Biochem. J. (1976) **156**, 561–568
Printed in Great Britain

A Sensitive Method for Measuring Protein Turnover Based on the Measurement of 2-^{3}H-Labelled Amino Acids in Protein

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(*Received* 16 *January* 1976)

A method for measuring the rate of protein degradation is described. The method measures the change in 2-^{3}H content of protein with time by racemization of the protein hydrolysate with acetic anhydride. The ^{3}H on C-2 of amino acids is stable in proteins but becomes labile, owing to the action of transaminases, once the amino acids are released by proteolysis. The specific measurement of 2-^{3}H in amino acids largely overcomes problems due to compartmentation and isotope recycling and evidence to support this claim is presented. Values for the half-life of *Lemna minor* (duckweed) protein determined by the new method are compared with values obtained by other methods.

We have described a method of measuring protein turnover (Humphrey & Davies, 1975) which depends on the rapid enzyme-catalysed incorporation of ^{3}H from ^3H_2O into free amino acids at C-2, together with the stabilization at this position that occurs when the amino acids enter protein. The speed of the exchange reaction permits short exposures to ^3H_2O, so that the incorporation of ^{3}H into amino acids is restricted to C-2 and C-3, and this largely eliminates the problems of metabolic pools and recycling of amino acids that are usually associated with measurements of protein turnover. However, the short exposure to ^3H_2O limits the sensitivity of the method by limiting the incorporation of ^{3}H into protein. Increasing the time of exposure to ^3H_2O increases the incorporation but also leads to ^{3}H entering non-exchangeable positions in the amino acids, thereby eliminating some of the advantages of the method. To increase the sensitivity of the method and preserve its special advantages, it is necessary to measure the incorporation of ^{3}H on C-2 of amino acids without interference from ^{3}H present in other positions. In the present paper we examine methods of selectively measuring the ^{3}H at the C-2 and report the application of these methods to the measurement of protein turnover in *Lemna minor* (duckweed).

The claim that the ^{3}H-labelling method largely eliminates the problem of recycling of amino acids assumes that when 2-^{3}H-labelled amino acids are released from protein, they are exposed to transaminases that rapidly exchange the 2-^{3}H for ^{1}H from ^1H_2O (hereafter called 'water'). In the present paper we provide evidence to support this assumption.

Materials and Methods

Materials

Plant material. Lemna minor (duckweed) was isolated from the River Yare by Dr. A. Trewavas (School of Botany, University of Edinburgh) and maintained in sterile culture in continuous light as described by Trewavas (1970) except that wide-necked (76mm) conical flasks replaced Roux bottles.

Chemicals. Dowex resins, pyridoxal hydrochloride, sodium glyoxylate, Triton X-100 and Tris base (under the trade name 'Trizma') were obtained from Sigma (London) Chemical Co., London S.W.6, U.K. The following radioactive compounds were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.: ^3H_2O (90mCi/μmol), L-[3-^{3}H]serine (5.3mCi/μmol), [2-^{3}H]glycine (2.8mCi/μmol), DL-[2-^{3}H]glutamate (3.6mCi/μmol), L-[4,5-^{3}H]lysine (19mCi/μmol), L-[5-^{3}H]arginine (16mCi/μmol), L-[*ring*-4-^{3}H]phenylalanine (11mCi/μmol), L-[4,5-^3H]isoleucine (12mCi/μmol), [U-^{14}C]glucose (65.7mCi/μmol) and L-[U-^{14}C]leucine (0.35mCi/μmol). PPO (2,5-diphenyloxazole) and acetic anhydride were obtained from BDH, Poole, Dorset, U.K. All chemicals were of the best grade available.

Methods

Preparation of 2-^3H-*labelled amino acids.* A number of 2-^{3}H-labelled DL amino acids were prepared from L-amino acids and ^3H_2O as described by Brundish *et al.* (1971).

Labelling with ^3H_2O. All manipulations were carried out under sterile conditions. For the determi-

nation of the effect of ^3H_2O on the subsequent growth of *Lemna minor*, fronds (0.1 g fresh wt.) were removed from the growth medium and floated on 10ml of ^3H-labelled growth medium (Humphrey & Davies, 1975) for 24h. The specific radioactivity of the growth medium was varied from 0 to 10mCi/ml. The fronds were removed from the ^3H-labelled medium, washed with 2 litres of ^3H-free medium and transferred to 1 litre of ^3H-free medium. For the measurement of protein turnover, fronds (1 g fresh wt.) were removed from the growth medium and floated on 100ml of ^3H-labelled growth medium (1mCi/ml) for 48h, then washed and transferred to either ^3H-free growth medium or water for periods of up to 10 days.

Counting of radioactivity of ^3H-labelled compounds. ^3H was measured by liquid-scintillation counting in an Intertechnique SL30 liquid-scintillation spectrometer, each sample being counted for radioactivity in toluene/Triton X-100/PPO scintillant (1000:500:7, v/v/w) until the standard error was below 3%. Counting efficiency was determined by the addition of an internal ^3H-standard.

Isolation and counting of radioactivity of ^3H-labelled free amino acids and amino acids incorporated into protein. The extraction and isolation of soluble amino acids and the extraction, hydrolysis and isolation of protein amino acids residues were as previously described (Humphrey & Davies, 1975).

Method of measuring 2-^3H in amino acids. In three of the four methods which were investigated, the ratios of the reactants were found to be critical. Hence to measure the 2-^3H content of an amino acid, its concentration must be determined and the other reactants added to give the ratios indicated below. With racemization with acetic anhydride it was possible to decrease the volume of water in which the ^3H-labelled amino acids were dissolved from 2.78 to 0.278mmol with no loss of recovery of 2-^3H.

(*a*) Oxidation with chloramine-T. The ^3H-labelled amino acid (1μmol)+chloramine-T (200μmol) were dissolved in 2.5ml of 1M-sodium citrate buffer (pH2.5) and shaken for 1h at 30°C to decarboxylate the amino acid. Excess of chloramine-T was removed by adding 0.25ml of a saturated solution of glycine and shaking for a further 1h. KOH (0.25ml; 4M) was then added, water was distilled off under vacuum and the ^3H radioactivity present in the water counted.

(*b*) Transamination with pyridoxal and copper. The ^3H-labelled amino acid (2μmol), pyridoxal hydrochloride (10μmol) and $CuSO_4$ (1μmol) were dissolved in 1.0ml of 20mM-disodium tetraborate buffer (pH9.5). The mixture was sealed into a test tube and heated at 100°C for 2h. After cooling, the tubes were opened, the contents acidified with conc. H_2SO_4 (0.01ml) and phenylhydrazine hydrochloride (10μmol) was added. Water was distilled off and ^3H radioactivity present in the water counted.

(*c*) Transamination with aluminium and glyoxylate. The ^3H-labelled amino acid (1μmol), sodium glyoxylate (100μmol) and $AlK(SO_4)_2$ (0.1μmol) were dissolved in 1.0ml of 0.1M-sodium citrate buffer (pH4.5). The mixture was sealed into a test tube and heated at 100°C for 18h. After cooling the tube was opened and 0.3M-NaOH (1ml) added, water was distilled off and ^3H radioactivity present in the water counted.

(*d*) Racemization with acetic anhydride. The ^3H-labelled amino acid (1.0μmol), water (2.78mmol) and acetic anhydride (5.6mmol) were heated in a sealed tube for 3h at 100°C. After cooling the tubes were opened and water (111.1mmol) was added to quench the reaction. The dilute acetic acid was distilled off and the ^3H radioactivity present was counted. In all cases the ^3H_2O was taken up in 15ml of toluene/Triton X-100/PPO scintillant (20:70:1, v/v/w) and counted for radioactivity. The distilled acetic acid from method (*d*) caused an initial chemiluminescence in the above scintillant, but this quickly died down.

Radioactivity counting of labelled protein. Protein was isolated and hydrolysed as described by Humphrey & Davies (1975). To determine the total ^3H content, the sample was evaporated to dryness, dissolved in 0.5ml of water and counted for radioactivity in 15ml of toluene/Triton X-100/PPO scintillant (1000:300:3, v/v/w). To determine the 2-^3H content, the amount of amino acid in the sample was determined by using an amino acid analyser, and the 2-^3H determined by racemization with acetic anhydride or by transamination with pyridoxal and copper.

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, NJ, U.S.A.). $^{14}CO_2$ was counted for radioactivity in 15ml of toluene/methanol/2-phenethylamine/PPO scintillant (900:400:200:7, v/v/v/w). ^3H_2O was counted for radioactivity in 15ml of toluene/Triton X-100/PPO scintillant (1000:500:7, v/v/w). The 2-^3H content was determined in duplicate samples by using the method of racemization with acetic anhydride.

Determination of ^{14}C and 2-^3H in free amino acids and protein amino acid residues. Lemna minor fronds (0.1g fresh wt.) were harvested from a growth medium, frozen with liquid N_2, then ground to a fine powder before being extracted with 5ml of 0.1M-potassium phosphate buffer (pH7.3). The extract was centrifuged at 10000*g* for 10min, the supernatant collected and protein precipitated by adding 5ml of trichloroacetic acid (10%, w/v). After standing overnight at 2°C, protein was collected by centrifuging at 20000*g*, for 10min, then hydrolysed on Dowex 50 (Humphrey & Davies, 1975). The supernatant containing the soluble amino acids was stored at −15°C until use.

The 2-^{3}H content of the protein extracted from *Lemna minor* after culture on medium containing [U-^{14}C]glucose and ^3H_2O was determined by racemization of the hydrolysates with acetic anhydride and the ^{14}C content was measured by oxidation.

The trichloroacetic acid-soluble fraction from the above *Lemna minor* was diluted by the addition of water (10ml) and passed through a column (5cm×1cm) of Dowex 50 (H^+ form). The column was washed with water (200ml) and the amino acids were eluted with 1.6M-NH_3 (5ml). The eluate was evaporated to dryness, and the amino acids were dissolved in water (0.5ml). The 2-^{3}H content of the amino acids was determined by racemization with acetic anhydride.

Where *Lemna minor* had been cultured on medium containing L-[U-^{14}C]leucine and ^3H_2O, the protein and free amino acids were extracted as described above and then dissolved in water (0.5ml) and stored at −15°C until use.

Samples (0.2ml) were loaded on the column of the amino acid auto-analyser for the separation of acidic and neutral amino acids. Since leucine appears near the end of the run, the processes were speeded up by decreasing the elution time with the first three buffers (Humphrey & Davies, 1975) and lengthening it with the last buffer. The eluate from the column was divided before the addition of ninhydrin so that 70% was collected as fractions (0.21ml) in a fraction collector and 30% was developed with ninhydrin to determine the leucine concentration. The fractions under the leucine peak were pooled and evaporated to dryness, then dissolved in 0.5ml of water. Duplicate samples (0.2ml) were taken; one was oxidized to give $^{14}CO_2$ for counting, the other was treated with acetic anhydride for determination of the 2-^{3}H content.

Results

Effect of ^3H_2O on growth and metabolism of Lemna minor

Lemna minor was cultured on ^{3}H-labelled medium (0–10mCi/ml) for 24h. The plants were then washed with ^{3}H-free medium and transferred to ^{3}H-free medium (1 litre) and culture was continued for 4 days. During this period the growth rate of the plants was measured. On completion of the culture period, the respiration rate and the mean fresh weight, frond area and root length were determined (Table 1). Amounts of ^{3}H above 2mCi/ml had an increasing effect on all the parameters examined, with root length and growth rate showing marked inhibition. For the labelling of protein for the subsequent determination of rates of degradation, *Lemna minor* was cultured on ^{3}H-labelled medium containing 1mCi/ml for 48h.

Comparison of methods of measuring 2-^{3}H in amino acids

Four methods of measuring 2-^{3}H in amino acids were examined for specificity and recovery. We used ^{3}H-labelled amino acids, supplied by The Radiochemical Centre, and some 2-^{3}H-labelled amino acids synthesized by the method of Brundish *et al.* (1971), as reference compounds. A comparison of the four methods for the reference compounds is shown in Table 2. Each method has advantages and disadvantages, and we have mainly used the method of racemization with acetic anhydride, as it gave better recovery from the ^{3}H-labelled amino acids, but have also made comparative determinations by using the method of transamination with copper and pyridoxal.

Table 1. *Effect of culture on medium containing ^{3}H on subsequent growth of Lemna minor*

Lemna minor was cultured on ^{3}H-labelled medium (0–10mCi/ml) for 24h. The plants were then washed and transferred to ^{3}H-free medium and cultured for 4 days. During this time the frond doubling-time was measured. After 4 days the plants were photographed *in situ* and the frond area and root length measured. They were then removed from the culture vessel, weighed, and the respiration rate measured by Warburg manometry.

^{3}H in culture medium (mCi/ml)	Frond doubling-time (h)	Respiration rate (μl of O_2/min per 100 fronds)	Fresh wt. (mg/10 fronds)	Frond area (cm^2)	Root length (cm)	
					Average	Range
0	37	5.7	10.0	0.25	4.4	0–11
2	37	5.7	9.9	0.23	3.21	0–11
4	39	5.0	8.0	0.20	1.93	0–4
6	48	4.8	7.7	0.18	1.57	0–3.5
8	59	3.3	6.7	0.15	0.97	0–2.7
10	71	3.1	6.8	0.15	0.89	0–3.0

Table 2. *Efficiency and specificity of a number of treatments for removal of 2-^{3}H from amino acids*

For details, see the text. Where the percentages are followed by numbers in parentheses, the figure is the mean of three experiments.

Amino acid	^{3}H on C-2 (%)	Treatment ... ^{3}H on C-2 (%) Chloramine-T	Pyridoxal and copper	Glyoxylate and aluminium	Acetic anhydride
DL-[2-^{3}H]Alanine	100	—	64	27	100
DL-[2-^{3}H]Aspartate	100	—	83	100	81
L-[5-^{3}H]Arginine	0	1	3 (±1)	2 (±0)	4 (±0)
DL-[2-^{3}H]Glutamate	100	90 (±10)	99 (±1)	98 (±2)	98 (±2)
[2-^{3}H]Glycine	100	15	48 (±3)	11 (±0)	90 (±10)
DL-[2-^{3}H]Isoleucine	100	—	23	9	100
L-[4,5-^{3}H]Isoleucine	0	3	3 (±0)	2 (±0)	5 (±1)
DL-[2-^{3}H]Leucine	100	—	86	26	99
DL-[2-^{3}H]Lysine	100	—	56	36	92
L-[4,5-^{3}H]Lysine	0	3	3 (±0)	2 (±0)	5 (±1)
DL-[2-^{3}H]Valine	100		31	10	99
[*ring*-4-^{3}H]Phenylalanine	0	7 (±0)	20 (±0)	31 (±3)	15 (±2)
^{3}H-labelled *Lemna minor* protein hydrolysate (20 min culture)		—	53 (±1)	53 (±1)	44 (±6)

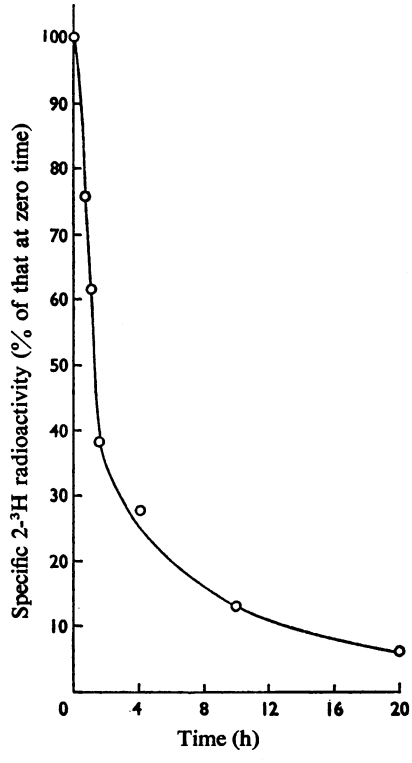

Fig. 1. *Loss of 2-^{3}H from free amino acids of Lemna minor during 'chase' on unlabelled medium*

Lemna minor was cultured on ^{3}H-labelled medium (1 mCi/ml) for 48 h then removed, washed and transferred to ^{3}H-free medium. Samples were taken at intervals, and the ^{3}H on the C-2 of the free amino acids measured by racemization.

Release of 2-^{3}H from free amino acids during the 'chase' period

The validity of the proposed method of measuring protein turnover requires that the cytoplasmic amino acids that become labelled with ^{3}H during the long exposure to ^3H_2O rapidly lose 2-^{3}H during the 'chase' period. To examine this point, *Lemna minor* was cultured on ^{3}H-labelled growth medium (1 mCi/ml) for 48 h, then washed and transferred to a ^{3}H-free medium. Free amino acids were isolated at intervals, and the 2-^{3}H content was determined by racemization with acetic anhydride. The results (Fig. 1) show a rapid removal of some 65% of the 2-^{3}H in the first 90 min, followed by a slower removal so that 80% is lost after 300 min.

Distribution of ^{3}H between protein amino acid residues

The distribution of ^{3}H between protein amino acid residues was determined after culturing the fronds on ^3H_2O-labelled growth medium for 48 h. The results (Table 3) are similar to those reported by Humphrey & Davies (1975) for short exposures (20 min) to ^3H_2O. However, the incorporation of ^{3}H is much greater, and is more evenly spread between the amino acid residues; in particular, arginine, which is not labelled during a 20 min exposure to ^3H_2O, becomes highly radioactive during the 48 h exposure.

Measurement of protein turnover

Lemna minor fronds (1 g fresh wt.) were grown on a ^3H_2O-labelled growth medium (1 mCi/ml) for 48 h, then transferred to either ^{3}H-free medium or water and grown for up to 7 days. Samples were taken at intervals, and the total ^{3}H and 2-^{3}H contents of the protein measured. When the former was measured, the specific radioactivity showed an increase during

Table 3. *Distribution of ^3H into the protein amino acids of Lemna minor after 48 h in ^3H-labelled (10 mCi/ml)*

For details, see the text.

Amino acid	Amino acid concn. (μmol/g fresh wt.)	$10^{-5}\times{}^3H$ in amino acids (d.p.m.)	$10^{-5}\times$Specific radioactivity (d.p.m./μmol)
Lysine	5.5	11.2	2.0
Histidine	2.3	3.4	1.4
Arginine	1.6	3.8	2.3
Aspartate	3.4	11.8	3.5
Threonine	1.5	3.9	2.7
Serine	2.8	7.8	2.8
Glutamate	4.1	27.1	6.6
Proline	3.1	23.8	7.7
Glycine	4.4	8.5	1.9
Alanine	5.1	39.4	7.7
Cystine	0.1	0.8	6.2
Valine	4.3	42.9	10.0
Methionine	0.1*	0.7	2.7
Isoleucine	5.4	66.7	12.4
Leucine	9.0	136.4	15.2
Tyrosine	2.5	13.9	5.5
Phenylalanine	2.9	25.7	8.9

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

the initial stage of the chase period (Fig. 2), suggesting that ^3H-labelled amino acids continue to enter protein after removal of *Lemna minor* from the ^3H_2O-containing growth medium. The initial increase in ^3H was not observed when the 2-^3H content of the protein was determined, suggesting that the rapid loss of 2-^3H from free amino acids (Fig. 1) prevents the continued entry of 2-^3H-labelled amino acids into protein. Instead there was a steady exponential decline in the 2-^3H content, from which the half-life of protein under standard growth conditions was estimated as 7.1 ± 0.5 days (Fig. 2*a*). Under conditions of starvation the half-life was estimated as 2 days (Fig. 2*b*).

A parallel experiment, in which the 2-^3H content of the protein was determined by transamination reaction with pyridoxal and copper, gave the same value for the half-life of *Lemna minor* protein.

Errors in measuring protein turnover due to recycling of amino acids

The suggestion that the ^3H-exchange method largely eliminates the problem of recycling of amino acids rests on the assumption that when amino acids are released from proteins, the 2-^3H rapidly exchanges with water (^1H_2O) so that if the amino acid re-enters

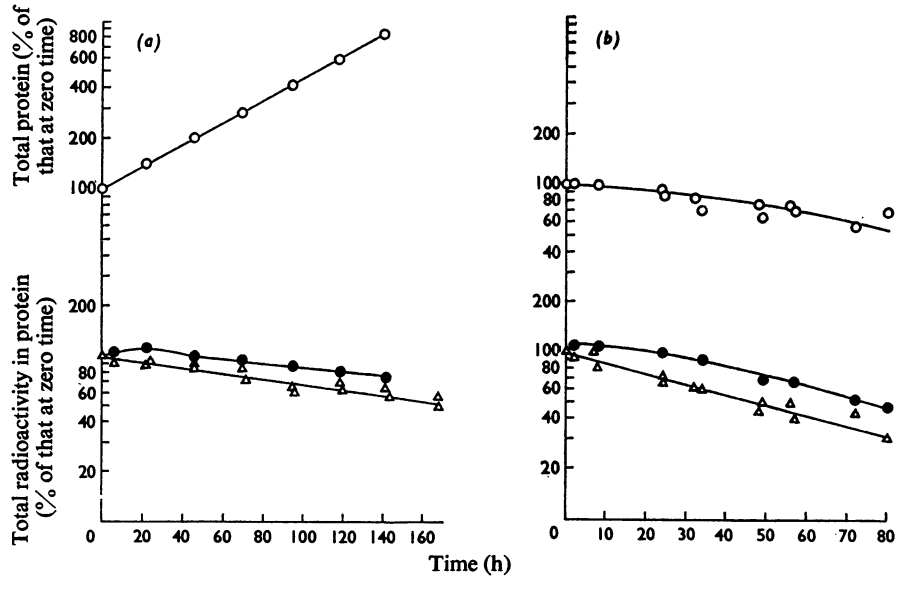

Fig. 2. *Comparison of protein degradation in Lemna minor growing in complete culture medium (a) and in water (b)*

Lemna minor was cultured on ^3H-labelled medium for 48 h, then removed, washed and transferred to either ^3H-free medium or water. Samples were taken at intervals, the protein was isolated and hydrolysed, and both the ^3H on the C-2 and the total ^3H content of the protein hydrolysates determined. The change in the ^3H content of the protein was used to determine rates of degradation. The lines through the points were determined by regression analysis. ○, Total protein in *Lemna minor*; ●, total ^3H radioactivity in *Lemna minor* protein expressed as a percentage of radioactivity at zero time; △, total 2-^3H radioactivity in *Lemna minor* protein expressed as a percentage of radioactivity at zero time.

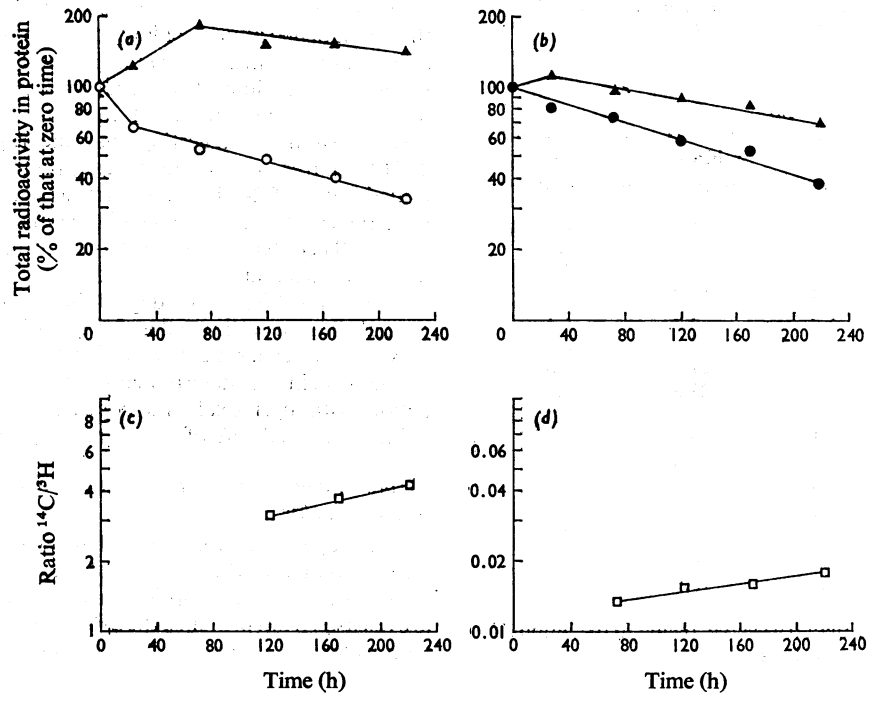

Fig. 3. *Comparison of rates of protein degradation in Lemna minor cultured on complete medium, obtained by various labelling methods*

Lemna minor was grown on medium containing [U-^{14}C]glucose for 48h either (*a*) in the presence of $^{3}H_2O$ (1mCi/ml) or (*b*) with [U-^{14}C]glucose alone, and then transferred to medium containing $^{3}H_2O$ (10mCi/ml) for 20min. The fronds were then washed and transferred to unlabelled medium, and samples taken at intervals. Soluble protein was extracted and its ^{14}C and ^{3}H content measured as described in the text. The change in the $^{14}C/^{3}H$ ratio was also measured for each experiment, and the data from (*a*) is shown in (*c*) and the data from (*b*) in (*d*). ▲, Total ^{14}C radioactivity in *Lemna minor* protein expressed as percentage of that at time zero; ○, total ^{3}H radioactivity in *Lemna minor* protein expressed as a percentage of that at time zero; ●, total 2-^{3}H radioactivity in *Lemna minor* protein expressed as a percentage of that at time zero; □, ratio (total ^{14}C)/(total 2-^{3}H) in *Lemna minor* protein.

protein it lacks ^{3}H on C-2. To test this assumption, we have double-labelled *Lemna minor* protein with ^{14}C and ^{3}H, then followed the loss of ^{14}C and ^{3}H from protein during the 'chase' period. If the ^{3}H-exchange reaction does not occur before the amino acid is re-incorporated into protein, the $^{14}C/^{3}H$ ratio in protein should not change. However, if the ^{3}H exchange is rapid, relative to the rate of incorporation of amino acids into protein, the ratio $^{14}C/^{3}H$ should increase with time. We have used three methods to double-label the protein:

(i) *Lemna minor* fronds (0.5g fresh wt.) were transferred to a growth medium (100ml) containing [U-^{14}C]glucose (10μCi) and grown under standard conditions. After 24h a further 10μCi of [U-^{14}C]-glucose was added and after another 24h the fronds were removed, washed with ^{14}C-free medium and then transferred to a growth medium (100ml) containing $^{3}H_2O$ (10mCi/ml). After 20min the fronds were removed, washed with standard growth medium and transferred to 1 litre of this medium. Samples were removed at intervals, protein was extracted and, after hydrolysis, ^{14}C and ^{3}H were determined by combustion to $^{14}CO_2$ and $^{3}H_2O$. The results (Fig. 3*a*) show that the ^{3}H content of protein (presumed to be 2- and 3-^{3}H) decreases more rapidly than the ^{14}C content. The observed loss of ^{3}H from protein suggests the existence of two main fractions with different half-lives.

(ii) *Lemna minor* fronds (0.5g fresh wt.) were transferred to growth medium (1 litre) containing $^{3}H_2O$ (1mCi/ml) and [U-^{14}C]glucose (10μCi). After 24h a further 10μCi of [U-^{14}C]glucose was added; after another 24h the fronds were removed and washed with 2 litres of standard growth medium, and batches of about 400 fronds transferred to the standard medium (1 litre) and cultured for several days. Samples (0.1g fresh wt.) were removed at intervals,

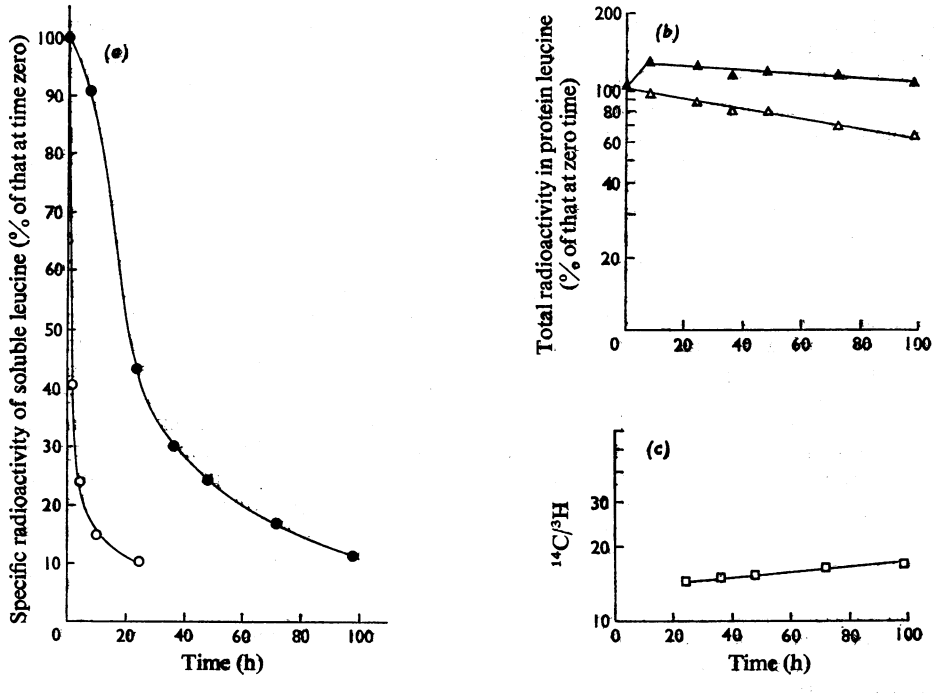

Fig. 4. *Change in the radioactivity of free and protein leucyl residues of Lemna minor during 'chase' on unlabelled medium*

Lemna minor was cultured on complete culture medium containing L-[U-^{14}C]leucine (0.01 μCi/ml) and $^{3}H_2O$ (1 mCi/ml) for 48 h. The fronds were then washed and transferred to unlabelled medium, and samples were taken at intervals. The ^{14}C and 2-^{3}H contents of the free (*a*) and protein leucyl residues (*b*) were measured as described in the text. The ratio of $^{14}C/2$-^{3}H of protein leucyl residues was also measured (*c*). ○, Specific 2-^{3}H radioactivity of free leucine expressed as a percentage of that at zero time; ●, specific ^{14}C radioactivity of free leucine expressed as a percentage of that at zero time; △, total 2-^{3}H radioactivity of protein leucyl residues expressed as a percentage of that at zero time; ▲, total ^{14}C radioactivity of protein leucyl residues expressed as a percentage of that at zero time; □, ratio $^{14}C/^{3}H$ in protein leucyl residues.

protein was extracted and, after hydrolysis, the ^{14}C content was determined by combustion to $^{14}CO_2$. Duplicate samples of the protein hydrolysate were treated with acetic anhydride to determine 2-^{3}H. The results (Fig. 3*b*) again show that 2-^{3}H is lost from protein more rapidly than is ^{14}C, but there is no evidence for two protein fractions with different half-lives.

(iii) The concentration of free leucine in plants is usually low (0.18 μmol/g fresh wt. in *Lemna minor* under our conditions of growth) and for this reason labelled leucine has been used by Kemp & Sutton (1971) to minimize the effect of storage pools. Since a large proportion of the 2-^{3}H in protein after culture on $^{3}H_2O$-containing growth medium was found in leucine (Table 2), we have carried out a double-label experiment with this amino acid.

Lemna minor fronds (0.5 g fresh wt.) were transferred to growth medium (1 litre) containing $^{3}H_2O$ (1 mCi/ml) and L-[U-^{14}C]leucine (10 μCi, 0.03 μmol). Further L-[U-^{14}C]leucine was added hourly at the rate of 0.7 μCi/h for 48 h. Fronds were removed, washed with 2 litres of standard growth medium and then transferred in 0.1 g samples to flasks containing 1 litre of standard growth medium. Samples (0.1 g fresh wt.) were removed at intervals, and the amounts of ^{14}C and 2-^{3}H determined in both soluble and protein-bound leucine. The results (Fig. 4) show that 2-^{3}H is rapidly lost from free leucine (60% in the first 2h), but suggest that there is more than one pool of free leucine. The loss of 2-^{3}H from protein leucyl residues is significantly greater than the loss of ^{14}C.

In all three experiments the $^{14}C/^{3}H$ ratio increased with time (Figs. 3*c*, 3*d* and 4*c*), supporting the view that 2-^{3}H was recycled to a significantly lesser extent than ^{14}C. The ratios were only calculated from the time that the total ^{14}C content of the protein showed a steady decline, as the increase in the total ^{14}C content before this gave rise to falsely high $^{14}C/^{3}H$ ratios.

Discussion

The pulse method of ^{3}H-labelling protein from $^{3}H_2O$ (Humphrey & Davies, 1975) minimizes the problems of metabolic pools and the recycling of

amino acids which are usually associated with measurements of protein turnover. These advantages are retained in the ^3H-labelling method described in the present paper by measuring specifically the 2-^3H content of protein. In most of the experiments we have used racemization in the presence of acetic anhydride to obtain ^3H_2O from 2-^3H-labelled amino acids. This has been our preferred method, since racemization occurs rapidly and the amino acids are protected by acetylation during the reaction. The chloramine-T method is unsatisfactory, since any small amounts of chlorine distilling over produce chemiluminescence during radioactivity counting. The transamination reactions yield oxo acids which are relatively unstable, and thus on theoretical grounds the methods are not entirely satisfactory.

The ^3H-labelling method described in the present paper is about 100 times more sensitive than the pulse method previously described (Humphrey & Davies, 1975). The increased sensitivity is due to the greater incorporation of ^3H which occurs with longer exposure to ^3H_2O. Thus in principle the sensitivity of the method can be adjusted to the needs of the particular investigation by varying the length of exposure to ^3H_2O, and means that the study of individual enzymes is possible. However, the sensitivity is decreased because it does not measure ^3H on C-3 of amino acids. In alanine, ^3H is incorporated at C-3 during transamination (Babu & Johnston, 1974) and our observation that only 50% of the ^3H incorporated into amino acids during pulse labelling from ^3H_2O is on C-2 suggests that exchange at C-3 may occur during transamination with most amino acids.

The prolonged labelling of protein should give a better estimate of the average rate of protein turnover than does the pulse method, which tends to overestimate the contribution of proteins with short half-lives. This effect is shown in Fig. 3, which contains a comparison of the two methods of ^3H labelling. The pulse method detects the protein fraction with a short half-life previously reported (Humphrey & Davies, 1975), but this fraction is not observed after prolonged exposure to ^3H_2O.

The assumption that the ^3H-labelling method minimizes problems of amino acid recycling has been examined by double-labelling protein with ^{14}C and ^3H, then comparing the rates at which 2-^3H and ^{14}C are lost. If amino acid recycling does not occur, or if recycling occurs without ^3H exchange, the ratio $^{14}C/2$-^3H in protein should be constant. The observation that the ratio increases with time is consistent with the view that the ^3H-exchange method decreases the error caused by amino acid recycling. This may be illustrated by comparing the apparent half-life of *Lemna minor* protein measured by the loss of ^{14}C from protein with that obtained for the same protein sample by the ^3H method.

The apparent half-lives measured after labelling with L-[U-^{14}C]leucine or [U-^{14}C]glucose were 13.0 days and 13.2 days respectively, whereas that measured by the ^3H method was 7 days.

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