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Biochemistry

General characteristics of protein degradation in diabetes and starvation

(protein catabolism/protein molecular size/protein isoelectric point/glycoproteins)

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ABSTRACT **The enhanced protein degradation associated with diabetes and starvation is fundamentally different from normal protein catabolism. In normal eukaryotic cells large molecular weight proteins tend to be degraded more rapidly than small proteins, acidic proteins tend to be degraded more rapidly than neutral or basic proteins, and glycoproteins tend to be degraded more rapidly than nonglycoproteins. All three of these general correlations are absent or markedly reduced in liver and muscle of diabetic and starved rats. In contrast, the correlations between protein size and half-life, between protein net charge and half-life, and between protein carbohydrate content and half-life are not affected in brain of diabetic or starved animals. These results suggest that diabetes and starvation alter the general characteristics of intracellular protein degradation in target tissues of insulin. Degradation of serum proteins is also affected in diabetes and starvation. In normal animals a general correlation exists between isoelectric points of serum proteins and their degradative rates. This relationship is abolished in diabetes and starvation, as it is among liver and muscle proteins. The implications of our findings are discussed with regard to possible mechanisms of the enhanced protein breakdown.**

Intracellular protein catabolism is a fundamental cellular process that has received increasing attention the past few years (1–4). Some of the general characteristics of the degradative process (2, 3) have suggested that polypeptide structure may influence how rapidly proteins are broken down within cells. For example, in a wide variety of tissues there is a general relationship between the molecular weight of a polypeptide and its half-life, with larger proteins having more rapid turnover rates than smaller proteins (2, 3, 5). Furthermore, a general correlation exists between protein isoelectric point and half-life, with acidic proteins turning over more rapidly than neutral or basic proteins (3, 6). We have confirmed and extended studies (7–9) which indicated that glycoproteins within cells are degraded more rapidly than nonglycoproteins (F. Kalish, N. Chovick, and J. F. Dice, unpublished data). None of these relationships can be explained by the others (ref. 6; J. F. Dice, E. J. Hess, and A. L. Goldberg, unpublished data), indicating that they are three distinct features of the degradative process.

The biochemical explanations for these characteristics of protein catabolism are unclear, but they are common to all eukaryotes examined, including several tissues of animals (5), plants (5, 10), and cells grown in culture (ref. 5; J. F. Dice, E. J. Hess, and A. L. Goldberg, unpublished data). However, these characteristics of protein catabolism have been demonstrated only under normal conditions and may or may not apply to the various pathological and physiological situations in which overall rates of protein degradation are altered.

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Nutrient deprivation is the most widely studied condition in which average rates of protein breakdown are increased. Various nutrients and hormones can affect protein degradative rates (3), but insulin is probably the most important single factor that regulates overall protein catabolism in mammals (3). In insulin-deficient states average rates of protein catabolism are accelerated 30–150% in liver cells, fibroblasts, adipocytes, skeletal muscle, and heart muscle (3).

Several studies have implicated lysosomal involvement in the enhanced protein catabolism due to nutrient deprivation. For example, lysosomes are larger and more fragile in livers that are perfused in the absence of insulin, in the presence of glucagon, or with inadequate levels of certain amino acids (11, 12). Similar changes in the lysosomal system have been reported for skeletal muscles (13) and heart muscle (14) perfused under poor nutritional conditions. Several investigators have concluded from these findings that lysosomes are also involved in basal levels of protein catabolism.

It is crucial for our understanding of the mechanisms and control of protein degradation to determine whether the enhanced catabolism in diabetes and starvation is simply an acceleration of normal breakdown processes or results from activation of different proteolytic mechanisms. We now report that three of the fundamental characteristics of protein degradation in normal tissues are absent or strikingly reduced in diabetes and starvation.

MATERIALS AND METHODS

Animals. Male rats (Simonsen Laboratories, Gilroy, CA), 160–180 g, were used for each of the studies. The animals were maintained on water and Purina Lab Chow (Ralston Co., St. Louis) for at least 4 days before use.

Diabetes. Severe, insulin-dependent diabetes was induced in rats by intravenous injection of streptozotocin (Upjohn) (150 mg/kg of body weight) as described (15). The rats were kept in wire metabolic cages and their diabetes was monitored by measurements of body weight, urine volume, urine glucose (Tes-tape, Lilly), urine ketones (Ketostix, Ames), and blood glucose (Dow Diagnostics). The diabetic animals were maintained with a single daily injection (at 5 p.m.) of 3–4 units of NPH U-40 insulin (Lilly), and the insulin was withdrawn on the day the experiment began. In this way the severity of the diabetes could be determined before the experiment, and acute insulin withdrawal could be achieved.

Double-Isotope Labeling and Preparation of Cytoplasmic Proteins. The experiments presented in this paper involve measuring relative rates of protein degradation by a modification of the double-isotope technique described by Arias *et*

Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; Con A, concanavalin A.

al. (16). In this procedure two isotopic forms of an amino acid, usually [^{3}H]leucine and [^{14}C]leucine, are used to establish two time points on the curve describing degradation of the protein. [^{14}C]Leucine is first administered to an animal, and then some days later [^{3}H]leucine is given to the same animal just before it is killed. The $^3H/^{14}C$ ratio of a protein fraction therefore reflects its degradative rate.

The double-isotope procedure as originally described is applicable only to animals under constant or "steady-state" conditions. By changing the protocol slightly, this technique can also be applied to animals undergoing physiological changes. The major modification of the experimental design involves using two animals instead of one. As an example, in one set of experiments a diabetic rat receiving insulin injections was given [^{14}C]leucine, its insulin was withdrawn, and the ^{14}C-labeled proteins were allowed to degrade *in vivo* for 3 days. A normal animal was injected with [^{3}H]leucine and killed 4 hr later. The appropriate tissues were pooled, and serum proteins (17) or cytoplasmic proteins (5, 6) were prepared. With certain assumptions discussed in the *Results*, the ^{14}C in a protein fraction reflects the amount of degradation during 3 days of diabetes, while the ^{3}H represents an initial point on the curve. Therefore, proteins that are degraded rapidly in diabetes will have high $^3H/^{14}C$ ratios. Control experiments consisted of using normal animals or diabetic animals receiving insulin in the same experimental protocol. In separate experiments, normal rats and diabetic rats maintained with insulin were indistinguishable in terms of the general characteristics of protein degradation (unpublished observation).

Sodium Dodecyl Sulfate ($NaDodSO_4$)/Polyacrylamide Gel Electrophoresis, Isoelectric Focusing, and Determination of Radioactivity. These procedures were carried out as described earlier (5, 6).

Separation of Glycoproteins from Nonglycoproteins. Glycoproteins were isolated by affinity chromatography using concanavalin A (Con A) linked to agarose (Miles Laboratories). Double-labeled proteins (10–12 mg) were applied to a column containing 2 ml of packed Con A-agarose. The column was then washed with 30 ml of salt solution (75 mM NaCl/1 mM $CaCl_2$/1 mM $MgCl_2$) to collect proteins that did not bind to the column. Glycoproteins that bound to the Con A were eluted with 20 ml of 5% α-methyl-D-mannoside in the salt solution described above. The protein fractions were precipitated in 10% trichloroacetic acid and their $^3H/^{14}C$ ratios were determined. Further details will be presented elsewhere (F. Kalish, N. Chovick, and J. F. Dice, unpublished data).

RESULTS

Diabetic and Starved Animals. Diabetic rats receiving daily insulin injections gained 4.0 ± 1.1% (n = 11) of their body weight daily compared to 4.4 ± 0.7% (n = 22) for normal rats. Ketonuria was consistently absent in diabetic rats receiving insulin, and their urine glucose levels (measured four times per day) were generally negative. Their blood glucose concentrations were 160 ± 40 mg/100 ml (n = 4) compared to 106 ± 26 mg/100 ml (n = 7) for normal animals.

When insulin was withdrawn from the diabetic rats, their blood glucose levels increased to 499 ± 48 mg/100 ml (n = 7), and polyuria, glucosuria, and ketonuria developed within 24 hr. Diabetic rats lost 30 ± 7% (n = 7) of their body weight during the 3 days of insulin withdrawal. Comparatively, starved rats lost 25 ± 6% (n = 5) of their weight.

Validation of Experimental Design. In order to correctly interpret the results of double-label experiments, four assumptions must be shown to be valid. Several investigators (16, 18–20) have examined these assumptions for liver or muscle of normal rats, but in the present study we examine them in diabetic and starved animals. Briefly, the assumptions are: (*i*) The rates of synthesis of proteins must be the same when both isotopes are given. (*ii*) The radioactive amino acid must not be metabolized to other compounds which are then incorporated into the proteins. (*iii*) The proteins under study must follow exponential decay kinetics. (*iv*) At the time the animal is killed, both the ^{3}H and ^{14}C must be on the exponential portion of the decay curve.

Our results (not shown) indicate that assumption *i* is valid for liver and muscle proteins from normal, diabetic, and starved animals. A normal rat was injected with [^{3}H]leucine and a diabetic rat maintained on insulin was given [^{14}C]leucine. Four hours later the animals were killed and the appropriate tissues pooled. Proteins separated either by $NaDodSO_4$ gel electrophoresis or by isoelectric focusing showed no greater variation in $^3H/^{14}C$ ratios than in a comparable experiment in which both isotopes were administered to a single, normal animal.

Assumption *ii* also appears to be valid for liver and muscle, since 90–100% of the ^{3}H and ^{14}C radioactivity in proteins is recoverable as leucine after the double-labeling procedure. This result applied to experiments using normal, diabetic, or starved animals.

Assumption *iii* is not strictly valid because the half-lives of proteins may be continually changing during the experimental period as the animal adapts to different durations of starvation and diabetes (21, 22). Therefore, the $^3H/^{14}C$ ratio of a protein fraction will reflect only its average degradative rate during the time interval studied. It is unlikely that unusual kinetics of breakdown have led us to erroneous interpretations, however, since the conclusions presented below are similar for either 2 or 3 days of diabetes or starvation.

Assumption *iv* is not valid even in normal animals for reasons discussed by Poole (19). Leucine is a reutilized amino acid, and leucine reutilization may change during diabetes and starvation, thereby complicating the interpretation of $^3H/^{14}C$ ratios of protein fractions. We have therefore developed a different double-isotope technique to estimate relative degradative rates of proteins in liver. In this method $NaH^{14}CO_3$ is given as the first isotope (the decay point) and [^{3}H]arginine as the second isotope (the incorporation point). The radioactivity in proteins after $NaH^{14}CO_3$ injection is primarily contained in glutamate, aspartate, and arginine in the liver (23), and the probability of reutilization of any of these labeled amino acids is very low (2, 23). Our initial experiments with this double-isotope procedure have fully confirmed the major conclusions presented in this paper regarding proteins from liver. Our results, at least in liver, therefore probably cannot be explained by artifacts associated with amino acid reutilization.

General Features of Protein Degradation in Diabetes and Starvation. If the degradative process in diabetes and starvation is qualitatively similar to that in normal animals, the general characteristics should be the same. That is, large proteins should be degraded more rapidly than small proteins, acidic proteins should be degraded more rapidly than neutral or basic proteins, and glycoproteins should be degraded more rapidly than nonglycoproteins. Double-labeled proteins obtained from various tissues of normal, diabetic, and starved animals were separated by subunit molecular weight using $NaDodSO_4$/polyacrylamide gel electrophoresis, by isoelectric point using isoelectric focusing, or by carbohydrate content using affinity chromatography with Con A-agarose.

Fig. 1 shows that a clear relationship exists between protein subunit size and half-life for cytoplasmic proteins from liver

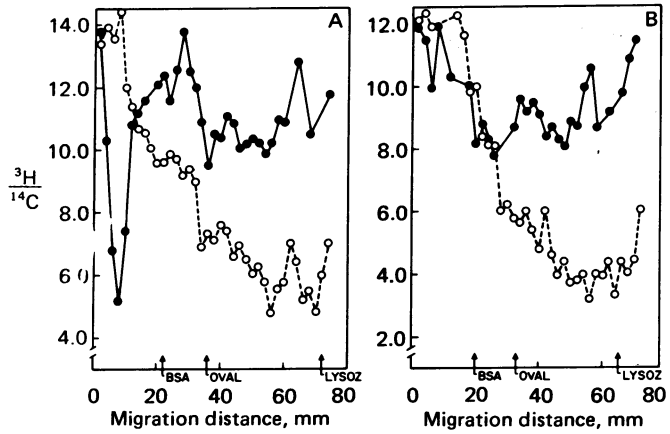

FIG. 1. Relative degradative rates of liver and muscle proteins in normal (○) and diabetic (●) rats: NaDodSO$_4$/polyacrylamide gel electrophoresis. A diabetic rat that had been maintained on insulin received 150 μCi of [^{14}C]leucine intraperitoneally, after which its daily insulin injections were withheld. Three days later it was killed and its tissues were pooled with those from a normal rat that had been injected with 750 μCi of [^{3}H]leucine 4 hr previously. Analogously, a normal rat was given 100 μCi of [^{14}C]leucine, and 3 days later its tissues were pooled with ^{3}H-labeled normal tissues. Separate experiments demonstrated that diabetic animals receiving insulin gave results identical to those of normal animals. Cytoplasmic proteins were isolated, 5 mg of protein was applied to a large-diameter (19 mm) gel, the gels were sliced, and radioactivity was determined. The direction of migration was from left to right, so the more slowly migrating, larger molecular weight proteins are on the left. Arrows indicate the position of marker proteins which were run simultaneously in small-diameter (5 mm) gels. BSA, bovine serum albumin (molecular weight 67,000); OVAL, ovalbumin (molecular weight 45,000); LYSOZ, lysozyme (molecular weight 14,000). (*A*) Cytoplasmic proteins from liver; (*B*) cytoplasmic proteins from muscle. Ratios are not plotted for fractions that contained less than 200 dpm of either isotope.

and muscle of normal animals, since the larger molecular weight polypeptides tend to have higher $^3H/^{14}C$ ratios. Identical results are obtained with diabetic animals receiving insulin injections instead of normal animals. In contrast, the relationship between protein size and degradative rate is absent among liver

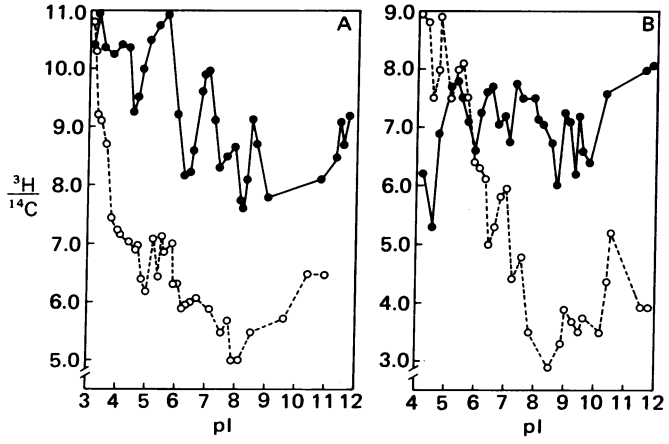

FIG. 2. Relative degradative rates of liver and muscle proteins in normal (○) and diabetic (●) rats: Isoelectric focusing. The double-labeled proteins were from the same preparation described in the legend of Fig. 1. Protein (40 mg) was focused in a 110-ml capacity isoelectric focusing column (LKB), fractions were collected, and radioactivity of each fraction was determined. (*A*) Cytoplasmic proteins from liver; (*B*) cytoplasmic proteins from gastrocnemius and thigh muscles. Ratios are not plotted for fractions that contained less than 200 dpm of either isotope.

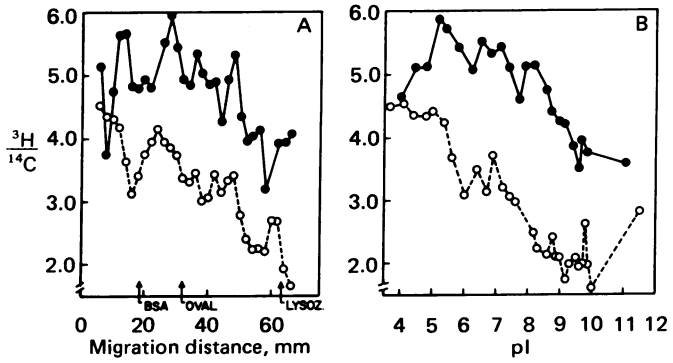

FIG. 3. Relative degradative rates of liver proteins in fed (○) and starved (●) rats. Two rats received intraperitoneal injections of 150 μCi of [^{14}C]leucine. Four hours later food was removed from one of the rats. Three days later both the "fed" and the "starved" rats were killed and their livers pooled with those from normal rats that had received 500 μCi of [^{3}H]leucine 4 hr previously. NaDodSO$_4$ gel electrophoresis (*A*) or isoelectric focusing (*B*) was performed, and radioactivity in different fractions was determined. Ratios were not plotted for fractions that had less than 200 dpm of either isotope.

and muscle proteins from diabetic animals that have had their insulin withdrawn.

Cytoplasmic proteins from liver and muscle of normal rats also exhibit a relationship between protein isoelectric point and degradative rate since acidic proteins tend to have higher $^3H/^{14}C$ ratios than neutral or basic proteins (Fig. 2). This relationship is markedly reduced among liver proteins and is abolished among muscle proteins from diabetic animals.

There is also a reduced correlation between protein size and half-life and between protein isoelectric point and half-life in liver and muscle proteins of starved animals. Fig. 3 shows these results for cytoplasmic proteins from liver. The effect of starvation on the characteristics of protein degradation appears to be less drastic than that of diabetes, which suggests that there may be more extreme imbalance of hormones and nutrients in diabetes.

As a control we have determined whether the changes in the general characteristics of protein degradation during diabetes and starvation are tissue specific. In the brain, an organ generally considered to be nonresponsive to insulin, the correlation between protein size and half-life is not affected by starvation (Fig. 4*A*) and that between protein isoelectric point and half-life is not affected by diabetes (Fig. 4*B*).

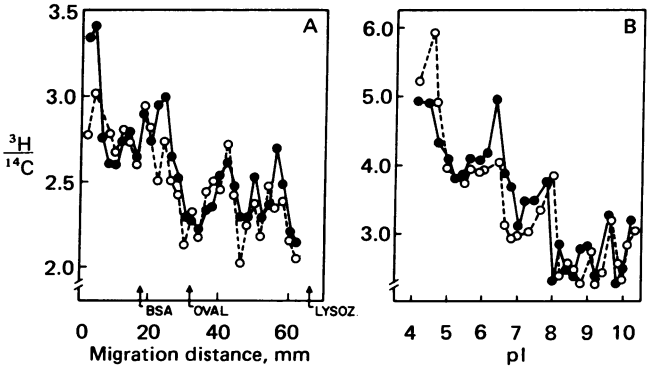

FIG. 4. Relative degradative rates of brain proteins in normal, diabetic, and starved rats. Proteins were derived from the same experiments described in the legends of Figs. 1 and 3. Proteins were separated by NaDodSO$_4$ gel electrophoresis (*A*) or by isoelectric focusing (*B*) and the radioactivity in various fractions was determined. Ratios are not plotted for fractions with less than 200 dpm of either isotope.

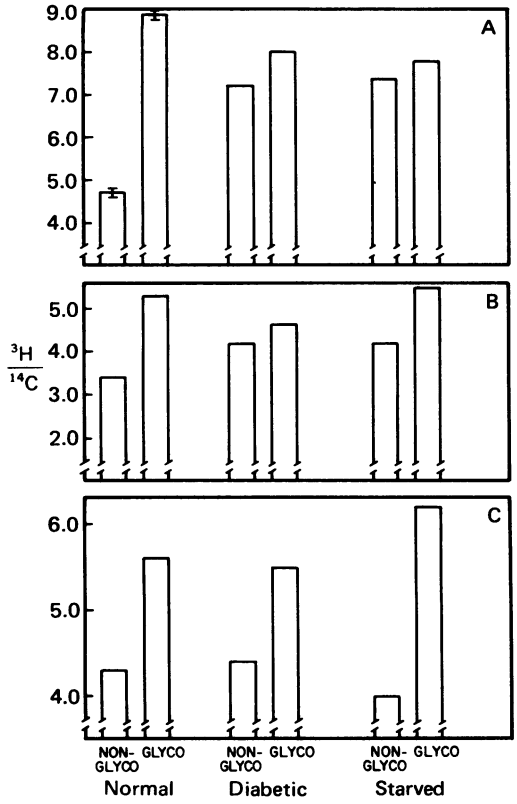

FIG. 5. Relative degradative rates of (*A*) liver, (*B*) muscle, and (*C*) brain proteins in normal, diabetic, and starved rats: Affinity chromatography with Con A-agarose. Double-labeled proteins were prepared as described in the text. Livers were extensively perfused to remove serum proteins before isolation of cytoplasmic protein components. Protein (10–12 mg) was applied to the column in each experiment. Nonglycoproteins (NONGLYCO) and glycoproteins that bind to Con A-agarose (GLYCO) were collected. Four to 6% of the total proteins from each of the tissues bound to Con A-agarose. $^3H/^{14}C$ ratios of nonglycoproteins and glycoproteins from normal liver are the averages ± 1 SD for three experiments with the same double-labeled proteins. Other $^3H/^{14}C$ ratios are from single experiments.

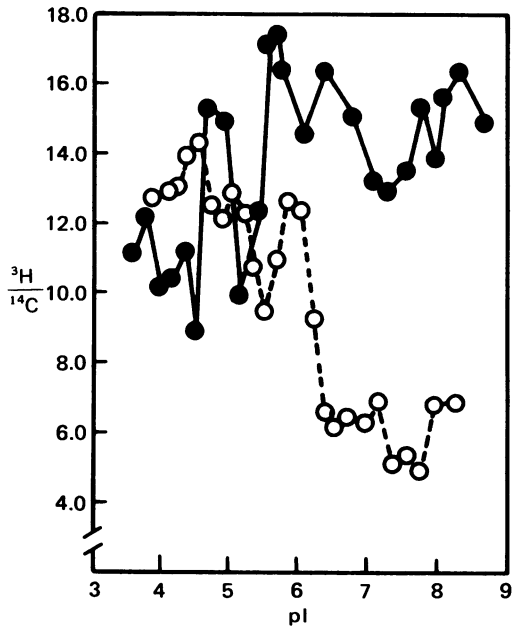

FIG. 6. Relative degradative rates of serum proteins in normal (○) and diabetic (●) rats: Isoelectric focusing. Serum proteins were derived from the same experiment described in the legend of Fig. 1. Serum proteins were collected and prepared as described (17). About 70 mg of protein was focused, and radioactivity was determined for each fraction. Ratios are not plotted for fractions with less than 200 dpm of either isotope.

Recent studies from our laboratory have shown that intracellular glycoproteins that bind to Con A or to other lectins are degraded more rapidly than nonglycoproteins in various normal tissues. Fig. 5 illustrates our results for liver, muscle, and brain proteins and also shows that the relationship between sugar content and protein half-life is reduced among liver and muscle proteins from diabetic and starved animals. In contrast, the relationship is not affected among brain proteins during diabetes and starvation.

Recently we suggested that degradation of serum proteins and intracellular proteins may share pathways or mechanisms that have at least some steps in common since some of the general characteristics of the degradative process for the two classes of protein are the same (17). It is intriguing that the overall catabolic rate of serum proteins is enhanced in diabetes and starvation (24–26) similar to the effect on breakdown of intracellular proteins in insulin-sensitive tissues. Furthermore, we find that the correlation between protein isoelectric point and half-life, which is a general characteristic of serum protein catabolism in normal animals (ref. 17; Fig. 6), is abolished in diabetes (Fig. 6) at the same time it is abolished for liver and muscle proteins (Fig. 2). Thus, the general characteristics of degradation of serum proteins and intracellular proteins appear to be similarly affected by diabetes.

DISCUSSION

The double-isotope method has proved to be very valuable in providing information about the general features of protein degradation in normal tissues, since catabolic rates of many proteins can be compared in a single experiment (2). Now that several features of normal protein catabolism are known (2, 3), it is possible to compare the normal breakdown processes with those in various abnormal conditions. The present study indicates that the double-isotope technique can be modified so that $^3H/^{14}C$ ratios of proteins reflect their relative rates of degradation during diabetes and starvation. The enhanced protein degradation associated with these conditions is fundamentally different from normal protein catabolism.

Other workers who have compared normal protein degradation with the accelerated protein catabolism during nutrient deprivation have found that breakdown of stable cell proteins is preferentially enhanced (3, 27–30). Our results are consistent with these findings. In fact, the rates of breakdown of three distinct classes of stable proteins, those of small molecular weight, those of neutral-basic isoelectric points, and those that are nonglycoproteins, approach that of the labile proteins, which are large or acidic or contain carbohydrate groups.

The preferential degradation of more stable proteins in times of need would seem to be advantageous to the organism. Since stable proteins tend not to be crucial enzymes in metabolic regulation (1, 3), they could perhaps be sacrificed with the least disruption of the cell's functioning.

Possible Mechanisms. The enhanced protein degradation seen in diabetes and starvation could be due to an alteration in the degradative mechanism(s), a change in the structure of proteins that renders them more susceptible to proteolytic attack, or both.

Perhaps the simplest explanation that could account for our results would invoke a degradative apparatus during nutrient deprivation which is composed of at least two components, as

originally hypothesized by Schimke (1) and recently in more detail by Knowles and Ballard (27). Since the enhanced degradation probably involves the lysosomal system (12), the two proteolytic systems might be: (*i*) the normal protein degradative machinery, which is very poorly understood but which results in considerable heterogeneity in rates of catabolism of various proteins, and (*ii*) lysosomal autophagy in which all types of proteins are degraded at equivalent rates.

Degradation of proteins with longer half-lives would be preferentially accelerated by diabetes or starvation since the relative contribution of autophagy in determining their half-lives would be greater. For example, if under normal conditions large proteins were degraded at 4%/hr and small proteins at 2%/hr and an additional 2%/hr for each class is superimposed due to lysosomal autophagy, the degradative rate of large proteins would be enhanced 50% while that of small proteins would be increased 100%. This type of scheme would tend to equalize the degradative rates of various protein classes but could not completely abolish the differences in half-lives unless autophagy accounted for a very large proportion of the protein catabolism. This may be the case in severe diabetes, but other explanations of our results are also possible.

For example, unique proteases may contribute to the enhanced protein degradation in diabetes and starvation. Many nonlysosomal proteases exist in muscle and in liver, and the activities of some of them increase during starvation and diabetes (3, 31, 32). If some of these proteases preferred small, basic nonglycoproteins as substrates, the normal correlations between protein size and half-life, between protein isoelectric point and half-life, and between protein carbohydrate content and half-life could be reduced or abolished.

The accelerated degradation of proteins might also result from an increase in the sensitivity of cell proteins to proteolytic attack. If the protease sensitivity of the more stable proteins were preferentially increased, the three characteristics of normal protein degradation that we have examined could be abolished.

Our finding that degradation of serum proteins may be affected by diabetes similarly to degradation of intracellular proteins is very exciting. Since the pathway of catabolism of serum proteins is much better understood than that of intracellular proteins, determination of the mechanisms by which nutrients and hormones affect serum protein catabolism may be more easily studied.

In closing, we wish to emphasize that the results presented in this report apply as a general rule only. There are specific enzymes in cells that turn over rapidly under normal conditions and also show enhanced degradation in diabetes and starvation (33). Furthermore, degradation of certain stable proteins may be little affected or even reduced under such conditions (34). In these cases changes in levels of specific substrates, cofactors, or regulatory molecules (1–4) may also contribute to the altered degradative rates during nutrient deprivation.

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