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A New Method for the Measurement of Protein Turnover

By THOMAS J. HUMPHREY and DAVID D. DAVIES
School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ, U.K.

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A new technique for the determination of rate constants of protein degradation is described. By using the method, half-lives of total soluble protein of *Lemna minor* during growth on full culture medium and distilled water were measured. The method involves incubating *Lemna* on a growth medium containing ^3H_2O. After a short exposure (20min) to ^3H-labelled culture medium, ^3H was found in soluble amino acids, especially aspartate, glutamate, glutamine and alanine. After transfer to a ^3H-free medium for 30min, 80% of the ^3H originally present in soluble amino acids was lost. These results suggest that ^3H enters and leaves amino acids at the α-carbon atom, a conclusion supported by the observed labelling of glutamate. The exchange of H and ^3H on the α-carbon atom is catalysed by transaminases and the speed of this exchange ensures that when the ^3H_2O is removed, the ^3H in free amino acids is rapidly lost, thereby eliminating problems connected with metabolic pools and recycling. After an exposure of 20min to ^3H-labelled medium all protein amino acids, except for arginine, were found to be radioactive. The loss of radioactivity from protein amino acids was used to measure protein degradation.

Most studies on protein turnover attempt to measure the incorporation of radioactive amino acids into protein and their subsequent loss from protein. In such studies, the existence of metabolic pools makes it difficult to allow for recycling of label and also to determine the specific radioactivity of the amino acid at the site of protein synthesis. In bacteria, pool exchange does not appear to be a serious problem (Pine, 1972), but in animals and animal-cell cultures difficulties are encountered (Munro, 1970). Pool exchange presents serious difficulties in plants, partly owing to the complex compartmentation of plant cells (Oaks & Bidwell, 1970) and partly because of the fact that extracellular amino acid traps to dilute the radioactive amino acids produced by protein degradation cannot be used (Oaks, 1965; Joy, 1969; Borstlap, 1972). Some authors have attempted to minimize the effects of recycling by using labelled glutamate (Holmsen & Koch, 1964), others have used labelled leucine to minimize the effect of storage pools (Kemp & Sutton, 1971), and others (Dice *et al.*, 1973) have used the double-labelling technique of Arias *et al.* (1969) to obtain comparative rates of protein degradation. The pioneering studies of Steward *et al.* (1956, 1958) identified all the difficulties associated with measuring protein turnover in plants, and subsequently Bidwell *et al.* (1964) attempted to analyse a large body of experimental data by using three equations derived by Hellebust & Bidwell (1963). We have recently questioned the validity of these equations and argued that a kinetic analysis of the experiments conducted by Steward and his colleagues is impossibly difficult (Davies & Humphrey, 1974). An alternative approach is necessary, and previous work from this laboratory (Trewavas, 1972) sought to overcome some of the difficulties by measuring the specific activity of aminoacyl-tRNA. The method has advantages over the more usual methods examined by Trewavas (1972), but is technically difficult, and we have explored alternative methods.

The method we suggest is based on the assumption that when cells are incubated with ^3H_2O, ^3H rapidly equilibrates with hydrogen on the α-carbon atoms of most amino acids, owing to an exchange reaction catalysed by transaminases (Hilton *et al.*, 1954). Some ^3H will also exchange with hydrogen on the β-carbon atoms of some amino acids owing to the keto–enol equilibrium of the oxo acid involved in the transaminase reaction. When cells are transferred back to water the transaminase ensures that the reverse sequence occurs and hydrogen replaces ^3H on the α-carbon. However, if an amino acid labelled with ^3H on the α-carbon atom is incorporated into protein, the ^3H is no longer exchangeable and remains in the protein when the cells are transferred back to water, but if the protein undergoes degradation to amino acids, transaminases ensure that ^3H is lost from the amino acids.

These exchange reactions, catalysed by transaminases, largely eliminate the problems of pools and recycling, so that to measure protein degradation it is only necessary to give cells a brief exposure to ^3H_2O, then transfer the cells back to water and measure the amount of ^3H present in protein as a function of time.

In the present paper we are concerned to check the validity of the assumptions and to apply the method to a plant (*Lemna minor*) which can be easily maintained in sterile culture.

Materials and Methods

Materials

Plant material. Lemna minor was isolated from the River Yare by Dr. A. Trewavas (School of Botany, University of Edinburgh) and maintained in a sterile culture in continuous light as described by Trewavas (1970).

Chemicals. Dowex resins, Triton X-100 and Tris base (under the trade name Trizma) were obtained from Sigma (London) Chemical Co., London S.W.6, U.K. L-[U-^{14}C]Glutamate (specific radioactivity 225μCi/μmol), DL-[2-^{4}H]glutamate (specific radioactivity 3.6mCi/μmol), L-[U-^{14}C]leucine (specific radioactivity 348mCi/μmol) and $^{3}H_2O$ (specific radioactivity 90μCi/μmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. 2,5-Diphenyloxazole (PPO) was obtained from British Drug Houses, Poole, Dorset, U.K., and Pronase, ribonuclease and deoxyribonuclease were obtained from Boehringer Corp. (London) Ltd., London W.5, U.K. C3 sulphonated polystyrene resin was obtained from Technicon, Basingstoke, Hants., U.K. Other chemicals were of the best reagent grade obtainable.

Methods

Labelling with ^{3}H. All manipulations were carried out under sterile conditions. *Lemna* fronds (0.2g fresh wt.) were removed from the growth medium and floated on 20ml of ^{3}H-labelled growth medium. The specific radioactivity was varied, 100mCi/ml being used to measure the incorporation of ^{3}H into the individual amino acids of protein, 20mCi/ml to study the incorporation into the individual soluble amino acids and 5mCi/ml for the routine determination of protein turnover.

In chase experiments, the fronds were removed from the tritiated medium, washed with 2 litres of ^{3}H-free medium and transferred to 1 litre of ^{3}H-free medium.

Counting of ^{3}H-labelled compounds. ^{3}H was measured by liquid-scintillation counting. Each sample was counted four times for 4min; the standard error never exceeded 5%. Counting efficiency was determined by adding an internal standard.

Isolation and counting of ^{3}H-labelled soluble amino acids. Lemna fronds (0.2g fresh wt.) were frozen in liquid N_2, ground to a fine powder and extracted with 3ml of trichloroacetic acid (5%, w/v). The extract was centrifuged at 20000*g* for 10min; the supernatant was decanted and stored, and the precipitate was extracted with a further 2ml of trichloroacetic acid. This extract was centrifuged as described above, and the supernatant was added to the first supernatant and stored overnight at 0°C. The combined extracts were clarified by centrifuging at 30000*g* for 10min and 20ml of water was added to the supernatant before passage through a column (5cm×1cm) of Dowex 50 (acid form). The column was washed with water until the eluate was free from ^{3}H (at least 100ml). The amino acids were eluted with 6ml of NH_3 (1.6M) and evaporated to dryness. When samples were to be stored at −15°C the amino acids were dissolved in 0.5ml of ethanol (80%, v/v) (Hempel, 1963). For immediate counting of radioactivity, samples were dissolved in 0.5ml of water, taken up in 15ml of a toluene–Triton X-100–PPO scintillant (1000:300:3, v/v/w) and counted in an Intertechnique SL 30 liquid-scintillation spectrometer. For the separation of amino acids, 0.1ml samples of the aqueous extract were resolved on a two-column Technicon amino acid analyser. Both columns contained C-3 resin; the column (12cm×0.6cm) for separating basic amino acids was run at 60°C and the column (50cm×0.6cm) for separating neutral and acid amino acids was run at 40°C. The basic amino acids were separated by using a single buffer [NaOH (0.6M), citric acid (0.1M), EDTA (disodium salt; 2.5mM) adjusted to pH5.20 with HCl]. The acidic and neutral amino acids were separated by stepwise elution with the following buffers, in which the final pH was adjusted with concentrated HCl.

(1) LiOH (0.2M)–citric acid (0.033M)–EDTA (disodium salt; 2.5mM)–2-methoxyethanol (2%, v/v), pH3.00.

(2) LiOH (0.2M)–citric acid (0.033M)–EDTA (disodium salt; 2.5mM), pH3.20.

(3) LiOH (0.3M)–citric acid (0.05M)–EDTA (disodium salt; 2.5mM), pH3.60.

(4) LiOH (0.3M)–citric acid (0.05M)–EDTA (disodium salt; 2.5mM), pH4.20.

The flow rate of both columns was 0.5ml/min and the effluent was split so that 25% passed through the analyser for development with ninhydrin and 75% was collected in 30s fractions for the determination of ^{3}H by being dissolved in 15ml of toluene–Triton X-100–PPO scintillant (1000:300:3, v/v/w) and counted for radioactivity in an Intertechnique liquid-scintillation spectrometer.

Isolation and counting of ^{3}H-labelled amino acids in soluble proteins. After exposure to a ^{3}H-labelled medium, *Lemna* fronds (0.2g) were frozen in liquid N_2, ground to a fine powder and extracted with 3ml of Tris buffer (pH9.7, 0.125M). The extract was centrifuged at 20000*g* for 10min, the supernatant was decanted into 3ml of trichloroacetic acid (10%, w/v) and stored overnight at 2°C. The suspension was

centrifuged at 30000*g* for 10min and the pellet washed by suspension in 10ml of acid–alcohol (ethanol–0.1M-HCl; 11:1, v/v), followed by centrifugation. The pellet was extracted with 1.0ml of NaOH (0.75M) for 2h at 37°C; Tris (4ml; pH9.7; 0.5M) was added and the solution transferred to a dialysis sac. Dialysis was continued for 2h at 40°C against a running solution of NaOH (10mM) at a flow rate of 1 litre/min then against running tap water for a further 15min. The protein solution was transferred to a centrifuge tube, 1ml of trichloroacetic acid (20%, w/v) was added and the suspension heated at 100°C for 30min (Schneider, 1957). After cooling, the suspension was centrifuged at 20000*g* for 10min and the pellet washed by suspension in 10ml of acid–alcohol followed by centrifugation. The proteins were mixed with 10ml of trichloroacetic acid (1%, w/v) and 5g of Dowex 50 (acid form). The mixture was poured into a column (10cm×1cm) and the washings were passed through twice to ensure complete adsorption of the protein. The packed column was washed with 50ml of water, sealed and heated at 100°C for 20h. The column was unsealed, washed with water (100ml) and then eluted with 6ml of NH_3 (1.6M). The eluate was evaporated to dryness and the amino acids were taken up in 0.5ml of ethanol (80%, v/v) and stored at −15°C. When the routine method of measuring protein turnover was involved, the sample was dissolved in 15ml of toluene–Triton X-100–PPO scintillant (1000:300:3, v/v/w) and counted for radioactivity in an Intertechnique liquid-scintillation spectrometer.

The amino acids were separated as described for the soluble amino acids except that different buffers were used and the columns were of different size.

Basic amino acids were eluted from a column (4.5cm×0.6cm) with a solution containing NaOH (0.37M), citric acid (0.1M) and EDTA (disodium salt; 2.5mM), adjusted to pH5.25 with concentrated HCl.

The neutral and acidic amino acids were eluted from a column (23.5cm×0.6cm) by stepwise elution with two buffers.

(A) NaOH (0.2M)–citric acid (0.1M)–2-methoxyethanol (6%, v/v)–EDTA (disodium salt; 2.5mM), adjusted to pH3.25 with HCl.

(B) NaOH (0.2M)–citric acid (0.1M)–EDTA (disodium salt; 2.5mM), adjusted to pH4.25 with HCl.

The effluent was split and analysed as described for the soluble amino acids.

Measurement of protein turnover by the method of Hellebust & Bidwell (1963). *Lemna* fronds were floated on a culture medium containing [U-^{14}C]-glutamate (0.5μCi/ml; 1.82μM) for 24h, then transferred to a ^{14}C-free culture medium. Weighed samples were removed at 0, 20 and 48h, frozen in liquid N_2, ground to a fine powder and protein and soluble amino acids separated by boiling in ethanol (80%, v/v) as described by Hellebust & Bidwell (1963). The separation and radioactivity counting of the amino acids by the method of Hellebust & Bidwell (1963) involves filter-paper chromatography. The Technicon amino acid analyser was used to separate amino acids. The method used was as described for the separation of protein amino acids except that the effluent was split so that 50% was passed through an anthracene-coil flow-cell placed in a Packard liquid-scintillation spectrometer for counting ^{14}C and the other 50% of the effluent was passed through the analyser for development with ninhydrin.

Measurement of protein turnover by the method of Kemp & Sutton (1971). *Lemna* fronds (0.5g) were floated on 50ml of culture medium containing [U-^{14}C]leucine (0.1μCi/ml; 0.29μM) for 7h. A sample was fractionated into soluble amino acids and protein as described by Kemp & Sutton (1971) except that a Technicon amino acid analyser was used. Since leucine emerges near the end of a normal run, the run time was shortened by omitting the first three buffers used for the separation of soluble amino acids. The column (50cm×0.6cm) was equilibrated with a solution containing LiOH (0.3M)–citric acid (0.05M)–EDTA (disodium salt; 2.5mM), adjusted to pH4.20 with concentrated HCl. The amino acids were applied to the column and eluted with the same buffer solution. The eluate was split, 50% for ninhydrin development and 50% passing through the anthracene coil.

Results

Effect of ^3H_2O on growth

When *Lemna* fronds were exposed to tritiated medium containing 5, 20 or 100mCi/ml for 20min, then transferred back to unlabelled medium, no effect on growth could be detected.

Uptake of ^3H_2O by Lemna

Lemna fronds (10g) were mixed with 10ml of ^{3}H-labelled culture medium (1mCi/ml) and the uptake of ^{3}H from the medium was measured. The movement of ^3H_2O into the fronds is rapid, 75% of the ^{3}H required to produce equilibrium entering within 1min (Fig. 1).

Incorporation of ^3H into soluble amino acids

Lemna fronds (0.2g fresh wt.) were floated on 20ml of ^{3}H-labelled culture medium (20mCi/ml) and the incorporation of ^{3}H into total soluble amino acids was determined at various time-intervals. Other samples were incubated with ^{3}H-labelled medium for various time-intervals, then transferred to a ^{3}H-free medium, and the amounts of ^{3}H remaining in the amino acids were determined at various time-intervals. The results shown in Fig. 2 indicate a rapid labelling of the amino acids during the incubation in

^{3}H-labelled medium, followed by a rapid loss of ^{3}H when the fronds were transferred to ^{3}H-free medium. Thus 80% of the ^{3}H incorporated into soluble amino acids during a 20min labelling period was lost during the 30min chase period.

The distribution of ^{3}H between the various soluble amino acids was determined after the fronds had been exposed to ^{3}H-labelled medium (20mCi/ml) for 20min and also after a further 30min on ^{3}H-free medium. The extensive labelling of alanine, aspartate,

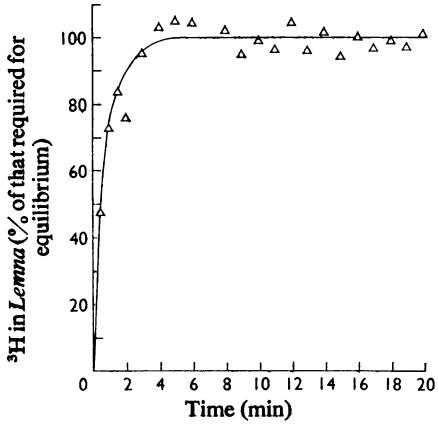


Fig. 1. *Movement of ^3H_2O into Lemna minor*

Fronds (10g fresh wt.) containing 94% water were mixed with 10ml of ^{3}H-labelled medium (1mCi/ml). Samples (10μl) were removed at the times indicated and counted for radioactivity.

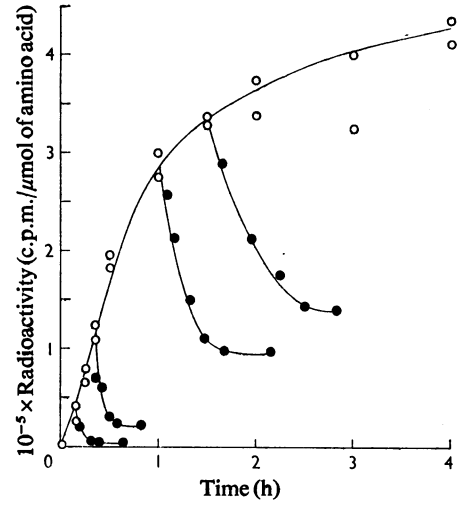


Fig. 2. *Incorporation of ^3H into soluble amino acids of Lemna and loss of ^3H from amino acids during chase on unlabelled medium*

For experimental details, see the text. ○, ^{3}H incorporated into amino acids during labelling period. ●, ^{3}H remaining in amino acids during chase period.

Table 1. *^3H incorporation into soluble amino acids of Lemma*

Lemna was incubated for 20min in ^{3}H-labelled medium (20mCi/ml) and chased for 30min in unlabelled medium.

Amino acid	Amino acid concentration (μmol/g fresh wt.)	Radioactivity after 20min labelling: 10^{-3} × ^{3}H in amino acids (d.p.m.)	Radioactivity after 20min labelling: 10^{-3} × Specific radioactivity (d.p.m./μmol)	Radioactivity after 30min chase: 10^{-3} × ^{3}H in amino acid (d.p.m.)	Radioactivity after 30min chase: 10^{-3} × Specific radioactivity (d.p.m./μmol)
Tyr	0.225	7.5	33.3	0	0
Phe	0.81	15.0	18.5	5.0	6.18
Lys	0.194	20.0	103.0	15.0	77.4
His	0.12	5.0	41.7	5.0	41.7
Arg	1.1	0	0	0	0
Asp	1.14	357.5	314.0	22.5	20.19
Thr	0.165	17.5	106.0	5.0	30.3
Ser	0.72	125.0	174.0	5.0	6.95
Asn	1.1	57.5	52.2	45.0	40.9
Glu	1.61	480.0	298.0	92.5	57.5
Gln	1.37	292.5	213.5	132.5	96.7
Gly	0.42	9.0	21.45	5.0	11.9
Ala	0.59	210.0	356.0	10.0	17.0
Cys	0.21	15.0	71.4	5.0	23.8
Val	0.135	20.0	148.0	10.0	74.0
Ile	0.142	0	0	0	0
Leu	0.18	12.2	67.8	0	0
10^{-3} × Total radioactivity (d.p.m.)		1643.2		357.5	

glutamate and glutamine, and the rapid loss of label during the chase period (Table 1), are consistent with the known metabolic reactions of these amino acids involving exchange of the hydrogen atoms on their α-carbon atoms. Similarly, the absence of label from arginine is consistent with the fact that the hydrogen on the α-carbon atom of arginine is derived by net synthesis from glutamate.

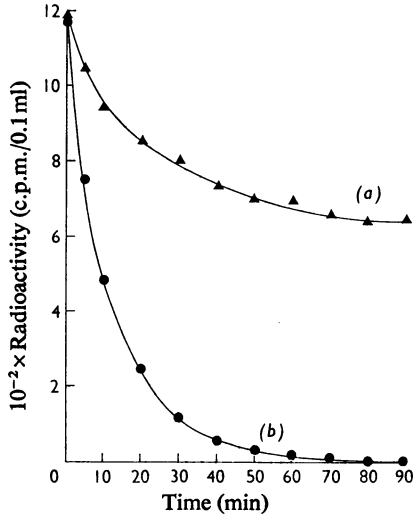


Fig. 3. *Effect of dialysis on ^3H-labelled protein*

Protein was precipitated and dialysed as described in the text. At intervals samples (0.1 ml) were removed and counted for radioactivity. (*a*) Protein labelled *in vivo* by exposing *Lemna* to ^3H-labelled medium (5 mCi/ml) for 20 min. (*b*) Protein extracted from *Lemna*, then equilibrated with ^3H_2O (5 mCi/ml) for 120 min.

Incorporation of ^3H into protein

Hydrogen atoms attached to the nitrogen atoms of peptide links are exchangeable (Englander *et al.*, 1972). Thus exposing *Lemna* to ^3H-labelled medium would be expected to label protein. However, high temperatures and alkaline conditions favour the exchange reaction, and it was found that dialysis under alkaline conditions completely removes the ^3H associated with peptide links. *Lemna* protein was labelled by incubating it with ^3H_2O (5 mCi/ml) for 90 min and also by exposing *Lemna* fronds to ^3H-labelled medium (5 mCi/ml) for 20 min. The loss of ^3H from both samples during dialysis is shown in Fig. 3.

The ^3H remaining after dialysis was assumed to be associated with protein. Evidence to support this assumption is as follows: (*a*) Treatment of the dialysed sample with Pronase (1 mg/ml) released ^3H into the trichloroacetic acid-soluble phase as the protein content (as determined by the microbiuret method) declined. (*b*) When the preparation was treated with ribonuclease or deoxyribonuclease (1 mg/ml), ^3H was not released into the trichloroacetic acid-soluble phase. (*c*) Some 87–94% of the ^3H associated with the preparation could be recovered in individual

Table 2. *Distribution of ^3H into the protein amino acids of Lemna after 20 min in ^3H-labelled medium (100 mCi/ml)*

For details see the text.

Amino acid	Amino acid concentration (μmol/g fresh wt.)	10^{-3} × ^3H in amino acids (d.p.m.)	10^{-3} × Specific radioactivity (d.p.m./μmol)
Lys	5.0	68.5	13.7
His	2.15	13.5	6.28
Arg	1.75	0	0
Asp	3.25	24.0	7.4
Thr	1.5	5.0	3.33
Ser	2.65	8.0	3.02
Glu	3.95	56.5	14.3
Pro	3.3	25.5	7.73
Gly	4.35	13.0	2.99
Ala	5.35	104.0	19.45
Cys	0.1	2.2	22.0
Val	4.2	41.5	9.9
Met	0.1*	1.5	15.0
Ile	5.2	22.0	4.23
Leu	8.1	168.5	20.8
Tyr	2.65	11.0	4.15
Phe	3.3	28.5	8.64

* Probably lower than true value owing to oxidation of methionine during hydrolysis.

amino acids after separation by amino acid analyser. The rate of incorporation of ^3H into protein was measured over a 4h period, and after an initial rapid rise was found to be linear with time.

Distribution of ^3H between protein amino acids

Lemna fronds (0.2g fresh wt.) were incubated with ^3H-labelled medium (100mCi/ml) for 20min. The protein was extracted and hydrolysed and the amino acids were separated in the Technicon amino acid analyser. The extensive labelling of alanine and glutamate, and the lack of label associated with arginine (Table 2), parallel the labelling of these amino acids found in the soluble amino acid fraction (Table 1). On the other hand, leucine, which is the most heavily labelled protein amino acid, was almost completely unlabelled in the soluble amino acid fraction.

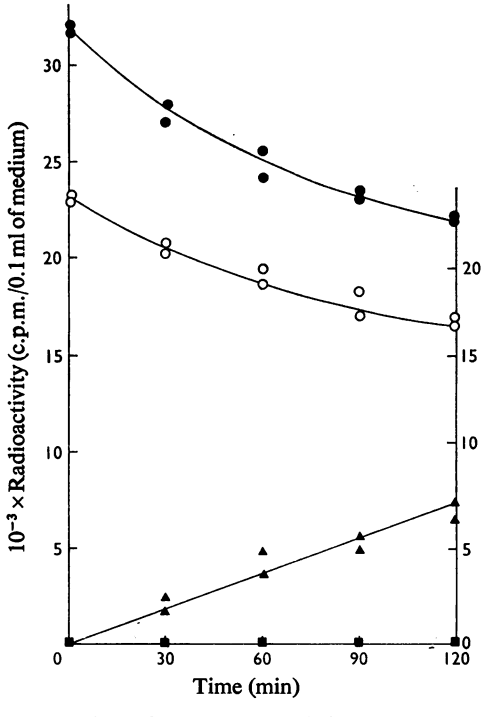


Fig. 4. *Uptake and incorporation of glutamate into Lemna protein*

Lemna fronds (0.2g fresh wt.) were incubated with 10ml of growth medium containing either [U-^{14}C]glutamate (265μCi/mol; 2.5μCi) or [2-^{3}H]glutamate (3.6mCi/μmol; 2.5μCi). Glutamate remaining in the medium was measured by isolating and counting the glutamate for radioactivity. Direct sampling of the external medium showed that ^3H in the external medium did not decrease, i.e. the ^3H lost from [2-^{3}H]glutamate appeared as ^3H_2O. ●, [U-^{14}C]Glutamate in external medium; ○, [2-^{3}H]-glutamate in external medium; ▲, ^{14}C incorporated into *Lemna* protein; ■, ^3H incorporated into *Lemna* protein.

Position of ^3H in labelled amino acids

Some 80% of the ^3H entering soluble amino acids during a 20min labelling period is lost during the subsequent 30min chase period. This is consistent with most of the ^3H being attached to the α-carbon atoms of the amino acids. Glutamate was isolated from the soluble amino acids labelled during the 20min exposure of *Lemna* to ^3H-labelled medium and degraded to succinate, CO_2 and water by oxidation with acid dichromate (Wolf, 1953), 70% of the tritium being recovered in the water.

An alternative check on the assumption that ^3H enters amino acids by exchanging with hydrogen on the α-carbon atoms can be achieved by reversing the labelling process, i.e. by examining the fate of [2-^{3}H]glutamate supplied to *Lemna*. The uptake of [U-^{14}C]glutamate and [2-^{3}H]glutamate by *Lemna* was measured together with their incorporation into proteins. The rate of uptake of both amino acids was the same, but no ^3H could be demonstrated in the extracted protein (Fig. 4).

Measurement of protein turnover in Lemna minor by ^3H exchange

Lemna fronds were floated on a ^3H-labelled medium (5mCi/ml) for 20min, then transferred to either ^3H-free medium or distilled water and cultured for 5 days. Samples were taken at regular intervals and the ^3H content of the proteins was determined. Initially we attempted to count the protein directly for radioactivity, but the reproducibility was poor and we found it necessary to hydrolyse the protein on Dowex 50 and to count the amino acids for radioactivity as described under 'Methods'. Where an oxidizer is available it may be more convenient to oxidize the sample and count the ^3H_2O. The loss of ^3H from the protein is shown in Fig. 5 and the results were used to determine a rate constant of degradation expressed as a half-life. The turnover of the total soluble protein in plants growing on the normal medium appears to consist of two main fractions, one with a half-life of 3 days, the other with a half-life of 7 days. Under conditions of starvation, the half-life of soluble protein is decreased to 2 days and there is no evidence for two fractions.

Measurement of protein turnover in Lemna minor by the method of Hellebust & Bidwell (1963)

Lemna fronds (1.8g fresh wt.) were floated on 100ml of culture medium containing [U-^{14}C]glutamate (0.5μCi/ml; 1.82μM) for 24h, then transferred to a ^{14}C-free culture medium. Samples were taken at regular intervals and the distribution of ^{14}C in the amino acids was determined. The values for the

specific radioactivity of glutamate, aspartate and proline were substituted in the equation:

$$CT = \frac{\Delta TA - \Delta C \cdot SA_s}{SA_s - SA_p}$$ (Hellebust & Bidwell, 1963)

where ΔC = change in amount of protein-bound amino acid; CT = protein turnover (degradation); ΔTA = change in radioactivity of the protein-bound amino acid; SA_s = specific radioactivity of the soluble amino acid; SA_p = specific radioactivity of the protein-bound amino acid. The calculated values for protein turnover are given in Table 3.

Measurement of protein turnover in Lemna minor by the method of Kemp & Sutton (1971)

Lemna fronds (0.5g fresh wt.) were floated on 50ml of ^{3}H-labelled culture medium containing [U-^{14}C]-leucine (0.1μCi/ml; 0.29μM) for 7h, then transferred to a ^{14}C-free medium. Samples were analysed to determine the specific radioactivity of free and protein-bound leucine. The absolute rate of protein synthesis was calculated by using the equation:

$$\text{Rate of synthesis} = \frac{\text{Specific radioactivity of protein-bound leucine}}{\text{Specific radioactivity of soluble leucine}}$$

By using this equation, the absolute rate of synthesis was found to be 14.7mg/h per g. The measured rate of net protein accumulation was 13mg/h per g, hence the rate of degradation was 1.7mg/h per g, which is equivalent to 4.1% /day or a half-life of 12.2 days.

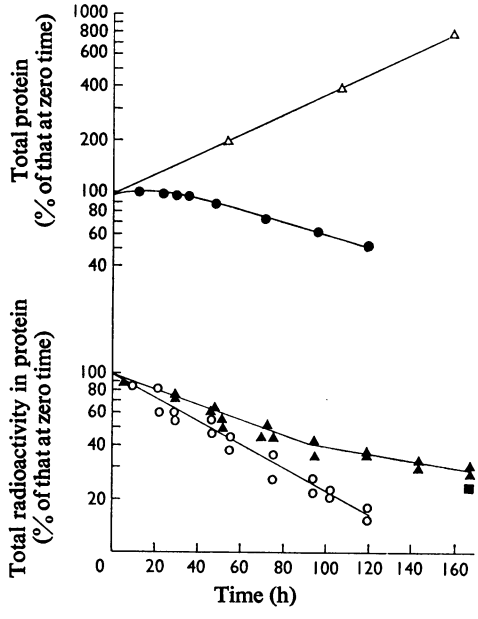


Fig. 5. *Comparison of protein degradation in Lemna growing in complete culture medium and in distilled water*

▲, Total radioactivity in *Lemna* protein grown on complete medium expressed as % of radioactivity at zero time; ○, total radioactivity in *Lemna* protein grown on water; △, total protein in *Lemna* grown on complete medium; ●, total protein in *Lemna* grown on water.

Discussion

The results presented in this paper show that when *Lemna* is exposed to ^3H_2O, many of the soluble amino acids rapidly become labelled with ^{3}H. When these amino acids are extracted from the plant, the ^{3}H is found not to be exchangeable with water, but when *Lemna* is transferred to water the ^{3}H is rapidly lost. These results suggest that the labelling of the amino acids *in vivo* is due to an enzyme-catalysed exchange reaction. The observation that 70% of the ^{3}H incorporated into glutamate is attached to the α-carbon atom suggests that the label is introduced at the α-carbon atom. The finding that when [2-^{3}H]glutamate is supplied to *Lemna* the ^{3}H is rapidly removed from the α-position suggests that the labelling of amino acids is largely due to enzyme-catalysed exchange reactions involving hydrogen atoms, e.g. transamination. The virtual absence of label from arginine is consistent with this interpretation, since arginine is made from ornithine, which involves net synthesis rather than exchange.

The 15–20% of ^{3}H that is not removed from the soluble amino acids during the 30min after *Lemna* is

Table 3. *Turnover rates of protein amino acids of Lemna minor obtained by the method of Hellebust & Bidwell* (1963)

Turnover rate is expressed as %/h. For details see the text.

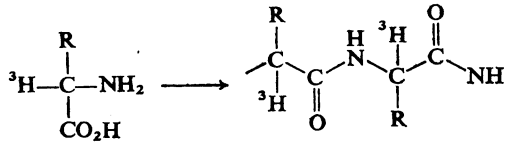
Amino acid	Turnover rate, 0–20h	Half-life (h)	Turnover rate, 0–48h	Half-life (h)
Glu	204.6	0.24	2.75	18.2
Asp	2.80	18.1	2.34	21.5
Pro	4.12	12.14	6.25	8

Table 4. *Comparison of half-lives of Lemna minor soluble protein obtained by various methods*

Condition	Half-lives (days) Hellebust & Bidwell (1963) method	Kemp & Sutton (1971) method	After Trewavas (1972)	Present method
Normal growth	0.01–0.9	12.25	7	Fraction A 3 Fraction B 7
Starvation	—	—	2	2

transferred back to an unlabelled medium may be due to ^3H entering positions that are not readily exchangeable, or the label may be introduced into the α-position of some amino acids by synthesis rather than exchange. Alternatively, some amino acids labelled in the α-position may enter the vacuole. Once in the vacuole the amino acid is separated from transaminases and consequently ^3H will not exchange with water when *Lemna* is transferred back to a ^3H-free medium.

During the 20min exposure to ^3H_2O, ^3H-labelled amino acids enter protein by net synthesis and the ^3H is in a stable position:



The non-exchangeability of ^3H incorporated into protein contrasts with the ready exchangeability of ^3H in the soluble amino acids and forms the basis for the method of measuring protein turnover which is presented in this paper. The method offers a number of advantages over other methods.

(1) The method is rapid and cheap.

(2) The problem of recycling is largely eliminated because ^3H incorporated into protein is stable, but when degradation occurs the ^3H of the amino acid is exchangeable and when the protein is re-formed it will not contain ^3H.

(3) The problem of compartmentation is largely eliminated because when *Lemna* is transferred back to unlabelled medium, the ^3H-labelled amino acids in metabolic pools rapidly lose ^3H. ^3H-labelled amino acids in storage pools (vacuoles) do not lose their ^3H. However, as soon as they leave the vacuole they are available to transaminases and thus equilibrate with water.

(4) The method labels a number of amino acids and offers advantages over methods using a single radioactive amino acid, such as the method of Kemp & Sutton (1971). When a single amino acid is supplied the heterogeneity of amino acid composition may yield different rates of degradation for the different amino acids supplied. The method of Hellebust & Bidwell (1963), although involving the administration of a single amino acid, measures the specific radioactivity of a number of individual amino acids and the results show large differences in apparent turnover rates (Table 3).

We have measured the rate of protein degradation in *Lemna* by three methods, and in Table 4 these values are compared with those obtained by Trewavas (1972) using the same growth conditions. The values determined by the method of Hellebust & Bidwell (1963) are much higher than the others. The validity of the equations developed by Hellebust & Bidwell (1963) has been questioned by Davies & Humphrey (1974) and it is clear that when the specific radioactivity of the protein amino acid approaches the specific radioactivity of the soluble amino acid, the equations yield very high estimates of protein turnover. The turnover rates measured by the ^3H-exchange method and by the method of Trewavas (1972) show good agreement. The ^3H-exchange method identifies two groups of proteins in growing *Lemna*, one group with a half-life of 3 days, the other with a half-life of 7 days. Under conditions of starvation the rate of protein degradation is increased, giving a half-life of 2 days. This result is at variance with the situation reported for carrot explants, where protein degradation is slow in slowly growing cells and rapid in rapidly growing cells (Bidwell *et al.*, 1964), but is consistent with the situation found in bacteria, in which protein degradation is slow in rapidly growing cells (Mandelstam, 1960).

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