The Measurement of Protein Turnover by Density Labelling

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A method for measuring the rate of protein degradation in plant tissue is described. The method uses density labelling to avoid difficulties associated with compartmentation and recycling of amino acids. Although the technique cannot be readily adapted to measure the rate of degradation of single proteins, it avoids difficulties of interpretation due to enzyme activation or inactivation. Values for the half-life of *Lemna minor* protein obtained by this method are compared with values obtained by a number of other methods. To obtain satisfactory results it was necessary to improve the method of isopycnic centrifugation in CsCl gradients. A considerable improvement was achieved by using KBr gradients, and the advantages of using KBr rather than CsCl for the separation of density-labelled protein are discussed.

The use of density labelling to distinguish between newly synthesized and pre-existing DNA was introduced by Meselson & Stahl (1958) and led to a similar method for the separation of newly synthesized from pre-existing protein (Hu *et al.*, 1962). A number of heavy isotopes have been used to obtain the necessary density shifts, but that most frequently used is deuterium, supplied as deuterium oxide. The method of isopycnic centrifugation has been used to separate 'heavy' and 'light' protein and so to demonstrate synthesis of enzymes *de novo* (Longo, 1968; Jacobsen & Varner, 1967; Schopfer & Hock, 1971; Sacher *et al.*, 1972; Quail *et al.*, 1973; Acton *et al.*, 1974) and in other cases to argue against the synthesis of particular enzymes *de novo* (Attridge & Smith, 1973; Attridge *et al.*, 1974).

The use of density labelling to measure the half-lives of individual enzymes was introduced by Williams & Neidhardt (1969), and a number of workers have measured half-lives for enzymes from higher plants (Zielke & Filner, 1971; Quail & Scandalios, 1971; Quail *et al.*, 1973; Acton *et al.*, 1974). A limitation which has not always been fully appreciated is that these methods do not differentiate between loss of enzyme activity and enzyme degradation; this is the other side of the coin which is currently being debated with respect to synthesis *de novo* of enzyme versus activation of pre-existing enzyme.

In the present paper we present a method for measuring the turnover of total protein. The method is not suitable for measuring the turnover of individual enzymes but on the other hand does not give erroneous results due to undetected enzyme activation or inactivation.

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Plants are first grown in a medium containing $^2\text{H}_2\text{O}$ and then in the same solution lacking $^2\text{H}_2\text{O}$. The heavy and light proteins respectively produced can be separated by isopycnic centrifugation. If a ^{14}C -labelled amino acid is given as a pulse at the end of the period in $^2\text{H}_2\text{O}$, ^{14}C label appears in heavy protein, and, if protein degradation occurs when the plants are transferred to H_2O , ^{14}C label is lost from the heavy protein and hence from the peak obtained on isopycnic centrifugation. If recycling of the amino acids occurs, label appears in the light protein peak. If there is no protein turnover, label will stay in the heavy protein peak. Protein degradation can therefore be measured by following the decrease in radioactivity associated with the heavy protein peak.

The method requires a particularly good separation of heavy and light protein, and initially, like most other workers, we adopted the CsCl gradients used by Meselson & Stahl (1958). It is perhaps surprising that a method developed for separation of nucleic acids should have been used so extensively for the separation of heavy and light protein. CsCl gradients did not give an adequate separation and KBr gradients were found to give a much better separation of protein.

Materials and Methods

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 as described by Trewavas (1970), except that in a number of experiments conical flasks replaced Roux bottles. It was found that optimum growth was obtained by using 1 litre of culture medium in 2-litre wide-necked (76mm) conical flasks. Under these

conditions the doubling time was 37 h, compared with 53 h in Roux bottles. Fronds were transferred to fresh medium every 5 days since this increased resistance to deuterium oxide.

For density labelling with deuterium oxide, the water in the growth medium was replaced with the required concentration of deuterium oxide. The medium was sterilized by filtration through a Millipore filter (GS, 0.22 μ m pore size), since autoclaving tended to precipitate salts from the medium with high deuterium oxide concentration.

Labelling procedure

(a) 80% deuterium oxide. *Lemna* fronds (1.0g fresh weight) were floated on 100 ml of culture medium containing deuterium oxide (80%), and after 30 min L-[U-¹⁴C]leucine (2.5 μ Ci, 7 nmol) or L-[4,5-³H]leucine (2.5 μ Ci, 4 nmol) was added and the culture continued for 3.5 h. The plants were removed by filtration, washed with sterile medium (500 ml) and transferred (about 400 fronds per flask) to flasks containing 1 litre of deuterium-free medium.

(b) 50% deuterium oxide. *Lemna* fronds were floated in the same manner on a culture medium containing deuterium oxide (50%) for either 6 days before adding L-[U-¹⁴C]leucine for 1 day or for 7 days before adding L-[U-¹⁴C]leucine for 2 days. After the period in deuterium oxide the plants were transferred to deuterium-free medium as described above.

Extraction of protein

Lemna fronds (0.5g fresh wt.) were removed from the growth medium, blotted dry, frozen with liquid N₂ and ground to a fine powder. Potassium phosphate buffer (2 ml, 50 mM, pH 7.0, containing mercaptoethanol, 20 mM) was added and the suspension clarified by centrifugation at 20 000g for 10 min. The supernatant was decanted and 1 ml desalted by passage through a column (10 cm $\times$ 1 cm) of Sephadex G-100 or G-200, previously equilibrated with the extraction buffer. The quantity of protein in the samples placed on the density gradient was determined by the micro biuret method of Goa (1951).

Isopycnic centrifugation

All centrifugation was carried out in a Beckman Spinco L2-65B preparative ultracentrifuge. CsCl gradients were made by slowly layering a CsCl solution (30%, w/v; 4 ml) containing the protein on to 2 ml of a dense CsCl solution (55%, w/v). KBr gradients were made by layering 3 ml of a KBr solution (67% saturated) containing the protein on to 5 ml of a saturated solution of KBr. In both cases the tubes were filled with paraffin oil. All salt solutions were in phosphate buffer (50 mM, pH 7.0, containing mercaptoethanol, 20 mM). Tubes were placed in a type 65 rotor (fixed angle) and centrifuged at 50 000 rev./min for 24 h at 5° or 15°C. On completion

of the run, fractions (7 drops) were collected by inserting a fine tube to the bottom of the centrifuge tube and pumping out the solution. Of every 35 drops, three were used to determine the refractive index with an Abbe refractometer. Refractive indices were converted into buoyant densities by using the equations

$$Q^{25} = (10.8601 \times \text{refractive index at } 25^\circ\text{C}) - 13.4974$$

for CsCl gradient

$$Q^{25} = (6.4786 \times \text{refractive index at } 25^\circ\text{C}) - 7.6431$$

for KBr

Fractions were collected in scintillation vials, and water (0.5 ml) was added to each vial to prevent salts coming out of solution when 15 ml of the scintillant fluid (toluene-Cellosolve-2,5-diphenyloxazole, 600:400:3, v/v/w) was added. The fractions were counted for radioactivity in a Intertechnique SL30 liquid-scintillation spectrometer.

Measurement of amino acid uptake and incorporation into protein

Lemna fronds (0.2g fresh wt.) were floated on medium (10 ml) containing L-[U-¹⁴C]leucine (1 μ Ci). At intervals samples (0.1 ml) were removed and taken up in 15 ml of scintillant and counted for radioactivity. To measure incorporation into protein, fronds were filtered, washed with water and placed in methanol (25 ml) and stored at 0°C overnight. The bleached plants were washed with methanol (100 ml), followed by water (100 ml), and the protein was extracted with 1 ml of NaOH (0.75 M) for 2 h at 37°C. A 0.5 ml portion of this protein extract was counted for radioactivity in vials containing 15 ml of scintillant.

Measurement of respiration and photosynthesis

Photosynthesis and respiration were measured by manometry as described by Umbreit *et al.* (1964).

Chemicals

Deuterium oxide (99.8%) was obtained from Prochem Ltd., Croydon, Surrey, U.K. Radioactive amino acids were supplied by The Radiochemical Centre, Amersham, Bucks., U.K. Sephadex G-100 and G-200 were obtained from Pharmacia, Uppsala, Sweden. All chemicals were of the best grade available.

Results

Toxicity of deuterium oxide

Growth. A visual effect of deuterium oxide on the growth of *Lemna minor* was observed when the fronds were transferred to a medium containing deuterium oxide; the fronds separated into individual fronds, the time taken for separation being 2 h on 80% ²H₂O, 4 h on 50% ²H₂O and 6 h on 40% ²H₂O.

Fig. 1. Effect of $^2\text{H}_2\text{O}$ on the uptake and incorporation of amino acids into protein by *Lemna minor*

(a) Effect of $^2\text{H}_2\text{O}$ on the uptake of leucine; (b) effect of $^2\text{H}_2\text{O}$ on the incorporation of leucine into protein. *Lemna* fronds (0.2g) were floated on medium (10ml) containing L-[U- ^{14}C]leucine ($1\mu\text{Ci}$) in the presence and absence of $^2\text{H}_2\text{O}$. Uptake and incorporation were measured as described in the text. ●, Medium containing H_2O ; △, medium containing 50% $^2\text{H}_2\text{O}$; ○, medium containing 80% $^2\text{H}_2\text{O}$.

Fig. 2. Comparison of separation of density-labelled protein by using CsCl (b) and KBr (a) gradients

Lemna was grown on a medium containing 50% $^2\text{H}_2\text{O}$ for 7 days. On day 7, L-[U- ^{14}C]leucine ($5\mu\text{Ci}/100\text{ml}$) was added and after 24h protein was extracted, low-molecular-weight substances were removed by passage through Sephadex G-200 and separated by isopycnic centrifugation. Control plants were grown in the absence of $^2\text{H}_2\text{O}$ and labelled by supplying the fronds with L-[4,5- ^3H]leucine for 24h. Details of the preparation of gradients and isopycnic centrifugation are given in the text. ○, L-[U- ^{14}C]Leucine in 'heavy' protein; ●, L-[4,5- ^3H]leucine in 'light' protein; □, refractive index at 25°C .

Overall growth was inhibited by $^2\text{H}_2\text{O}$: 80% $^2\text{H}_2\text{O}$ completely inhibited growth, and 50% $^2\text{H}_2\text{O}$ increased the doubling time from 53h to 76h.

Amino acid uptake. The uptake of L-[U- ^{14}C]phenylalanine was not significantly inhibited by 80%

$^2\text{H}_2\text{O}$, but this concentration inhibited the rate of uptake of L-[U- ^{14}C]leucine by 50%. Only slight inhibition (5–10%) of the uptake of L-[U- ^{14}C]leucine was produced by 50% $^2\text{H}_2\text{O}$ (Fig. 1).

Incorporation into protein. The inhibition of protein

Fig. 3. Measurement of protein turnover by using a 24 h exposure to [^{14}C]leucine to label protein

Lemna fronds were density-labelled for 6 days with 50% $^2\text{H}_2\text{O}$, and on day 7 L-[^{14}C]leucine ($5\mu\text{Ci}/100\text{ml}$) was added. After 24h plants were transferred to a medium lacking both $^2\text{H}_2\text{O}$ and [^{14}C]leucine. Samples were removed at intervals, protein was isolated and separated by isopycnic centrifugation as in Fig. 2. $\circ$, L-[^{14}C]leucine in 'light' protein; —, L-[4,5- ^3H]leucine in 'light' protein centrifuged with each sample as an internal marker. (a) 10 h after transfer; (b) 24 h after transfer; (c) 72 h after transfer; (d) 96 h after transfer; (e) 120 h after transfer.

synthesis, as measured by incorporation of amino acids, was measurably greater than that of amino acid uptake. Incorporation of L-[^{14}C]leucine ceased after 4h on 80% $^2\text{H}_2\text{O}$ and was inhibited 45–50% on 50% $^2\text{H}_2\text{O}$ (Fig. 1).

Respiration and photosynthesis. Both respiration and photosynthesis were markedly affected by 80% $^2\text{H}_2\text{O}$, respiration being inhibited by 30% and photosynthesis by 40%. Only slight inhibition (5%) of respiration was produced by 50% $^2\text{H}_2\text{O}$.

Recovery from toxic effects of $^2\text{H}_2\text{O}$. *Lemna*

rapidly recovers from exposure to $^2\text{H}_2\text{O}$. Thus, on transfer to $^2\text{H}_2\text{O}$ -free medium after 4h on 80% $^2\text{H}_2\text{O}$, respiration and photosynthesis returned to normal rates after 90 min; protein synthesis as measured by incorporation of L-[^{14}C]leucine returned to normal after 6h and a normal growth rate was resumed after 1 day.

Improved separation of density-labelled protein by the use of KBr gradients

To obtain maximum density differences many

workers have used 80% $^2\text{H}_2\text{O}$. In view of the toxic effects of 80% $^2\text{H}_2\text{O}$, it seemed desirable to lower the concentration of $^2\text{H}_2\text{O}$ to 50%. The consequent loss of resolution of heavy and light protein peaks on a CsCl gradient was unacceptable, but the improved separation obtained with a KBr gradient permitted the use of 50% $^2\text{H}_2\text{O}$ in the growth medium.

Heavy protein (labelled with [^{14}C]leucine) obtained by growing *Lemna* in 50% $^2\text{H}_2\text{O}$ was completely separable from light protein (labelled with [^3H]leucine) obtained by growing *Lemna* in H_2O when a KBr gradient was used for isopycnic centrifugation, but not when a CsCl gradient was used (Fig. 2). The buoyant density of *Lemna* soluble protein grown on H_2O was 1.3006 and after 7 days on 50% $^2\text{H}_2\text{O}$ the buoyant density was 1.3263. The peaks produced on a KBr gradient were taller and sharper than those on CsCl. The improvement produced by KBr can be measured by comparing the ratio peak height/peak area. This ratio for the H_2O protein peaks shown in Fig. 2 was 1.73 when using KBr and 1.21 with CsCl.

An alternative index can be obtained by measuring the square root of the mean standard deviation from the central point at the base of the peak. The radioactive counts in each peak fraction were fed into a QMC system (MK 2J) computer and the root of the mean square deviation was computed. For the data of the H_2O peaks shown in Fig. 2, KBr gave a value of 3.09 compared with a value of 4.67 for the CsCl gradient.

Measurement of protein turnover

A series of experiments with 80% $^2\text{H}_2\text{O}$ gave unsatisfactory results. In our first experiments using

Fig. 4. Measurement of protein degradation in *Lemna* by using a 24h exposure to [^{14}C]leucine to label protein

Data obtained as in Fig. 3 are plotted as the logarithm of the normalized peak area against time. The line is fitted by the method of least squares.

50% $^2\text{H}_2\text{O}$, *Lemna* fronds were density-labelled for 6 days and on day 7 L-[U- ^{14}C]leucine (5 μCi) was added to the deuterated medium. After 24h in the presence of [^{14}C]leucine plants were rinsed with deuterium-free medium and transferred to deuterium-free medium. Samples were removed at intervals and the distribution of ^{14}C in 'heavy' and 'light' protein was determined after isopycnic centrifugation. The results shown in Fig. 3 indicate that after transfer to deuterium-free medium there is a continued incorporation of [^{14}C]leucine into proteins of slowly decreasing density, which complicates the analysis. However, by using half-peak areas, that is the half peak with the greater density, the problem of incorporation of [^{14}C]leucine into proteins of lower density is largely avoided. The heavier-half area of the peak of heavy protein is measured for every sample and corrected for the amount of protein introduced on to the gradient and for the amount of growth as measured by frond number. The logarithm of the normalized peak areas gave a linear relationship with respect to time from which the half-life of the total soluble protein can be shown to be 97 h (Fig. 4).

In subsequent experiments the labelling regime was changed, so that after 7 days in 50% $^2\text{H}_2\text{O}$ L-[U- ^{14}C]leucine (5 μCi) was added, and after 8 h the plants were filtered, washed with 50 ml of growth medium containing 50% $^2\text{H}_2\text{O}$ and transferred back to 100 ml of the deuterium-containing medium for a further 40 h. The plants were then washed with deuterium-free medium and maintained in deuterium-free medium for periods up to 6 days. 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. The almost complete incorporation of [^{14}C]leucine into 'heavy' protein before transfer to the deuterium-free medium simplified the results, as shown in Fig. 5. The logarithm of the normalized peak areas gave a linear relationship with respect to time from which the half-life of the total soluble protein was shown to be 101 h (Fig. 6).

Discussion

Protein turnover may be defined in various ways (see Huffaker & Peterson, 1974). Koch (1962) made a private poll which suggested that for most biochemists protein turnover was equivalent to enzyme degradation, and this is the meaning employed in the present paper.

The rate of protein turnover is usually calculated from changes in the specific radioactivity of protein and amino acids after supplying a tissue with a radioactive intermediate, usually an amino acid. However, attempts to measure the rates of synthesis and degradation with radioactive isotopes are complicated by the presence of different pools of amino acids in the cell, only a small portion of the soluble amino acid

Fig. 5. Measurement of protein turnover by using an 8h exposure to $[^{14}\text{C}]$ leucine to label protein

Lemna fronds were density-labelled for 7 days with 50% $^2\text{H}_2\text{O}$, and on day 8 L-[U- ^{14}C]leucine ($5\mu\text{Ci}/200\text{ml}$) was added. After 8h, plants were transferred to a medium lacking $[^{14}\text{C}]$ leucine. After a further 40h, plants were transferred to a medium lacking both $^2\text{H}_2\text{O}$ and $[^{14}\text{C}]$ leucine. Samples were removed at intervals, protein was extracted, low-molecular-weight substances were removed by passing through Sephadex G-100 and the heavy and light fractions separated by isopycnic centrifugation in KBr . $\circ$, L-[U- ^{14}C]leucine in protein; $\bullet$, immediately after transfer from $^2\text{H}_2\text{O}$ medium; (b) 8h after transfer; (c) 24h after transfer; (d) 48h after transfer; (e) 72h after transfer; (f) 96h after transfer; (g) 144h after transfer; (h) 144h after transfer.

Fig. 6. Measurement of protein degradation in *Lemna* by using an 8 h exposure to [¹⁴C]leucine to label proteins

Data obtained in Fig. 5 are plotted as the logarithm of the normalized peak area against time. The line is fitted by the method of least squares.

being available for protein synthesis. Isotope-decay-type experiments largely used in mammalian tissue are unsuitable in plants, where the radioactive precursor can be stored in the vacuole and slowly released. A further problem in the application of these methods is the re-utilization of radioisotope released by protein degradation. The method presented in this paper attempts to avoid these difficulties by separating newly synthesized from previously synthesized protein. Thus, if recycling of a labelled amino acid occurs, the labelled amino acid will leave ‘heavy’ protein and reappear in newly synthesized ‘light’ protein. Thus, by isolating ‘heavy’ protein and measuring its radioactivity, the problems of storage pools and recycling are largely avoided.

The conditions used in the first density-labelling experiment reported in this paper only partially solved these difficulties. Thus it is clear from the data of Fig. 3 that, after transfer from ²H₂O to H₂O, free leucine continued to be incorporated into protein. The experimental conditions used in the second experiment involved growing *Lemna* for 40 h on ²H₂O after removing the fronds from the [¹⁴C]leucine-containing medium. During this period free or soluble [¹⁴C]leucine was almost completely absent and thus did not complicate the analysis when the fronds were transferred to H₂O.

Toxic effects of ²H₂O

The toxic effects of ²H₂O on higher plants are well documented, but little attention has been paid to toxicity in the design and interpretation of density-labelling experiments. Sacher *et al.* (1972) found that exposure of potato discs to ²H₂O produced lowered activity for a number of enzymes. However, the recognition that deuterium oxide inhibits protein synthesis does not prevent the experimenter from drawing valid qualitative conclusions about synthesis of

Table 1. Comparison of half-lives of *Lemna minor* soluble proteins obtained by various methods

Lemna was grown under constant conditions and protein turnover measured by using the methods listed below, except that the data for Trewavas (1972) were taken directly from his paper, since the *Lemna* strain and growth conditions were identical.

Author	Half-life (days)
Bidwell <i>et al.</i> (1964)	0.88
Kemp & Sutton (1971)	12–25
Trewavas (1972)	7
Humphrey & Davies (1975)	Fraction A 3
	Fraction B 7
Heavy-water method	
Data of Fig. 4	4.0
Data of Fig. 6	4.2

enzymes *de novo*. On the other hand, the toxic effects must raise doubts about the quantitative interpretation of labelling experiments. In the experiments reported in this paper, the severe toxic effects of ²H₂O on amino acid uptake and protein synthesis observed with 80% ²H₂O precludes the use of 80% ²H₂O in experiments designed to measure protein turnover. The lower concentration of 50% ²H₂O proved much less toxic and the recovery on transfer to a ²H₂O-free medium was relatively rapid. Consequently, if one wishes to measure the rate of protein degradation, it is advisable to carry out the labelling schedule in the sequence reported in this paper.

Comparison of different methods of measuring protein turnover

We have measured the rate of protein degradation in *Lemna* by density labelling and by three other methods. These values are compared with those obtained by Trewavas (1972), who used the same strain of *Lemna* and the same growth conditions as in Table 1.

The values determined by the method of Hellebust & Bidwell (1963) are much higher than those obtained by the other methods. The validity of the equations used by Hellebust & Bidwell (1963) have been questioned by Davies & Humphrey (1975) and in particular their interpretation of label-chase kinetics. The turnover rates measured by the method of Trewavas (1972) and by the ³H-exchange method of Humphrey & Davies (1975) are in good agreement, but the density-labelling method gives a significantly shorter half-life for *Lemna* proteins. This discrepancy may be due to the toxic effect of ²H₂O. However, other explanations are possible. For example, the method described here involves the use of a column of Sephadex G-200 or G-100 to remove low-molecular-weight proteins; this step is necessary because such proteins

do not band well during isopycnic centrifugation. However, Dice *et al.* (1973) have shown that in stem segments of *Pisum sativum* larger proteins are more labile than smaller proteins. Thus the use of Sephadex G-200 may increase the relative concentration of proteins of high molecular weight and so produce an apparently faster turnover.

Use of KBr gradients for isopycnic centrifugation

After the success of CsCl gradients for the resolution of 'heavy' and 'light' DNA, a number of other salts were examined for their possible use in isopycnic centrifugation (Ifft *et al.*, 1961; Hu *et al.*, 1962). However, despite its shortcomings CsCl has become something of a fashion and is extensively used for isopycnic centrifugation. We have found KBr gradients to give much better separations of proteins than CsCl. KBr provides a shallower gradient, but of much greater significance is the lesser band width of the protein peaks.

One reason why KBr gradients have not been used may be the report (Hu *et al.*, 1962) that β -galactosidase (EC 3.2.1.23) is labile in a KBr gradient. We have observed that lactate dehydrogenase (EC 1.1.1.27) is stable in a KBr gradient. If this finding can be extended to other enzymes, then the very much better separation obtained with KBr compared with CsCl suggests that KBr should replace CsCl in the isopycnic centrifugation of proteins.

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