Effect of a Nutritional Shift on the Degradation of Abnormal Proteins in the Mouse Liver

DECREASED DEGRADATION DURING RAPID LIVER GROWTH

By RICARDO AMILS,* RUBEN D. CONDE† and OSCAR A. SCORNIK
Department of Biochemistry, Dartmouth Medical School, Hanover, NH 03755, U.S.A.

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1. The intravenous injection of puromycin to mice 0.5 min after administration of radioactive leucine resulted in the release of labelled ribosome-bound nascent protein chains within the next 0.5 min. 2. During the subsequent 13 min, 40% of the liver protein radioactivity disappeared. The rate of this process was already maximal 0.5 min after the injection of puromycin, with no apparent lag. 3. Evidence is presented that this phenomenon represents the selective degradation of puromycinyl-peptides: (a) the magnitude of this fraction corresponded to the calculated proportion of protein radioactivity in nascent chains at the time of the puromycin effect; (b) the size distribution of the proteins disappearing between 2 and 14 min was smaller than that of those retained at 14 min; and (c) when the injection of puromycin was delayed for 5 min, or when the leucine pulse was interrupted by the injection of cycloheximide (rather than puromycin), the fraction disappearing within 14 min was much smaller. 4. The degradation of puromycinyl-peptides was much slower in the rapidly growing livers of animals recovering from a protein depletion than in the protein-depleted controls. It is concluded that the large decrease in the overall rates of total liver protein degradation previously described during liver growth is a general phenomenon, also affecting the rate of scavenging of abnormal proteins.

A detailed study of the synthesis, export and degradation of total protein during liver growth after a partial hepatectomy (Scornik, 1974*a,b*; Scornik & Botbol, 1976) or after a nutritional shift (Conde & Scornik, 1976) convinced us that the net protein gain in these situations is due predominantly to a decreased rate of protein degradation.

In many cases changes in the rate of degradation of individual proteins can determine their concentration in the cell (Schimke, 1970). It can be argued that decreased overall rates of protein degradation during liver growth represent the sum of specific regulatory effects on the cellular content of each individual protein, rather than a general effect on the degradation of all proteins. Abnormal proteins, produced in cells by mutation (Goldschmidt, 1970; Platt *et al.*, 1970; Lin & Zabin, 1972; Adams *et al.*, 1972; Capecchi *et al.*, 1974; Kuehl & Scharff, 1974),

amino acid analogues (Rabinovitz & Fisher, 1964; Goldberg, 1972; Kanai & Sugimura, 1974; Knowles *et al.*, 1975; Bradley *et al.*, 1976; Prouty, 1976), or the premature release of nascent chains by puromycin (Morris *et al.*, 1963; Pine, 1967; Baglioni *et al.*, 1969; Goldberg, 1972; Kemshead & Hipkiss, 1974; McIlhinney & Hogan, 1974; Hendil, 1976) are known to be rapidly degraded *in vivo*. This degradation is essentially a scavenging process, and it seems unlikely that its rate should be under a specific control related to cell growth. For this reason, we became interested in the degradation of puromycinyl-peptides in livers of protein-depleted and re-fed animals, conditions in which we have observed the largest difference in the overall rate of degradation of total liver proteins (Conde & Scornik, 1976).

In the present paper we offer evidence that puromycinyl-peptides are degraded during rapid liver growth in re-fed mice at a much slower rate than in the liver of normal and protein-depleted animals, a finding that supports the view that liver growth is accompanied by a general decrease in the rate of degradation of liver proteins. The data also suggest that the mechanism degrading abnormal proteins may

* Present address: Department of Chemistry, Columbia University, New York, NY 10027, U.S.A.

† Present address: Departamento de Química Biológica, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Buenos Aires, Argentina.

be the same one that degrades all or most liver proteins.

Experimental

Diets

Purina Rat Chow (pellets) containing 23% (w/w) protein was obtained from Purina Foods Co., St. Louis, MO, U.S.A. The Protein Depletion Diet, U.S.P. XV (powder), was purchased from ICN Pharmaceuticals, Cleveland, OH, U.S.A.

Chemicals

L-[1-¹⁴C]Leucine (53 μ Ci/ μ mol) and L-[4,5-³H]-leucine (5mCi/ μ mol) were obtained from New England Nuclear Corp., Boston, MA, U.S.A. Non-radioactive L-leucine was purchased from Calbiochem, San Diego, CA, U.S.A. Puromycin and cycloheximide were bought from Sigma Chemical Co., St. Louis, MO, U.S.A. Evan's Blue was purchased from J. T. Baker Chemical Co., Phillipsburg, NJ, U.S.A. Blue Dextran 2000 and Sephadex-G-200, particle size 40–120 μ m, were obtained from Pharmacia Inc., Piscataway, NJ, U.S.A. Protein standards and all other reagents were of the highest purity available.

Animals

CD-1 mice (male, 6 weeks old), were purchased from Charles River Breeding Laboratories, Wilmington, MA, U.S.A. After arrival the animals were housed, five per cage, in a 25°C room illuminated from 06:00 to 18:00h, and fed with Purina Rat Chow and water *ad libitum*. They were kept for a minimum of 1 week under these conditions. Protein deprivation and re-feeding with the normal diet were carried out as described previously (Conde & Scornik, 1976). Re-feeding was permitted for 16h.

Analytical procedures

Many of the experiments described in the present paper required very short intervals between injections and collection of the tissue. The livers were immersed in iced water 5 ± 1 s after decapitation of the animals. The procedure by which livers were collected has been examined in detail in previous publications (Scornik, 1974a,b). The determination of RNA and the measurement of the protein radioactivity in liver homogenates and ribosome-bound nascent protein chains were carried out as previously reported (Scornik, 1974a). Plasma volume was calculated by the Evan's-Blue method (Furth & Sobel, 1946) as previously described (Scornik & Botbol, 1976). The average value was 5.0 ± 0.1 ml/100g body wt. in normal mice, and 6.8 ± 0.1 ml/100g body wt. in protein-depleted animals; in re-fed mice it was the same, expressed per weight of the animals at the time

of re-feeding. Total plasma protein radioactivity was determined as described by Scornik & Botbol (1976).

Injected solutions

L-[1-¹⁴C]Leucine was injected in either trace or massive amounts. In the first case, the radioactive amino acid was diluted to 5.5 μ Ci/ml in 0.9% (w/v) NaCl containing 0.2% (w/v) Evan's Blue, and adjusted to pH 7.4 with NaOH. The amount of solution injected in the tail vein was 0.2ml (unless otherwise indicated). In the second case, a solution of 3.4 μ Ci/ml (specific radioactivity 0.048 μ Ci/ μ mol) was prepared by mixing sufficient amounts of radioactive and non-radioactive leucine in 0.9% NaCl containing 0.2% Evan's Blue, and the pH was adjusted to 7.4; 1ml of this solution was used intravenously. L-[4,5-³H]Leucine was prepared with a specific radioactivity of 58 μ Ci/ml, and 0.5ml was injected. Puromycin was diluted to 5mg/ml in 0.9% NaCl and the pH was adjusted to 7.0. The amount injected per mouse was 0.2ml, intravenously. Cycloheximide (10mg/ml) was prepared in 0.9% NaCl at pH 7.0; 0.3ml per mouse was injected intraperitoneally or intravenously, as indicated in each case.

Size distribution of liver radioactive proteins

Extracts of radioactive proteins were prepared and analysed by chromatography at 20°C in a column (16.5cm $\times$ 1.35cm) of Sephadex G-200 equilibrated with 0.1M-sodium phosphate buffer (pH 7)/0.05M-NaCl/0.1% 2-mercaptoethanol/0.01% sodium dodecyl sulphate/6M-urea/0.1% thymol. Livers were homogenized in 9vol. of 0.3M-sucrose and centrifuged for 10min at 900g to remove nuclei and cell debris. A portion of the supernatant (5ml) was mixed with 1ml of 10% (w/v) sodium deoxycholate and centrifuged for 90min at 150000g in a Spinco ultracentrifuge at 4°C. The pellet was discarded, and 2ml of supernatant was mixed with 56mg of solid sodium dodecyl sulphate and 720mg of solid urea. The samples were then heated for 30min at 100°C and left at room temperature for 3 days. Before chromatograph they were dialysed for 1h at room temperature against the column buffer. The ³H- and ¹⁴C-labelled samples were mixed in equal proportions; 0.25ml of this pool was mixed with 25 μ l of glycerol and 50 μ l of a solution of Blue Dextran (20mg/ml) and applied to the column; 1.5ml fractions were collected. Blue Dextran was measured by its absorption at 625nm; 0.1ml of a non-radioactive 1:3 (w/v) liver homogenate was then added, and protein was precipitated with 40% (w/v) trichloroacetic acid added to give a final concentration of 10% (w/v). The usual procedure for determination of liver protein radioactivity was followed (Scornik, 1974a). The position of the protein standards was determined separately under the same conditions.

Results and Discussion

Degradation of puromycinyl-peptides in the livers of normal animals

Puromycin reacts both *in vivo* and *in vitro* with nascent peptidyl-tRNA bound in the ribosomal P site, to release puromycinyl-peptides (Allen & Zamecnik, 1962). Puromycinyl-peptides are degraded very rapidly *in vivo*. This has repeatedly been shown in bacteria (Pine, 1967; Goldberg, 1972; Kemshead & Hipkiss, 1974) and cultured mammalian cells (Morris *et al.*, 1963; Baglioni *et al.*, 1969; McIlhinney & Hogan, 1974; Hendil, 1976). We permitted the incorporation of radioactive leucine for 30s, a time when more than half of the protein radioactivity was still in ribosome-bound nascent protein chains (Scornik, 1974a) and then injected unlabelled puromycin. The release of nascent chains from the ribosomes *in vivo* was essentially completed 30s after the injection of puromycin (Fig. 1). To study the fate of the resulting material it was necessary to prevent continuing incorporation of the radioactive leucine; we used cycloheximide for this purpose. Puromycin itself permitted an incorporation at more than 20% of the control rate. Cycloheximide is a more powerful inhibitor; its full effect is instantaneous when injected

intravenously and it becomes effective within 30s when administered intraperitoneally. The combination of puromycin and cycloheximide decreased incorporation of the tracer to negligible values (Fig. 2a).

In normal animals, 30% of the newly synthesized protein in the liver is exported as plasma proteins (Scornik & Botbol, 1976). We measured total plasma-protein radioactivity in every case and added the observed value to the total liver-protein radioactivity (see legends to Fig. 2 and Fig. 4 below). In most experiments, however, we limited our observation to a period of 14min, within which labelling of the plasma proteins was still negligible (Figs. 2a, 2b and 2c and Fig. 4a below). Only in one case, when the measurements were made 60min after the tracer injection, was the total plasma-protein radioactivity considerable (Fig. 4a below).

The injection of puromycin shortly after the tracer resulted in the rapid disappearance of a substantial fraction of liver protein radioactivity. This was determined in normal mice (i.e. mice fed with a normal diet throughout) in two experiments. In the first experiment (Fig. 2a) the protein was labelled by injecting a trace amount of radioactive leucine, followed 0.5 min later by puromycin. Cycloheximide was injected 1.5 min after puromycin. The total liver-protein radioactivity was measured 2, 3, 8 and 14 min after the tracer injection. In the second experiment the initial events were examined in greater detail (Fig. 2a); the animals were killed 1, 2 and 3 min after injection of the radioactive leucine. Because only a relatively small fraction of the total radioactivity disappeared in this short time, we sought to improve the accuracy of the measurements by injecting a massive rather than a trace amount of leucine, and by adjusting the results to the RNA content of each liver. The amount of precursor was sufficient to expand the pool of free leucine to the point where endogenous sources became negligible. In doing this, the accuracy of the measurement was improved because we avoided individual variations in the specific radioactivity of the precursor pool. It has been shown previously that this procedure does not interfere with liver protein synthesis (Scornik, 1974a). In this experiment the s.e.m. for each group of ten animals was approx. 4% of the average, whereas when trace amounts of leucine were injected it was approx. 8%.

In Fig. 2(d), the results of both experiments are expressed as percentages of the initial value and as a function of the time lapse after the injection of puromycin (after a small correction for plasma-protein radioactivity and the continuing incorporation of leucine, as explained above); 40% of the protein radioactivity disappeared within 14 min without any appreciable lag. We believe that this phenomenon

Fig. 1. Release of ribosome-bound nascent protein chains by puromycin

Normal (○), protein-depleted (▲) and re-fed (■) mice received an intravenous injection of a trace amount of L-[1-¹⁴C]leucine. Then 30s later (zero time) some animals were killed, whereas the others received a second injection of puromycin and were killed 15 or 30s after this second injection. The radioactivity of the ribosome-bound nascent protein chains was determined as described previously (Scornik, 1974a), calculated as d.p.m. per mg of rRNA, as a percentage of the value at zero time under each condition. Each point represents the result from a group of three animals.

Fig. 2. Rapid disappearance of liver-protein radioactivity after the injection of puromycin into normal mice

Total liver-protein radioactivity and total plasma-protein radioactivity were determined as described in the Experimental section. Each point represents the average result for a group of mice (number of animals is indicated in parentheses). The bars indicate S.E.M. (a) In one experiment the mice received an intravenous injection of a trace amount of L-[1-¹⁴C]leucine (at zero time) followed by puromycin 0.5 min later. Cycloheximide was injected intraperitoneally 2 min after the tracer injection. ●, Total liver protein radioactivity; ▲, total plasma-protein radioactivity. In a parallel control experiment (□), puromycin and cycloheximide were injected 2 min and 0.5 min respectively before the tracer injection. All values are expressed as total d.p.m. (left ordinate). In a second experiment, a massive amount of L-[1-¹⁴C]leucine was injected at zero time, followed by puromycin 0.5 min later. Cycloheximide was injected intraperitoneally 1 min after the tracer injection. Total liver-protein radioactivity (○) is expressed as d.p.m./mg of RNA (right ordinate). (b) At zero time the mice received the intravenous injection of a trace amount of L-[1-¹⁴C]leucine. Cycloheximide was injected intravenously 0.5 min later. ▲, Total liver-protein radioactivity; ○, total plasma-protein radioactivity. In a parallel control experiment, cycloheximide was injected together with the tracer and the total liver-protein radioactivity determined at various times thereafter (△). (c) At zero time the mice received a trace amount of L-[1-¹⁴C]leucine followed by puromycin 5.5 min later. Cycloheximide was injected intraperitoneally 7 min after the tracer injection. ■, Total liver-protein radioactivity; ●, total plasma-protein radioactivity. □, same control experiment as in (a). (d) In each of the experiments described in (a)–(c), the values in the control experiment were subtracted from, and those of the plasma-protein radioactivity were added to, the corresponding values of liver protein radioactivity. In each case the result was expressed as percentage of the actual or extrapolated value at 0.5 min after injection of puromycin (○, ●, ■) or cycloheximide (▲). The symbols are the same as those used to represent the liver-protein radioactivity in the corresponding experiments in (a)–(c).

represented the selective degradation of puromycinyl-peptides for the following reasons.

(1) The magnitude of the disappearing fraction was in accordance with our expectations. The release of nascent chains from the ribosomes is half completed 15s after the injection of puromycin (Fig. 1), or 45s after the injection of the radioactive leucine, the moment at which nascent chains constitute 45% of the total liver-protein radioactivity, as shown previously (Scornik, 1974a).

(2) In a control experiment puromycin was injected 5.5 min after the tracer, at which time more than 90% of the radioactive proteins had been completed and released from the ribosomes (Scornik, 1974a). In this case the fraction of the total protein radioactivity which disappeared in the subsequent 14 min was much smaller (Figs. 2c and 2d).

(3) In another control experiment the radioactive pulse was interrupted after 0.5 min by the intravenous injection of cycloheximide, and puromycin was omitted (Fig. 2b). Cycloheximide alone was not as effective in abolishing continued incorporation of the precursor as when it was combined with puromycin. Although the residual incorporation was only about 1% of the control, after 14 min it amounted to one-third of the initial value, which represented a 30s pulse (Fig. 2b). The total protein radioactivity was corrected for this incorporation, as shown in Fig. 2(d). Most of the protein radioactivity was retained in the liver.

(4) Because puromycinyl-peptides are released from the ribosomes prematurely, their size distribution is smaller than that of the normal newly synthesized proteins. Thus if the disappearance of protein radioactivity represents the selective degradation of puromycinyl-peptides, one should expect an increase in the size distribution of the remaining (normal) proteins. This was found to be the case in the experiment shown in Fig. 3. Liver proteins were labelled with either [^3H]leucine or [^{14}C]leucine, followed by puromycin and cycloheximide in the same way as described for the experiment in Fig. 2(a). The ^3H -labelled animals were killed 2 min after the tracer injection, and the ^{14}C -labelled animals were killed 12 min later. Extracts from their livers were mixed and analysed by gel filtration in the presence of urea and sodium dodecyl sulphate as shown in Fig. 3. The selective degradation of low-molecular-weight proteins between 2 and 14 min is expressed as a lower $^{14}\text{C}/^3\text{H}$ ratio at the right-hand side of the chromatogram, a difference that was not apparent in the control (Fig. 3b). It can also be shown by calculating the size distribution of the difference between both labels (Fig. 3a). The ^{14}C -labelled proteins (representing stable components remaining in the liver after 14 min) had an average mol.wt. of 40000. The difference between ^3H - and ^{14}C -labelled proteins (representing

Fig. 3. Size distribution of liver proteins 1.5 and 13.5 min after injection of puromycin

Normal mice received the intravenous injection of either L-[4,5- ^3H]leucine (▲) or L-[1- ^{14}C]leucine (●), followed by puromycin 0.5 min later. Two animals injected with [^3H]leucine were killed 2 min after the tracer injection. Another two, injected with [^{14}C]leucine, were killed 12 min later. Extracts from each of the four livers were mixed, and the size distribution of the labelled proteins was analysed by gel filtration in Sephadex G-200, as described in the Experimental section. (a) For comparison, the total ^3H radioactivity (2678 d.p.m.) was assigned the value of 1.0 (left ordinate). The total ^{14}C radioactivity (610 d.p.m.) was assigned the value of 0.7 because under these conditions the liver-protein radioactivity at 14 min was 70% of that at 2 min (Fig. 2a). The difference between both tracers expressed in this way (Δ) was plotted in a scale (right-hand ordinate) expanded in such a way that the total area under the (Δ) curve is the same as that under the (●) curve. The arrows indicate the following markers (from left to right): Blue Dextran (V_0), conalbumin (mol.wt. 79000), bovine serum albumin (mol.wt. 67000), ovalbumin (mol.wt. 45000) and lysozyme (mol.wt. 15000). (b) The $^{14}\text{C}/^3\text{H}$ ratio in each fraction is expressed in a relative scale where total ^{14}C d.p.m./total ^3H d.p.m. = 1.0. ■, Ratios of the fractions in the experiment shown in (a); □, ratios of the fractions in a control experiment (not shown) in which the same ^3H -labelled extracts were compared with ^{14}C -labelled livers obtained 2 min after the tracer injection.

the material that had disappeared between 2 and 14 min) had an average mol.wt. of 27000.

It is noteworthy that the rate of disappearance was already maximal 0.5 min after the injection of puromycin (Fig. 2d). It took 0.5 min for the completion of the puromycin effect (Fig. 1). Therefore it appears

that degradation of puromycinyl-peptides occurred with no lag, or with a lag shorter than 0.5 min. This fact should be taken into consideration when a mechanism for the degradation of puromycinyl-peptides is proposed. It seems difficult to accept a two-step mechanism, involving the inclusion of the protein in an autophagic vacuole which would subsequently fuse with lysosomes (de Duve & Wattiaux, 1966; Knowles & Ballard, 1976). Direct uptake by the lysosomes (Hayashi *et al.*, 1973; Dean, 1976) or degradation by soluble proteinases (Hayashi & Natori, 1976; Goldberg & St. John, 1976) would better explain the absence of an appreciable lag.

Decreased rates of degradation of puromycinyl-peptides during rapid liver growth

The results described above convinced us that the rapid disappearance of a fraction of the liver-protein radioactivity observed after the injection of puromycin represented the selective degradation of puromycinyl-peptides. We next observed this phenomenon in the livers of protein-depleted and re-fed animals, situations in which we previously demonstrated a large difference in the rate of degradation of liver proteins (Conde & Scornik, 1976). For this purpose the animals received the intravenous injection of a trace amount of L-[1-¹⁴C]leucine followed by puromycin and cycloheximide in the same way as described in the experiment in Fig. 2(a). The animals were killed from 2 to 60 min after the injection of the tracer. We believe that in both protein-depleted and re-fed mice, puromycinyl-peptides represent the same fraction of the total liver-protein radioactivity, because (a) the proportion of liver-protein radioactivity in nascent chains 0.5 min after the tracer injection was essentially the same in both conditions (in two experiments we measured it to be 59 and 61 % in protein-depleted mice and 61 and 67 % in re-fed animals), and (b) the rate at which nascent chains were released from the ribosomes by puromycin in both situations was also the same in both conditions (Fig. 1).

The results are shown in Fig. 4. A dramatic difference was observed. Whereas in protein-depleted mice, degradation of puromycinyl-peptides proceeded at a rate equal to or greater than that in normal animals, in re-fed animals the degradation of puromycinyl-peptides was much slower. Between 2 and 8 min after the tracer injection, the livers of protein-depleted mice lost 45 % of their total radioactivity, whereas in re-fed mice the loss in that interval was negligible.

In a previous study we have found that the degradation of total liver protein was virtually halted 16 h after re-feeding protein-depleted mice with a normal diet. This decreased rate of degradation was the predominant reason for the rapid net liver-

Fig. 4. Decreased rates of degradation of puromycinyl-peptides during protein re-feeding

Protein-depleted (▲) or re-fed (■) mice received at zero time an intravenous injection of a trace amount of L-[1-¹⁴C]leucine, followed by puromycin 0.5 min later. Cycloheximide was injected intraperitoneally 2 min after the tracer injection. The total liver-protein radioactivity under each condition was added to the corresponding plasma-protein radioactivity (shown separately in a). In a parallel control experiment the inhibitors were injected before the tracer (as in Fig. 2a, □); the incorporation into liver protein was negligible (not shown). The sum of liver- and plasma-protein radioactivities are plotted as percentage of the value at 2 min in each condition (b). Bars represent the S.E.M. and the numbers of mice in each group are given in parentheses.

protein gain in this condition (Conde & Scornik, 1976). We have now shown that the degradation of at least one class of abnormal proteins, puromycinyl-peptides, is affected in the same way.

The degradation of abnormal proteins is believed to represent a scavenger function of the degradation mechanisms (Pine, 1967; Schimke, 1970; Siekevitz, 1972; Goldberg & St. John, 1976). It has been suggested that in bacteria the scavenging mechanism may be different from the mechanism degrading normal proteins. This was inferred from the finding that bacterial mutants which fail to degrade incomplete β -galactosidase molecules (Bukhari & Zipser, 1973) continued to degrade normal bacterial proteins at the same rate as the wild type (Goldberg & St. John, 1976). The situation in the liver seems to be different. It is difficult to imagine the selective advantage of a separate mechanism degrading puromycinyl-peptides (and presumably other abnormal proteins) at a lower rate during growth. More

likely, the degradation pathways of normal and abnormal proteins share at least one rate-limiting step, and the mechanisms controlling it during growth neglect the scavenging function of protein degradation in favour of a more rapid and efficient protein gain. In any case, under most conditions, abnormal proteins probably occur in cells very rarely, and, even at a much slower rate, they can eventually be degraded.

Variable rates of degradation of individual proteins can play a central role in determining their concentration in the cell (Schimke, 1970). It could be that decreased rates of degradation during growth represent the sum of the effects of the individual regulation of the protein components of the cell. It is, however, unlikely that the cell has specific control mechanisms for the degradation of abnormal proteins. The dramatic decrease in the rate of degradation of puromycinyl-peptides during re-feeding suggests the general regulation of a single mechanism responsible for the degradation of normal and abnormal proteins.

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