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Amino Acid Recycling in Relation to Protein Turnover[1]

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ABSTRACT

Methods of measuring amino acid recycling in *Lemna minor* are described. The extent to which the recycling of individual amino acids may underestimate protein turnover has been measured for a number of amino acids. The methods have been used to study the relationship between protein turnover and amino acid recycling during nitrogen starvation. It is concluded that following the removal of nitrate from the environment, protein turnover is enhanced, the partitioning of amino acids between protein synthesis and amino acid metabolism is relatively constant, but the total amount of amino acids recycling is increased.

When growth is reduced by depriving the plant of an essential nutrient, it is reasonable to suppose that the life span of the protein might be increased with a corresponding decrease in the rate of synthesis of new protein. Bidwell *et al.* (1) have interpreted their experiments to mean that protein turnover is slow in slow growing cultures of carrot phloem explants. We have argued elsewhere (4) that the kinetic data of these experiments have been misinterpreted, because the kinetic treatment appropriate for a "label" experiment was applied to a "chase" experiment, so that the analysis not only gives the wrong rate, but the inverse of the true rate.

Measurements of protein degradation based on tritium labeling of protein have shown that when *Lemna minor* is deprived of nitrogen its growth rate is greatly reduced and protein degradation is increased (10). This suggests an alternative strategy which the plant might employ to adapt to the deprivation of nitrate. The plant could change its enzyme complement by rapidly synthesizing new proteins from amino acids formed by degradation of old proteins.

The reutilization of amino acids—amino acid recycling—is one of the main contributing factors to underestimating the rate of degradation of proteins. Protein half-lives are most frequently measured by labeling cells with radioactive amino acids and then following the decrease in trichloroacetic acid-precipitable radioactivity. The error in this method, which is produced by amino acid recycling, is well documented (13) and attempts have been made to evaluate the extent of amino acid recycling in animal tissues (3, 16). Koch (14) concluded that "no matter how an isotope experiment is conducted the true half-life cannot be calculated without information additional to the time course of activity in the component under study."

We have adapted the technique of density labeling pioneered by Varner (18) to measure amino acid recycling. This method produces visually attractive results, but lacks precision. We have therefore concentrated on measuring the true half-life of protein by a tritium-labeling technique (11) and have applied the method to study the effect of nitrogen starvation on amino acid recycling in *L. minor* and *Schizosaccharomyces pombe*.

MATERIALS AND METHODS

Plant Materials. *L. minor* was isolated from the River Yare by A. Trewavas (School of Botany, University of Edinburgh) and maintained in a sterile culture as described by Trewavas (17) except that wide necked (76-mm) conical flasks replaced Roux bottles. *S. pombe* 972h⁻ was provided by A. Coddington (School of Biological Sciences, University of East Anglia) and was cultured in complete minimal medium (6) at 30 C.

Chemicals. Deuterium oxide (99.8%) was obtained from Prochem, Ltd., Croydon, Surrey, U.K. The following radioactive compounds were obtained from The Radiochemical Centre, Ltd., Amersham, Bucks., U.K.: ^3H_2O (90 mCi/μmol), L-[U-^{14}C]arginine monohydrochloride (0.3 mCi/μmol), L-[U-^{14}C]glutamic acid (0.3 mCi/μmol), L-[U-^{14}C]isoleucine (0.27 mCi/μmol), L-[U-^{14}C]leucine (0.32 mCi/μmol), DL-[4,5-^{3}H]leucine (27 mCi/μmol), and L-[U-^{14}C]lysine monohydrochloride (0.33 mCi/μmol). PPO (2,5-diphenyloxazole) and acetic anhydride were obtained from B.D.H., Poole, Dorset, U.K. All other chemicals were obtained from local sources and were of the best grade available.

Density Labeling with ^2H_2O. The radioactive and density labeling of *L. minor* protein and the subsequent extraction and isopycnic centrifugation on KBr gradients were as previously described (2).

Labeling with ^3H_2O and ^{14}C-Amino-Acids. *Lemna* fronds were grown on a complete medium containing ^3H_2O (1 mCi/ml) and 1 μCi/ml of either L-[U-^{14}C]leucine, L-[U-^{14}C]arginine, L-[U-^{14}C]lysine, L-[U-^{14}C]glutamic acid, L-[U-^{14}C]isoleucine, or L-[U-^{14}C]lysine for 48 hr. The fronds were then transferred to a complete growth medium lacking ^{3}H and ^{14}C-cultured for up to 9 days. Samples were taken at intervals and the 2-^{3}H and ^{14}C content of soluble protein measured.

S. pombe was grown from a small inoculum in medium containing ^3H_2O (1 mCi/ml) and L-[U-^{14}C]leucine (1 mCi/ml) for 2 hr. The cells were then harvested by centrifugation, washed with ^{14}C and ^{3}H-free medium, and transferred to either complete minimal medium or K-phosphate buffer (0.1 M, pH 7). Samples were taken at hourly intervals for 12 hr and the 2-^{3}H and ^{14}C content of the extracted protein measured.

Counting of Radioactive Labeled Protein. Protein labeled with ^3H_2O and ^{14}C was isolated and hydrolyzed as described previously (10). In double-label experiments, the amino acid hydrolysates were oxidized to $^{14}CO_2$ and ^3H_2O in a biological material oxidizer (R. J. Harvey Instrument Corp., Hillsdale, N.J.). $^{14}CO_2$ was counted for radioactivity in 15 ml of toluene-methanol-2-phenylethylamine/PPO scintillant (900:400:200:7, v/v/v/w). The 2-^{3}H content was determined in duplicate samples by using the method of racemization with acetic anhydride as described by Humphrey and Davies (11). Protein labeled with ^2H_2O and ^{14}C was counted for ^{14}C as described previously (2).

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RESULTS

Measurement of Amino Acid Recycling and Protein Degradation Using Density Labeling. *Lemna* fronds (1 g fresh wt) were floated on 100 ml of culture medium containing deuterium oxide (50%). After 7 days L-[U-^{14}C]leucine (2.5 μCi) was added and after 8 hr the plants were filtered, washed with 50 ml of growth medium containing 50% ^2H_2O. The fronds were transferred back to 100 ml of the deuterium-containing medium for a further 40 hr. The plants were removed by filtration and washed with 500 ml of sterile medium before being transferred (about 400 fronds/flask) to flasks containing 1 liter of deuterium-free growth medium with or without nitrate. At various time intervals, samples were removed, protein was extracted, and the distribution of ^{14}C in "heavy" and "light" protein determined after isopycnic centrifugation (Fig. 1).

The heavier-half area of the peak of heavy protein was measured for every sample and corrected for the amount of protein introduced onto the gradient and for the amount of growth as measured by frond number. The logarithm of the normalized half-peak areas gave a linear relationship with respect to time from which the true half-life of the total soluble protein can be obtained (Fig. 2). The plot of the logarithm of the normalized total peak area against time gives an apparent half-life of the total soluble protein which is slower than the true rate, due to the recycling of amino acids which is to be seen (Fig. 1) in the transfer of radioactivity from the heavy fraction to the light fraction.

The results of a similar experiment in which after labeling with deuterium and [^{14}C]leucine the fronds were transferred to a growth medium from which nitrate had been omitted, are shown in Figure 3.

Measurement of Amino Acid Recycling and Protein Degradation Using Tritiated Water and ^{14}C-labeled Amino Acids. The method of determining the rate constants of protein degradation by measuring the loss of ^{3}H from C-2 of protein amino acids (11) minimizes the effect of amino acid recycling due to the speed of the transaminase reaction. Assuming that the ^{3}H method gives the "true" half-life of protein degradation, feeding ^{14}C-labeled amino acids will give the "apparent" half-life of protein degradation in which the longer half-life is due to recycling of the individual amino acids.

Lemna fronds (1 g fresh wt) were floated on 100 ml of growth medium containing ^3H_2O (1 mCi/ml) and a ^{14}C-labeled amino acid (1 μCi/ml) and cultured for 48 hr. The fronds were washed with 2 liters of unlabeled growth medium and then transferred to unlabeled growth medium for periods up to 9 days. At various intervals, samples were removed and the amount of 2-^{3}H and ^{14}C present in the protein determined. The

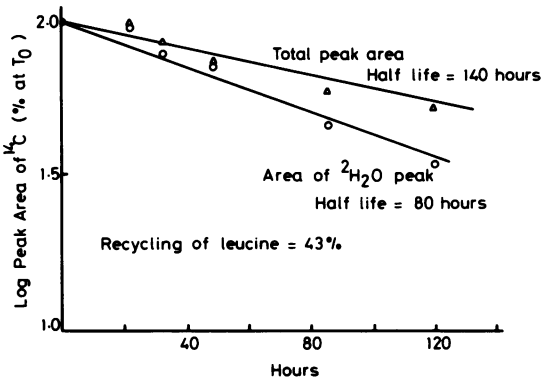

FIG. 2. Measurement of protein degradation in *Lemna* grown on complete medium. Data obtained in Figure 1 are plotted as the logarithm of the normalized peak areas against time. △——△: total peak area, which gives an apparent rate of degradation; ○——○: heavy peak area, which gives the true rate of degradation.

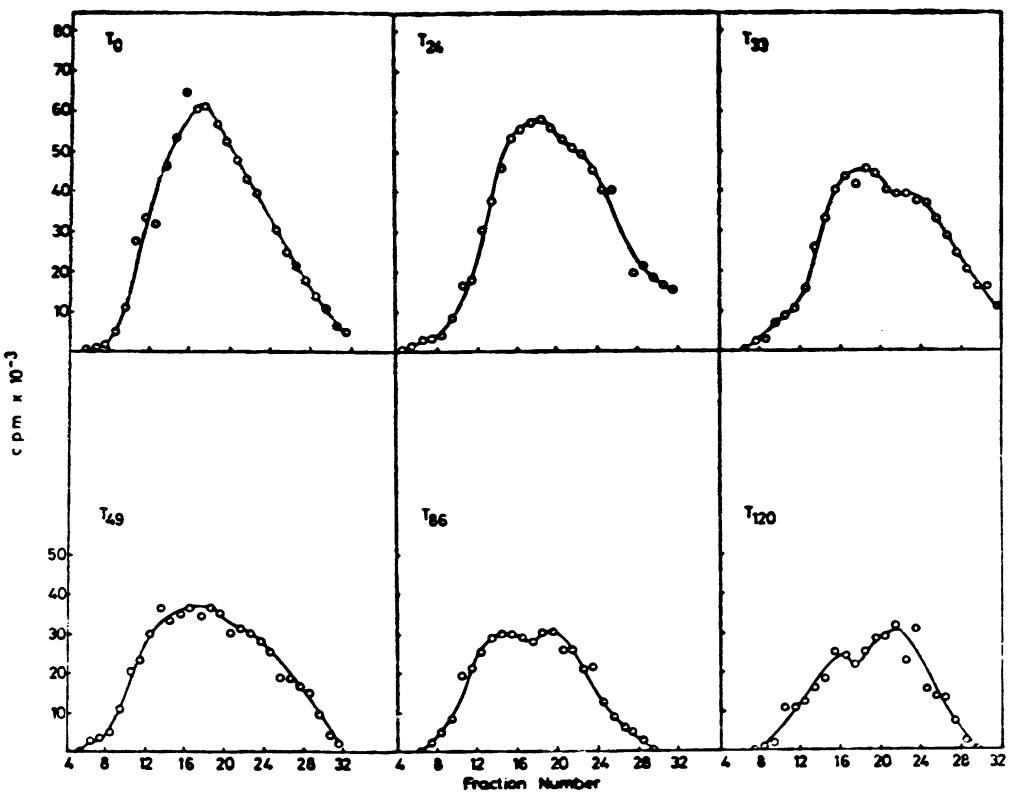

FIG. 1. Measurement of protein turnover by density labeling coupled to exposure to [^{14}C]leucine to label protein. *Lemna* fronds were maintained in a culture medium containing NO_3^- throughout the experiment. Proteins were isolated and fractionated as described in the text.

results presented in Figure 4 show that the apparent half-life of protein varies with the amino acid used.

The effect of nitrate deprivation on the rate of protein degradation and on the apparent rate of protein degradation was determined by labeling with ^3H_2O and L-[U-^{14}C]leucine then transferring one sample to an unlabeled growth medium lacking nitrate, the other to a complete medium. The results are shown in Figure 5.

Protein Turnover and Amino Acid Recycling in *S. pombe*. The true and apparent rate of protein degradation in *S. pombe* was determined by labeling cells with ^3H_2O and L-[U-^{14}C]leucine, then transferring them to unlabeled complete growth medium or to phosphate buffer (pH 7, 0.1 M). The

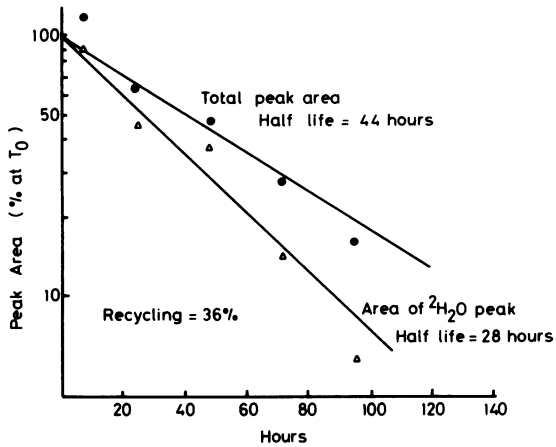

FIG. 3. Measurement of protein degradation in *Lemna* grown on a medium lacking nitrogen. Data were obtained as in Figure 1 except that when the fronds were transferred from the deuterium-containing medium they were cultured in a nitrogen-free growth medium. △——△: total peak area; ●——●: heavy peak area.

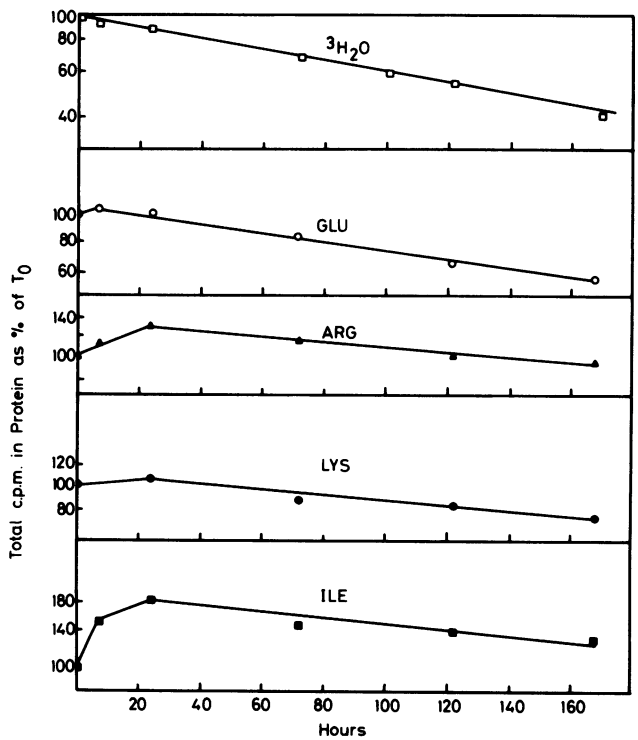

FIG. 4. Comparison of apparent rates of protein degradation in *L. minor* obtained with various ^{14}C-amino-acids and the rate determined by the 2-^{3}H method of Humphrey and Davies (10).

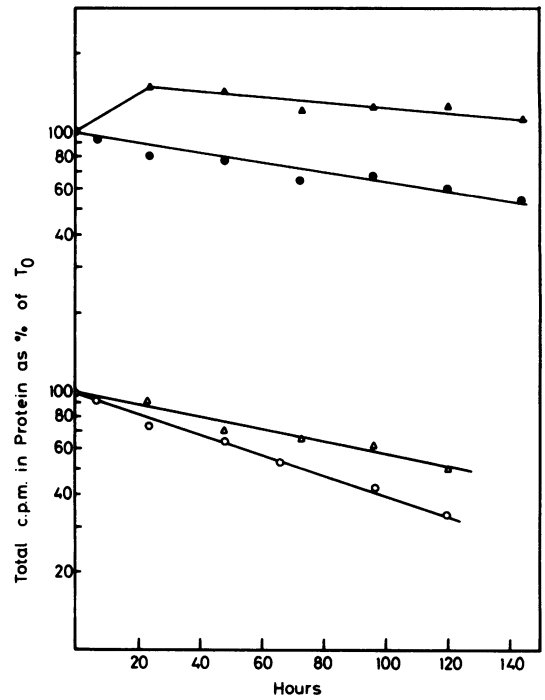

FIG. 5. Comparison of rates of protein degradation in *L. minor* cultured on a complete medium and on a nitrogen-deficient medium. ▲——▲: [^{14}C]leucine-complete growth medium; ●——●: ^3H_2O-complete growth medium; △——△: [^{14}C]leucine-NO_3; ○——○: ^3H_2O-NO_3.

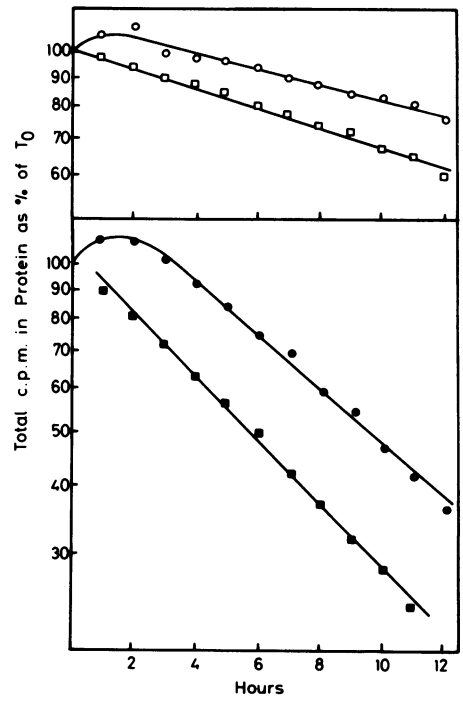

FIG. 6. Comparison of rates of protein degradation in *S. pombe* cultured on a complete growth medium and on K-phosphate buffer (0.1 M, pH 7.4). ○——○: [^{14}C]leucine-complete medium; □——□: ^3H_2O-complete medium; ●——●: [^{14}C]leucine-K-phosphate; ■——■: ^3H_2O-K-phosphate.

effect of starvation on protein turnover and amino acid recycling is shown in Figure 6.

DISCUSSION

The methods of measuring the "true" rate of protein degra-

dation used in this paper depend on the rapid transaminase-catalyzed incorporation of ^3H or ^2H at C-2 of free amino acids (15) and assume stabilization of this attachment when the amino acids enter protein. The rationalization for this assumption is that the protein-bound amino acids do not undergo transamination. However, the assumption can be questioned on the grounds that since H on the amide N of the peptide link rapidly exchanges with water (5), there must be *some* exchange of the H on the 2-C atom. The question is, at what rate does this exchange take place? In unpublished experiments we have attempted to measure the rate of exchange of hydrogen at the 2-C atom of protein-bound arginine and were unable to measure any significant exchange. Fortunately work with fossils shows that the half-life of racemization is very long (*e.g.* for aspartate, 15,000 years [7]) and we can therefore neglect exchange reactions at the 2-C position of protein-bound amino acids.

A simple model of protein turnover and amino acid recycling is given by

$$\begin{array}{c} \text{Carbohydrates} + NH_4^+ \\ \downarrow k_4 \\ \text{Protein} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{Amino acids} \xrightarrow{k_3} CO_2 + NH_4^+ \end{array}$$

in which the rate constants are the summation of many individual rate constants. The kinetic analysis of even this simple model leads to complex rate equations. However, we can obtain a simple expression for the fraction of amino acid recycling if we assume that the protein precursor pool of amino acids is small. This assumption has been previously employed by Kemp and Sutton (12) in their method for measuring protein turnover in tobacco leaves and evidence that the protein precursor pools of leucine and valine are small in soybean hypocotyls has been obtained by Holleman and Key (8).

The true rate of protein degradation equals

$$P'e^{-k't}$$

where P' represents ^{14}C in the heavy protein fraction (Fig. 1) or ^3H in protein labeled by the ^3H_2O method and k' is a first order rate constant.

The apparent rate of protein degradation equals

$$P''e^{-k''t}$$

where P'' represents the ^{14}C in total protein and k'' is a first order rate constant.

The proportion of amino acid not recycled equals

$$\frac{k_3}{k_2 + k_3} = \frac{k''}{k'}$$

The proportion of amino acid recycled equals

$$1 - \frac{k''}{k'} = 1 - \frac{\text{true half-life of protein}}{\text{apparent half-life of protein}}$$

Amino acid recycling is visually apparent in the density labeling experiment (Fig. 1) in which the rate of protein degradation is seen as the decrease in counts in the "heavy" peak and recycling of leucine is seen as the rise in counts in the "light" peak. Thus if the percentage amino acid recycling was 100% then the increase in the light peak would equal the decrease in the heavy peak, so there would be no decrease in the total peak area and the apparent rate of protein degradation would be zero.

$$\%\ \text{amino acid recycling} = \left(1 - \frac{\text{half life from "heavy" protein}}{\text{half life from total protein}}\right) \times 100$$

In the double labeling experiments employing ^3H_2O and a ^{14}C-amino-acid, the determination of the apparent half-life of protein by the ^{14}C method requires that the first 24 hr be ignored, due to the continued incorporation of ^{14}C-amino-acids into protein in the early part of the "chase" period. Subsequently, the plot of log percentage counts remaining in protein against time is linear, giving an apparent half-life for protein. Hence,

$$\%\ \text{amino acid recycling} = \left(1 - \frac{\text{half-life by } ^3H_2O \text{ method}}{\text{half-life by } ^{14}C\text{-amino-acid}}\right) \times 100$$

Table I. Recycling of amino acids in *Lemna minor* growing in a complete medium

Method	Amino acid	Recycling %
Density labeling	Leucine	43
^3H + ^{14}C labeling	Leucine	50
^3H + ^{14}C labeling	Glutamate	29
^3H + ^{14}C labeling	Arginine	48
^3H + ^{14}C labeling	Lysine	45
^3H + ^{14}C labeling	Isoleucine	50

Table II. Effect of environmental changes on protein turnover and amino acid recycling

Organism	Growth conditions	Protein half life	Leucine recycled
		hr	%
Lemna minor	Full growth medium	143	50
	$-NO_3^+$	73	42
	Full growth medium + ^2H_2O	80	43
	$-NO_3 + {}^2H_2O$	28	36
Schizosaccharomyces pombe	Full medium	24	30
	K phosphate buffer	6.2	20

Data on amino acid recycling implicit in Figures 2, 4, and 5 are collected in Table I. This table provides quantitative confirmation of the amount of recycling which would be anticipated for individual amino acids based on their involvement in general metabolism. Thus glutamate would be expected to be more involved in amino acid metabolism than leucine so that the percentage of glutamate recycled would be less than that of leucine. Hence the choice of glutamate by Holmsen and Koch (9) to minimize amino acid recycling is justified by the data in Table I, whereas the choice of leucine for use in protein degradation studies by Kemp and Sutton (12) clearly leads to an underestimation of the rate of protein degradation. The table also provides a correction factor to enable the apparent half-life of protein determined by the usual method employing ^{14}C-amino-acids to be converted into a true half-life.

The relationship between protein turnover and amino acid recycling during starvation has been examined from *L. minor* deprived of nitrogen and *S. pombe* deprived of nutrients. The results are collected in Table II.

Within the limitations of the simplified model of amino acid recycling and the assumption of a small pool of protein precursor amino acids, it is clear that the percentage of amino acid recycling is not increased by starvation. On the other hand the *amount* of amino acid recycled is greatly increased during starvation, for example the ratio:

$$\frac{\text{amount of leucine recycled in absence of } NO_3}{\text{amount of leucine recycled in full medium}}$$

is from the data of Table II seen to be 1.7 for *Lemna* and 2.6 for *Schizosaccharomyces*. This increase in amino acid recycling is consistent with a strategy to exchange one set of enzymes for another set, to meet a change in the environment.

LITERATURE CITED

1. Bidwell GSR, RA Barr, FC Steward 1964 Protein synthesis and turnover in cultured plant tissues: sources of carbon for synthesis and the fate of the protein breakdown products. Nature 203: 367-373
2. Boudet A, TJ Humphrey, DD Davies 1975 The measurement of protein turnover by density labeling. Biochem J 152: 409-416
3. Dallman PR, EC Manies 1973 Protein deficiency: turnover of protein and reutilization of amino acid in cell fractions of rat liver. J Nutrition 103: 257-266
4. Davies DD, J Humphrey 1976 Protein turnover in plants. *In* N Sunderland, ed, Perspectives in Experimental Biology, Vol 2, Botany. Pergamon Press, New York

5. ENGLANDER SW, NW DOWNER, H TEITELBAUM 1972 Hydrogen exchange. Annu Rev Biochem 41: 903-924
6. GUTZ H, H HESLOT, U LEUPOLD, N. LOPRIENO 1974 *Schizosaccharomyces pombe. In:* RC King, ed, Handbook of Genetics, Vol 1. Plenum Press, New York, pp 395-446
7. HELFMAN PM, JL BADA 1975 Aspartic acid racemization in tooth enamel from living humans. Proc Nat Acad Sci USA 72: 2891-2894
8. HOLLEMAN JM, JL KEY 1966 Inactive and protein precursor pools of amino acids in the soybean hypocotyl. Plant Physiol 42: 29-36
9. HOLMSEN JW, AL KOCH 1964 An estimate of protein turnover in growing tobacco plants. Phytochemistry 3: 165-172
10. HUMPHREY TJ, DD DAVIES 1975 A new method for the measurement of protein turnover. Biochem J 148: 119-127
11. HUMPHREY TJ, DD DAVIES 1976 A sensitive method for measuring protein turnover based on the measurement of 2-^{3}H-labeled amino acids in protein. Biochem J 156: 561-568
12. KEMP JD, DW SUTTON 1971 Protein metabolism in cultured plant tissues. Calculation of an absolute rate of protein synthesis, accumulation and degradation in tobacco callus *in vivo*. Biochemistry 10: 81-88
13. KLEVECZ RR 1971 Rapid protein catabolism in mammalian cells is obscured by reutilization of amino acids. Biochem Biophys Res Commun 43: 76-81
14. KOCH AL 1962 The evaluation of the rates of biological processes from tracer kinetic data. 1. The influence of labile metabolic pools. J Theor Biol 3: 283-303
15. MITRA R, J BURTON, JE VARNER 1976 Deuterium oxide as a tool for the study of amino acid metabolism. Anal Biochem **70:** 1-17
16. SWICK RW 1958 Measurement of protein turnover in rat liver. J Biol Chem 231: 751-764
17. TREWAVAS A 1970 The turnover of nucleic acids in *Lemna minor*. Plant Physiol 45: 742-751
18. VARNER JE 1971 The control of enzyme formation in plants. SEB Symp 25: 197-205