*).

the concentration used in our routine assay conditions. Moreover, the activity was quantitatively recovered on combining homogenates from the two groups in various proportions. Both sets of findings thus seem to rule out the possibility mentioned above.

Increased secretion or leakage into the blood was also considered as a possible explanation for the decrease in hydrolase activities in the liver. Cycloheximide has indeed been reported to enhance the release of cathepsin D from cultured limb-bone rudiments into the medium (Hille *et al.*, 1970), although no such an effect could be detected with rat embryo fibroblasts (Amenta *et al.*, 1978b); moreover, the release of cathepsin D from organ-cultured hearts was instead inhibited by cycloheximide (Wildenthal & Griffin, 1976). Fig. 3 shows the blood plasma activity of two lysosomal hydrolases in cycloheximide-treated rats, compared with alanine aminotransferase. For the latter reference enzyme, activity more than doubled over the experimental period, indicating some degree of injury to the liver cells (cf. Ch'ih *et al.*, 1976); however, β -galactosidase and β -N-acetylglucosaminidase activities showed the opposite change, falling to 25–30% of the initial values. These results argue against a loss of lysosomal hydrolases into the plasma in cycloheximide-treated animals. Moreover, the minimal change in aminotransferase activity (in absolute values) along with the decrease in lysosomal enzyme activities in the plasma indicate that no significant cell death occurred during the experimental period (cf. Zuretti & Baccino, 1976), in agreement with ultrastructural observations (F. M. Baccino & D. Cantino, unpublished work).

Endogenous proteolysis

Since only a proportion of tissue proteinases are lysosomal, we also determined whether the general proteolytic potential towards endogenous substrates changed in the liver after starvation and the administration of cycloheximide. For this purpose, liver proteins were labelled *in vivo* with [3 H]valine; the total particulate fraction of liver homogenates (Mortimore *et al.*, 1973) was then incubated *in vitro* under different conditions of pH (acidic or neutral) and particle integrity (with or without Triton X-100), and the release of perchloric acid-soluble radioactivity was measured after different time periods. Proteolytic rates (Fig. 4) were clearly higher at pH 5.0 than at pH 7.4 and were markedly stimulated by Triton X-100, reflecting the unrestricted accessibility of substrates to proteolytic enzymes, particularly lysosomal cathepsins. It is probable that the acceleration of proteolytic rates at pH 5 in the absence of Triton X-100 is also due to progressive loss of compartmentation of the lysosomal enzymes (Mortimore & Ward, 1981). Con-

Fig. 3. Effect of the administration of cycloheximide on blood plasma enzyme activities β -N-Acetylglucosaminidase (○), β -galactosidase (□) and alanine aminotransferase (●) activities in plasma are shown at various experimental time periods. For experimental details see the text. Each point corresponds to the mean for four rats, except for t_0 values (six animals); vertical bars denote S.E.M.

Table 4. Effects of starvation and the administration of cycloheximide on lysosome osmotic fragility

Livers were homogenized to 10% (w/v) in cold 0.25 M-sucrose with a Potter-type homogenizer. The free activity of β -N-acetylglucosaminidase was evaluated directly in these homogenates (initial free activity) or after hypo-osmotic shock in 0.15 M-sucrose for 30 min at 0°C (Zuretti & Baccino, 1976), and is expressed as percentage of total activity, assayed in the presence of 0.1% (w/v) Triton X-100. There were eight animals in each group. Significance of the differences was as follows: starved or cycloheximide-treated versus fed: $^aP < 0.05$, $^bP < 0.001$; cycloheximide-treated versus starved: $^cP < 0.05$, $^dP < 0.001$.

	Initial free activity	Hypo-osmotic shock	Difference
Fed controls	9.7 ± 1.34	24.6 ± 2.45	$+15.0 \pm 2.77$
Starved	11.3 ± 1.49^a	33.9 ± 6.86^b	$+22.7 \pm 5.57^b$
Cycloheximide-treated	9.7 ± 1.12^c	$20.5 \pm 2.02^{b,d}$	$+10.8 \pm 2.34^{b,d}$

sistent with this view, the free activity of β -N-acetylglucosaminidase indeed increased from an initial 15% to approx. 60% of total activity by the end of the 60 min incubation, with only minor differences among the three groups (results not shown). The endogenous proteolytic rates at pH 5 were somewhat higher for starved than for fed rats. Moreover, under all assay conditions the proteolytic rates were markedly lower with preparations from cycloheximide-treated rats, the largest difference occurring at pH 5 in the absence of Triton X-100.

Lysosome fragility

Further information on the state of the lysosomal system in the cycloheximide-treated rats was obtained by measuring the fractional free activity of β -N-acetylglucosaminidase in liver homogenates either osmotically protected or submitted to hypo-

osmotic shock (Baccino & Zuretti, 1975; Zuretti & Baccino, 1976). Lysosome fragility was augmented by starvation (difference column in Table 4), whereas the administration of cycloheximide decreased lysosomal fragility to below the control values seen in fed animals. Since the osmotic fragility mainly reflects the size distribution of particles (Deter & de Duve, 1967), these findings suggest an increase in lysosomal size in starved rats and a decrease in size in cycloheximide-treated rat livers. In agreement with this conclusion, the results of morphometric investigations (Cantino *et al.*, 1979; F. M. Baccino & D. Cantino, unpublished work) showed that autophagic vacuoles increased in number in starved animals and virtually disappeared after the administration of cycloheximide. Also, the size distribution of dense bodies shifted towards lower values in the cycloheximide-treated group.

Fig. 4. *Effect of the administration of cycloheximide on endogenous proteolysis in rat liver cell-free preparations*
The general experimental protocol was as in Table 2, rats being given [^3H]valine intravenously 48 h before being killed. Livers were homogenized to 10% (w/v) in ice-cold 0.25 M-sucrose containing 1 mM-EDTA, pH 7.4, with a Potter homogenizer. After centrifugation at 2°C up to an integrated field-time of 6×10^6 g·min (r_{av} 5.4 cm), the pellets were gently suspended in 0.25 M-sucrose; these suspensions were incubated at 37°C under vigorous shaking in the presence of 5 mM-L-valine and 50 mM-buffer, either sodium acetate, pH 5.0 (a and b) or sodium phosphate, pH 7.4 (c and d), with (b and d) or without (a and c) 0.1% (w/v) Triton X-100; the final concentration of these cytosol-free preparations was as in the original homogenate. At the times shown, perchloric acid was added to a final 0.27 M concentration, the radioactivity of the acid-soluble phase then being counted. For full experimental details see the text. The net release of acid-soluble radioactivity was expressed as a percentage of the initial protein-bound radioactivity (on the ordinate; note the 5-fold difference in scale between upper and lower panels). Symbols: □, fed controls; ○, 24 h-starved rats; △, cycloheximide-treated rats. Each point corresponds to the mean for 12 animals, vertical bars denoting S.E.M.

Discussion

Quite exceptionally among animal tissues, the liver is subjected to pronounced circadian fluctuations of protein mass, probably related to its central homeostatic role in metabolism. These fluctuations, which depend on the feeding state, are dramatized in rats trained to eat a single 3–4 h meal per day (Waterlow *et al.*, 1978, pp. 698–708; Khairallah, 1978, 1981) or starved for 24 h (Garlick

et al., 1975), such as in the present work. Khairallah (1978, 1981) has pinpointed the modulation of protein turnover peculiar to this 'early starvation phase' (Goodman *et al.*, 1980); between the beginning and the end of a 3 h feeding in meal-fed rats, he was able to demonstrate a 4-fold suppression of liver protein breakdown, related to changes in autophagocytic rate. In the present experiments, 24 h-starved rats showed a 25–30% decrease in total liver protein. As shown by measurements on slow-

turnover proteins, which form the bulk of tissue protein (Garlick *et al.*, 1976), the apparent degradation rate over the 24h period almost doubled in starved compared with fed animals. By simple calculation, based on data obtained with [^{14}C]-bicarbonate (Table 2), this change can be shown to account for most of the protein loss (k_s , k_d and k_g are the synthesis, degradation and growth rate constants):

$$k_s = k_d = 0.32 \text{ day}^{-1} \quad (1)$$

in fed rats, assuming steady state ($k_g = 0$), and

$$k_g = k_s - k_d = 0.32 - 0.54 = -0.22 \text{ day}^{-1} \quad (2)$$

in starved rats.

In eqn. (2) k_s has the value found in fed animals, although it is known to be slightly decreased over a 24h starvation (Garlick *et al.*, 1975). Furthermore, in eqn. (2) [and eqn. (3) below] k_g has the value observed for total protein, rather than that for slow-turnover proteins, as in the case of k_d . We assume that such a value of k_g may be valid for slow-turnover proteins too; as a first approximation, this seems justified by considering that data for tubulin, taken as an individual paradigm for 'fixed' slow-turnover proteins, seem to fit well with those for total protein (Table 1). In fact, the hepatic content of total tubulin paralleled the fall in total protein content in starved rats; moreover, the proportion of unpolymerized tubulin was increased, which may be indicative of a higher rate of tubulin breakdown (Chafouleas *et al.*, 1981).

Compatible with the view that hyperautophagia develops in the early starvation phase (Khairallah, 1978, 1981; Pfeifer & Strauss, 1981) is the present finding that the state of the lysosomal system was modified in starved rats, as indicated by the enhanced response to the osmotic-fragility test (cf. Deter & de Duve, 1967). With regard to the observation that in starved rats the total activity of lysosomal enzymes per liver generally showed little change (though in most cases specific activities were slightly increased), it is worth noting that regulation of autophagocytosis as such does not seem to require quantitative changes in the proteolytic enzyme system of cells, as both suggested by its rapid activation or suppression (Neely *et al.*, 1974; Amenta *et al.*, 1976a, 1977b, 1978a; Pfeifer, 1978; Mortimore & Ward, 1981) and indicated by measurements of enzyme activities in cells and tissues (Deter & de Duve, 1967; Amenta *et al.*, 1978a).

The paradoxical protection of the liver protein mass afforded by cycloheximide, an inhibitor of protein synthesis, against the starvation-induced protein loss can be attributed to the fact that protein degradation was maintained at a low rate. The same degree of protection was afforded in the case of total

tubulin; in addition, the proportion of polymerized tubulin was restored to normal values, which may be indicative of decreased tubulin degradation (Chafouleas *et al.*, 1981). Consistently, all the present data indicate that cycloheximide altered the liver from being 'poised' in a proteolytic direction to a protein-sparing condition. A number of mechanisms appeared to be operative in achieving this state: (i) the osmotic fragility of lysosomes was maintained at a low level; (ii) rates of endogenous proteolysis were lowered in the total particulate fraction of liver homogenates, (a) either intact or (b) lysed by Triton X-100; (iii) the activity of lysosomal proteinases was definitely lowered in terms of both total hepatic activity and specific activity.

The observations based on intact lysosomes [(i) and (ii)(a) above] as well as morphometric data (Cantino *et al.*, 1979; F. M. Baccino & D. Cantino, unpublished work) indicate that starvation-related autophagocytosis was suppressed in cycloheximide-treated animals. This inference fits with the well-documented ability of cycloheximide in short-term experiments to inhibit the hyperautophagic proteolytic response elicited by serum deprivation in cultured cells (Amenta *et al.*, 1976a, 1977b, 1978b; Hendil, 1977), by isolation procedures in dissociated hepatocytes (Seglen *et al.*, 1981) and by perfusion (Woodside, 1976) or glucagon (Rumpelt & Weissbach, 1978; Zuretti *et al.*, 1980, 1982) in the liver [although cycloheximide failed to prevent this glucagon effect in hepatocyte monolayers (Hopgood *et al.*, 1980) or in the perfused liver (Woodside *et al.*, 1974)].

On the other hand, the observations based on lysed lysosomes [(ii)(b) above] and proteinase activities [(iii) above] point to lowered activity of the proteolytic enzymes of cells as an additional mechanism accounting for the cycloheximide effect. However, the present data do not rule out the possibility that the fall in proteinase activities is merely coincidental and may not be directly related to the decrease in protein catabolism. Although undoubtedly attractive, the hypothesis that lysosomal proteinase activity may be one of the limiting factors controlling protein catabolism (Wildenthal & Griffin, 1976; Amenta *et al.*, 1977a, 1978b; Lockwood & Shier, 1977; DeMartino & Goldberg, 1978; Millward *et al.*, 1980; Hopgood & Ballard, 1980; Baccino *et al.*, 1981) must be accepted with reservations.

Of interest, at any rate, is the observation that cycloheximide, although stabilizing total protein and tubulin content, caused a decrease in many lysosomal hydrolase activities, particularly proteinases and acid ribonuclease. Our data do not indicate that activities were decreased either by inhibitors or by enzyme loss from liver cells into the blood. Increased bulk exocytic discharge into bile seems

unlikely, in that the daily biliary output of three lysosomal hydrolases has been estimated to average 3% of their content in the liver (LaRusso & Fowler, 1979). Therefore inhibition of synthesis is in all likelihood the main mechanism leading to decreased hydrolase activities after the administration of cycloheximide, suggesting that inactivation (if not degradation) of lysosomal enzymes is regulated differently from general cell protein catabolism (in agreement with Amenta *et al.*, 1978b). In this regard, it is worth noting that protein synthesis was not totally inhibited in cycloheximide-treated rat livers, as both inferred from the marked increase in tyrosine aminotransferase activity (Table 3) and suggested by protein-turnover data after labelling with [^{14}C]bicarbonate; indeed, by calculation as above:

$$k_s = k_g + k_d = -0.12 + 0.31 = 0.19 \text{ day}^{-1} \quad (3)$$

in cycloheximide-treated rats [a similar calculation based on [^3H]valine data gives $k_s = 0$, which seems unlikely (Yeh & Shils, 1969; Baccino *et al.*, 1977)]. As a final consideration, the present results suggest, though do not prove, that the turnover of some lysosomal enzymes such as acid ribonuclease and proteinases, particularly cathepsin B, may be faster than currently supposed (cf. Bohley *et al.*, 1979).

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