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Degradation of intra- and extrahepatic protein by livers of normal and diabetic mice: Differential responses to starvation

(endocytosis/protein synthesis/streptozotocin)

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ABSTRACT **Rates of total hepatic proteolysis were measured in normal and streptozotocin–diabetic mice during feeding and over 48 hr of starvation, during which livers in the two groups lost 37% and 54% of their protein content, respectively. Measurements were made in 15-min *in situ* cyclic perfusions from the linear accumulation of free valine in the presence of cycloheximide; rates were corrected for turnover of short-lived proteins because these components contribute negligibly to alterations in liver protein content. During deprivation, corrected rates, expressed on a per liver basis, remained constant in normal mice but increased markedly in the diabetic group, attaining twice prestarvation values by 48 hr. By contrast, degradation rates of long-lived intracellular proteins, calculated from the sum of their synthesis and the linear decrease in protein content, decreased predictably in both groups and in parallel with absolute rates of protein synthesis. The extra proteolysis, representing the difference between total and long-lived protein degradation, was small in fed animals but increased progressively during starvation. With diabetic mice, however, the increase was ≈5 times that of the normal and, in absolute terms, roughly equaled the total loss of liver protein. We suggest that this fraction arose from intrahepatic breakdown of proteins that were ultimately derived from sources outside the liver. Acceleration of this novel process could play an important interim role in providing endogenous glucogenic substrate under conditions in which the demand for this substrate is high.**

The protein content of liver is highly labile and, in small animals such as the rat or mouse, 30–40% may be lost in 48 hr of starvation (1, 2). Because the loss is proteolytic and the degradative mechanism is acutely responsive to amino acids at physiological concentrations (3, 4), it could serve as an immediate source of amino acids for gluconeogenesis and other ongoing metabolic processes in the postabsorptive period (3). We know little, however, of the way protein content is regulated *in vivo* or the interplay between its determining functions, synthesis and degradation. The RNA content does diminish during starvation (5, 6); thus, absolute rates of protein synthesis per liver would be expected to decrease (5). Equivalent estimates of cellular protein breakdown have not yet been reported, but they too may decrease. However, because the cells steadily lose protein, degradative rates will remain higher than those of synthesis.

These questions assume considerable importance in uncontrolled diabetes, in which the demand for glucogenic substrates is elevated and the steady-state content of liver protein quite possibly is reduced as a consequence of lower rates of protein synthesis (7–9) and increased rates of breakdown (7, 10). Here, the sudden withdrawal of food might accelerate existing proteolytic processes and even induce catabolic responses not usually manifest under normal circumstances. In this study, we have evaluated both the degradation of resident intracellular proteins and total rates of hepatic proteolysis in normal and streptozotocin–diabetic mice during 48 hr of starvation. The findings support the above predictions concerning intracellular protein degradation but also show that starvation can enhance hepatic degradation of extrinsic proteins in both groups of mice. In the diabetic mouse, this appears to become a major fraction of proteolysis in liver.

EXPERIMENTAL PROCEDURES

Animals. Male 7-week-old CD-1 mice (Charles River Breeding Laboratories), initially weighing 32–34 g, were used. They had free access to water and standard Charles River chow and were maintained in an environmentally controlled room (light on 7 a.m. to 7 p.m.). Diabetes was induced in fed animals by administration of streptozotocin (200 mg/kg of body weight), given intravenously by tail vein early in the day. The agent was dissolved to 20 mg/ml in ice-cold 0.9% NaCl/0.05 M Na citrate, pH 4.3, and used within 2 hr. Semiquantitative measurements of urine glucose (Tes-Tape, Eli Lilly) and ketones (Ketostix, Ames, Elkhart, IN) and of blood glucose (Dextrostix, Ames) were used to monitor the severity of the diabetes. Plasma glucose and insulin were measured by using a Technicon Auto-Analyzer and a radioimmunoassay kit (Becton Dickinson, Rutherford, NJ); plasma glucagon was determined as described by Cherrington *et al.* (11).

Liver Perfusion. Livers were cyclically perfused *in situ* (3, 12) at a flow of 3 ml/min with modifications for mouse liver (13); the perfusion medium was Krebs–Ringer bicarbonate buffer, pH 7.4/4% bovine plasma albumin (Pentex, fraction V; Miles)/10 mM glucose/0.27% washed bovine erythrocytes (3). Other additions to the perfusate were made as indicated. Where analyses were to be carried out, livers were quickly excised and frozen between clamps previously cooled in liquid N_2.

Protein Synthesis. Synthesis was determined from the incorporation of L-[U-^{14}C]valine (295 mCi/mmol; 1 Ci = 3.7×10^{10} becquerels; New England Nuclear) into liver protein during 2 hr of cyclic perfusion with 10 times the normal concentrations of mouse plasma amino acids (see Table 2) and 5 or 10 mM valine in a total perfusate volume of 60 ml. The high valine concentration and large perfusate volume maintained optimal precursor specific activity (14) and minimized dilution of the label over the 2-hr period; sampling over the 2-hr course showed that the specific radioactivity did not change. Incorporation was computed by dividing the total radioactivity in liver by the measured specific radioactivity of perfusate plasma valine.

Calculation of Long-Lived Protein Synthesis. Because liver contains a small pool of short-lived proteins (<1% of total pro-

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tein; for discussion, see ref. 4) and some export protein in transit to points of secretion, both of which would become maximally labeled early in the incorporation period, the above procedure overestimates rates of synthesis of long-lived proteins. The degree of overestimation can be expressed as follows:

$$\frac{\text{Observed incorporation}}{\text{Actual incorporation (long lived)}} = \frac{A + B}{C\ (\text{min of incorporation})} + 1, \quad [1]$$

where A is percentage of total protein turnover as secretory protein in intracellular transit, B is percentage of total protein turnover as short-lived protein, and C is percentage of long-lived protein, expressed on the same basis. It is evident that the ratio will approach unity as the sum of A and B becomes very small or the duration of incorporation becomes very long. Since neither condition prevailed in these experiments, the ratio was calculated from the following considerations: Nominal values for total protein turnover by synthesis, 81%/day or 0.056%/min, and its fractional distribution into long-lived (0.58), secretory (0.26), and short-lived (0.16) components were taken from Scornik *et al.* (15, 16) and from our own work (unpublished data); reasonable agreement was found among the three sets of data. The value for A, 0.32% was computed from the fractional synthesis of secretory proteins, expressed as percentage of the total and multiplied by an estimated transit time of 22 min; the latter represents the lag between the start of labeling and the establishment of a steady state of label secretion (ref. 12; unpublished data). B, 0.15%, was determined from the fraction of total synthesis comprising short-lived proteins divided by the average rate constant of turnover, $\approx 0.06\ \text{min}^{-1}$ (unpublished data). C was computed from these data to be $0.056\%\cdot\text{min}^{-1} \times 0.58 = 0.032\%\cdot\text{min}^{-1}$. Substituting these numerical values for A, B, C, and time (120 min) in Eq. 1, we obtain a ratio of 1.12, a result indicating that the actual rate of long-lived protein synthesis is 1/1.12 or 0.89 of the observed incorporation. Although this calculation has not been validated in diabetes, it would appear to be applicable in starvation (16). All data have been corrected by this factor, but possible exceptions will be noted in the text.

Determination of Proteolysis. Rates of total proteolysis were estimated during brief cyclic perfusions from the linear accumulation of valine in the presence of 18 μM cycloheximide. The initial volume of medium was 17 ml, and approximately four 0.5-ml samples were taken between 5 and 15 min. This cycloheximide procedure, which was adapted from a method previously validated in rat livers (3, 4, 17), provides an estimate of the combined breakdown of long-lived, short-lived, and endocytosed proteins (3, 4). Because the proteolytic effects of physiological regulators such as amino acids, insulin, and glucagon are not immediate but delayed 15–20 min (17), the initial rates will reflect proteolytic activity that exists in liver at the start of perfusion.

Proteolytic rates were computed by least-squares regression from changes in total valine accumulation. Control studies showed that valine did not enter erythrocyte water during these brief perfusions, and no changes in free intracellular valine in liver between 5 and 15 min of perfusion were observed under any metabolic condition. All rates were corrected for residual protein synthesis (3).

Analytical Procedures. Perfusate plasma valine was assayed by the competitive tRNA binding method of Rubin and Goldstein (18); amino acid analyses of liver extracts, perfusate mixtures, and acid hydrolysates of liver protein (19), and analyses for the determination of plasma valine specific radioactivity were carried out by ion exchange chromatography as described (3). RNA was measured as detailed by Fleck and Munro (20). Liver protein was isolated as described (21, 17) and assayed for protein content against desiccated crystalline bovine albumin standards (22). Aliquots of protein powder were dissolved in 0.1 M NaOH and radioactivity was determined in a Beckman model LS 8000 liquid scintillation spectrometer. Samples were counted in formula 947 scintillation cocktail (New England Nuclear); quenching was corrected for by the use of an external standard. Data were evaluated statistically with Student's distribution of t and expressed as mean ± SEM; values of $P > 0.05$ were considered not significant.

RESULTS AND DISCUSSION

***In Vivo* Response to Streptozotocin.** Three days after injection of streptozotocin, plasma insulin decreased from a normal range of 60–100 microunits/ml to 4 ± 1.5 microunits/ml in four mice; mean plasma glucose was 32.4 ± 1.7 mM. In day 7 diabetic mice, plasma glucagon averaged 394 pg/ml in a pool of five mice as compared with 80 pg/ml for normal animals. These hormonal levels are in close agreement with reported values in severely diabetic streptozotocin-treated rats (23). The mice were hyperphagic, glucosuric, polyuric, and occasionally mildly ketotic and the incidence of diabetes was virtually 100%. As long as food was provided, they were active and apparently healthy without insulin treatment. As shown in Table 1, body and liver weights in normal animals increased ≈1%/day; corresponding weights in animals five days after injection were ≈88% and 80%, respectively, of control values. The stability of the diabetes produced by streptozotocin in this study is in agreement with findings in BALB/c Dub mice (9) but in contrast to experience with rats in which, reportedly, an insulin-dependent state is readily produced (10, 23).

Effects of Diabetes on Hepatic Protein Turnover in Fed Mice. Both protein synthesis and the protein content of livers from fed day 5 diabetic animals were decreased significantly over control values (Tables 1 and 2). The reduction in content

Table 1. Effects of streptozotocin on mouse body and liver weight and on liver RNA, protein content, and protein turnover

	Control	Diabetic	Change %	Change Direction
Body weight, g	34.7 ± 0.87 (11)	30.7 ± 0.77* (16)	12	Decrease
Liver weight, g	1.98 ± 0.10 (11)	1.74 ± 0.10 (16)	12	Decrease
Liver RNA, mg/g of liver	8.23 ± 0.22 (4)	7.18 ± 0.20† (4)	13	Decrease
Liver protein, μmol of valine per liver	182.8 ± 6.8 (18)	146.3 ± 3.2‡ (8)	20	Decrease
Fractional protein synthesis, hr^{-1}	0.017	0.015	12	Decrease
Fractional protein degradation, hr^{-1}	0.024	0.026	8	Increase

Male CD-1 mice weighing 32–34 g were given streptozotocin on day 0. A weight-matched group served as normal controls and the animals were killed on day 5. Liver protein is expressed in valine equivalents based on the average valine content of acid-hydrolyzed (19) protein samples (0.458 μmol/mg); this value was found to remain the same in diabetes, starvation, and refeeding. Nominal rates of fractional synthesis and degradation of long-lived proteins were computed from absolute rates per liver of fed animals (Table 2 and Fig. 2) divided by liver protein content (line 4). Values in parentheses represent n.

*$P < 0.005$.
†$P < 0.02$.
‡$P < 0.001$.

to 80% of control can be explained by the decreases in liver RNA and fractional rate of protein synthesis (both to ≈88% of control), possibly combined with a small increase in fractional degradation of liver proteins (Table 1). It should be pointed out that, while absolute rates of proteolysis in fed diabetic mice were lower than those in normal controls (Table 3), protein turnover was increased, a relative effect attributable to the absolute decrease in liver protein.

It is doubtful that proteolytic rates measured during the day, as these were, would be maintained at this level in feeding animals throughout a full 24-hr period. Autophagic activity in rats is known to be suppressed completely shortly after food intake and to be accelerated during daylight hours when the animals normally eat very little (24). Because this cellular process, in both its overt and its basal form, may be the main mechanism for degrading long-lived proteins (4, 25), it is reasonable to predict that rates of proteolysis will fluctuate strongly during the normal feeding cycle. Whether or not such a cycle would be altered in hyperphagic diabetic mice is not currently known.

Effects of Starvation on Hepatic Protein Synthesis, Degradation, and Content in Normal Mice. When food was withdrawn from the control mice, liver protein content decreased 37% over the subsequent 48 hr (Fig. 1) at an average rate of 1.40 μmol of valine·hr^{-1}; linearity of the decrease was substantiated in a separate control study (unpublished data) in which additional points were obtained. Although rates of protein synthesis per gram of liver did not change significantly, absolute rates per liver diminished in direct proportion to the decrease in protein content; thus, the fractional rate of synthesis remained constant. These observations may be interpreted in light of recent data by McNurlan *et al.* (5), who reported proportionate decreases in total liver RNA and absolute rates of protein synthesis in 48-hr starved rats. In contrast to our results, however, their protein losses were disproportionately small, and fractional rates tended to decrease.

Estimates of long-lived protein breakdown over the course of starvation based on the sum of the rates of protein loss and the absolute rates of protein synthesis are shown in Fig. 2. Because the loss of protein was linear, the degradation of long-lived proteins would be expected to decrease over the 48-hr period in parallel with synthesis. By contrast, absolute rates of total proteolysis remained comparatively constant (Fig. 2 and Table 3). This seemed to be so whether or not the rates were corrected for the breakdown of short-lived proteins. Subtracting the long-lived components from the corrected total left an unexplained remainder (Fig. 2) that progressively increased during starvation.

Effects of Starvation in Diabetic Mice. Despite their lower initial content of liver protein (Fig. 1), diabetic mice lost protein at a somewhat greater rate than did control mice (1.65 versus 1.40 μmol of valine·hr^{-1}). This difference probably reflects the greater spread between fractional rates of protein synthesis and breakdown that existed at the time food was withdrawn (Table 1). As shown in Table 2, rates of incorporation per gram of liver actually increased significantly in 48-hr starved diabetics as compared with fed animals. The effect tended to minimize the deprivation-induced decrease in absolute synthesis per liver previously observed in normal mice (Table 2 and Fig. 2). While we have no clear explanation for the phenomenon, it could indicate that less RNA was removed; alternatively, it might mean that less export and more resident (long-lived) protein was synthesized. The latter possibility is supported by at least two reports indicating that secretory protein biosynthesis is preferentially suppressed in diabetic rats (8, 26). If this were the case here, the correction factor of 0.89 used for protein synthesis would be too low. However, even if we assume that secretory protein synthesis is nil and the correction factor is unity, the effect on results in Fig. 2 and the conclusions will be virtually negligible.

The most striking finding in this study was the large unexplained release of valine that appeared in the starved diabetic mice (Fig. 2 and Table 3), a value 5 times larger than that in normal animals. Possible derangement of branched-chain amino acid metabolism was excluded by a more extensive analysis of the amino acids released. Reliable determinations of 11 others besides valine could be made; these included alanine, glutamine, glycine, isoleucine, histidine, leucine, lysine, phenylalanine, serine, threonine, and tyrosine. In two experiments, the mean percentage increment in their individual concentrations over the 5- to 15-min perfusion interval was 126

Table 2. Synthesis of long-lived proteins in livers of normal and diabetic mice before and after 48 hr of starvation

			Long-lived protein synthesis	
	Incorporation, μmol of valine·hr^{-1}·(g of liver)$^{-1}$	Liver weight, g	Absolute, μmol of valine·hr^{-1} per liver	Fractional, hr^{-1}
Normal				
Fed (11)	1.71 ± 0.03	1.98	3.01	0.017
Starved (3)	1.85 ± 0.22	1.19	1.96	0.017
Diabetic				
Fed (8)	1.38 ± 0.04*	1.74	2.14	0.015
Starved (4)	1.79 ± 0.13†	1.14	1.82	0.027

Day 5 diabetic mice and their controls (Table 1) were starved 48 hr. Incorporation of valine into perfused liver protein and derived rates of long-lived protein synthesis were determined on day 5 (fed) and day 7 (48-hr starved) animals. The 2-hr perfusions used 5 or 10 mM labeled valine and an amino acid mixture having the following percentage molar composition: alanine, 9.88; arginine, 5.24; asparagine, 1.48; aspartic acid, 2.29; cysteine, 1.70; glutamine, 11.07; glutamic acid, 6.61; glycine, 9.74; histidine, 1.88; isoleucine, 3.15; leucine, 5.75; lysine, 10.74; methionine, 1.85; phenylalanine, 2.26; proline, 3.23; serine, 3.83; threonine, 6.15; tryptophan, 2.47; tyrosine, 2.63; valine, 8.03 (i.e., 10 times normal mouse plasma amino acid concentrations except for valine, for which the normal concentration is 3.28 mM). Fractional rates of synthesis represent absolute rates per liver divided by the corresponding liver protein content shown in Fig. 1. Values in parentheses represent *n*.

*$P < 0.001$ versus fed normal.

†$P < 0.02$ versus fed diabetic.

Table 3. Rates of total proteolysis in livers of normal and diabetic mice before and during 48 hr of starvation

	Proteolysis, μmol of valine·hr^{-1} per liver	
	Total	Total minus short-lived fraction
Normal		
Fed (30)	5.40 ± 0.50	4.57
Starved 24 hr (7)	5.04 ± 1.08	4.35
Starved 48 hr (7)	4.92 ± 0.54	4.38
Diabetic		
Fed (18)	4.44 ± 0.58	3.85
Starved 24 hr (8)	7.20 ± 1.20*	6.65
Starved 48 hr (21)	9.04 ± 1.14†	8.54

Animal weights and treatment were as in Tables 1 and 2. Total proteolysis was determined as described in *Experimental Procedures* and corrected for breakdown of short-lived proteins (taken to equal 0.16/0.58 or 27.6% of long-lived synthesis). For the correction, rates of synthesis (Table 2) were multiplied by 0.276 and the product was subtracted from fed and 48-hr values; the 24-hr corrections were interpolated from fed and 48-hr values. Values in parentheses represent *n*.
*$P < 0.05$ versus fed diabetic.
†$P < 0.001$ versus fed diabetic; $P < 0.005$ versus 48-hr normal starved.

± 31% ($P < 0.005$ by pair difference); all increased except alanine, and the average compared closely with that for valine (120%). When considered together with earlier results, these findings indicate that the enhanced release of valine was proteolytic in nature and that the breakdown of some fraction other than cellular proteins was accelerated.

Nature of the Proteolytic Responses to Caloric Deprivation in Normal and Diabetic Mice. Of the three known sources of proteolysis in liver, (*i*) long-lived proteins, (*ii*) short-lived proteins, and (*iii*) endocytosed proteins, only the latter provides a reasonable explanation for the extra amino acids released during starvation. The first appears to have been adequately excluded by our measurements of protein degradation. The additional possibility that short-lived-protein turnover was accelerated in the diabetic group would require that total protein synthesis had increased 4-fold over the 48-hr period—a highly improbable occurrence. More direct evidence along this line was obtained in cyclically perfused livers that had been labeled *in vivo* with [^{14}C]valine 18 and 4 hr previously. In four paired experiments with fed and 48-hr starved diabetic mice, the release of label in the presence of 15 mM unlabeled valine showed no significant differences in the breakdown of short-lived and long-lived proteins (data not shown). Of additional interest was the fact that rates of long-lived-protein degradation, which averaged 6.8% of total liver label per hr, were virtually the same as results obtained with normal fed mice. Both sets of data were obtained in the absence of restriction to proteolysis (i.e., no amino acid additions); thus, they show that the potential for degrading intracellular proteins had not diminished during starvation.

In view of the high concentration of glucagon and low level of insulin, we may ask why liver protein degradation in our diabetic mice and those of Bond (9) was not appreciably enhanced over control rates. Earlier studies with streptozotocin- and alloxan-diabetic rats exhibited evidence of increased protein breakdown (7, 10) or enhanced autophagy (23) shortly after insulin withdrawal or administration of streptozotocin. Part of the answer may lie in species differences. The severely diabetic rat appears to require insulin to prevent ketoacidosis and sustain a reasonable level of health (7, 10, 23); the mouse, at comparably low levels of insulin, does not (9). It is thus possible in the mouse to induce starvation in a highly insulin-deficient, but metabolically stable, state without the added disturbance of acute insulin withdrawal. A second and possibly more pertinent factor is the strong modulating effect of amino acids themselves on the

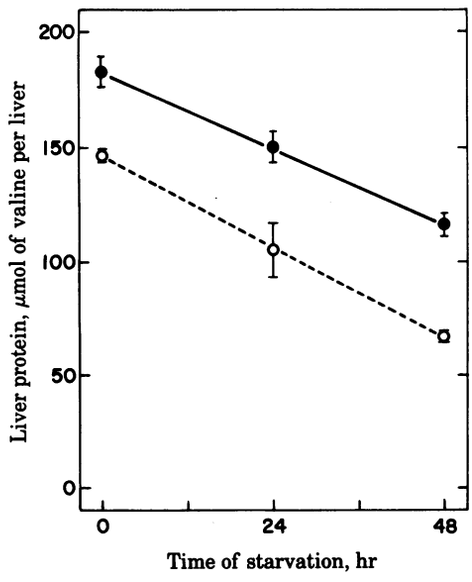

FIG. 1. Loss of total protein during 48 hr of starvation in normal (●——●) and streptozotocin-diabetic (○----○) mice. Animal weights and treatment were as in Tables 1–3. Average rates of valine loss were 1.40 and 1.65 μmol per hr per liver for normal and diabetic mice, respectively.

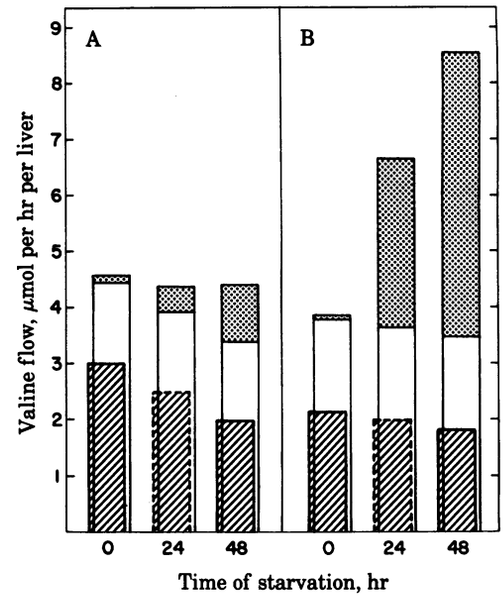

FIG. 2. Flow of valine into and out of resident liver protein in comparison with rates of total (corrected) proteolysis during starvation in normal (*A*) and streptozotocin-diabetic (*B*) mice. ▨, Absolute rates of synthesis of long-lived proteins from data in Table 2; the 24-hr rate was interpolated from initial and final values. Offset bars represent absolute rates of protein degradation; □, long-lived proteins, calculated as described by Conde and Scornik (16) from the sum of synthesis and the average rate of net protein valine loss at each point (Fig. 1); ▩, Extra proteolysis unexplained by the breakdown of cellular proteins, computed as the difference between the degradation of long-lived proteins and total proteolysis (corrected for short-lived components as described in Table 3).

catabolic action of glucagon. In the perfused rat liver, glucagon-induced autophagy and proteolysis can be completely inhibited by physiological concentrations of amino acids (3). Based on these findings, we believe that the quantity of extra amino acids generated proteolytically during starvation would be sufficient to suppress these catabolic responses.

The source(s) of the extra protein degraded during starvation is not known, but some general possibilities should be mentioned. Plasma proteins have been shown to be degraded at increased rates by perfused livers of alloxan–diabetic rats (7). However, because normal rates are quite low and appear to be reduced rather than accelerated in nutritional deprivation (27), it seems unlikely that this source would contribute more than a small fraction to the total. Small intestine, thymus, and other lymphoid tissues are known to involute markedly during starvation. The latter cells are lysed by a glucocorticoid-facilitated action (28), followed by the release of immunoglobulins and possibly other cellular proteins. The fate of intestinal mucosal cells and their proteins when the former are shed into the lumen of an involuting gut is not at all certain, but there is evidence to suggest that, under similar circumstances, polypeptides can escape digestion locally and pass directly into the circulation by pinocytosis (29). Whether or not the mouse liver does, in fact, take up these and other proteins in quantities sufficient to explain the observed rates of degradation is a question that merits further investigation.

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